Europ. J. Cancer Vol. 11, pp. 1–8. Pergamon Press 1975. Printed in Great Britain

Antitumour Immunity—1. Differential Response of Neuraminidase-Treated and X-Irradiated Tumour Vaccine*

P. K. RAY†, V. S. THAKUR and K. SUNDARAM
Medical Division, Cancer Immunobiology, Bhabha Atomic Research Centre, Trombay, Bombay–400 085, India

Abstract—*A dimethyl-benz-dithionapthene-induced fibrosarcoma shows reduced transplantability in syngeneic Swiss mice when treated with* Vibrio cholerae *neuraminidase (VCN). Reduced transplantability of fibrosarcoma has also been observed if they are X-irradiated. Inoculation of VCN treated cells leads to the development of strong antitumour immunity, whereas comparable results are not obtained with X-irradiated cells. However, inoculation of cells treated with VCN followed by X-irradiation can also establish lasting antitumour immunity. It is suggested that 'tumour vaccine' produced in this way may be very effective for the immunotherapy of tumour.*

INTRODUCTION

VARIOUS laboratories [1–10] have reported that *Vibrio cholerae* neuraminidase (VCN) treated tumour cells have reduced transplantability in an otherwise susceptible host. Animals previously inoculated with VCN treated tumour have been shown to be refractory to normal tumour challenge. Several mechanisms have been proposed as to how VCN treated tumour cells induce tumour specific immunity [1–10]. In a recent review, Weiss [11] has pointed out that absence of tumour or the presence of small, slowly-growing tumour in animals receiving VCN-treated tumour cells might be due to the fact that less than LD_{100} doses of undamaged viable cells were given. He mentioned that animals receiving inocula containing similar proportions of living and dead cells not treated with neuraminidase may also lead to comparable results. Moreover, comparisons of the lethality of inoculated BW 10232 mouse mammary carcinoma cells fail to reveal a significant difference between X-irradiated cells and those incubated with neuraminidase after exposure to 5000 R [12]. In this review, Weiss [11] raised the question 'is neuraminidase really necessary?' He stated that X-irradiated cells might exhibit an immunogenic response equal to that shown by those treated with neuraminidase. We tested this phenomenon using VCN treated tumour cells and X-irradiated tumour cells, since X-irradiated vaccines of tumour cells sometimes [13, 14], but not invariably [15], show increased immunogenic activity.

This paper reports that VCN-induced increased immunogenicity of tumour cells cannot be achieved with lethally X-irradiated tumour cells. Tumour specific immunity, however, can be achieved with VCN treated and X-irradiated tumour cells indicating that VCN modulates the tumour cell surface in a specific way to induce antitumour immunity unlike the X-irradiated cells.

Accepted 19 September 1973.

*Part of this report was presented at the DAE Symposium on 'Biological approach to problems of medicine, industry and agriculture', Trombay, 12–14 March, 1974.

†Address reprint requests to: Dr. P. K. Ray, Medical Division, Cancer Immunobiology, Bhabha Atomic Research Centre, Trombay, Bombay–400 085, India.

MATERIAL AND METHODS

Animals

Swiss mice originally obtained from Jackson Laboratory have been maintained here by brother–sister mating. Male mice 8–10 weeks old were used. Animals were housed, 4–5 animals/cage; food and water were supplied *ad libitum*.

Tumours

A dimethyl-benz-dithio napthene-induced fibrosarcoma has been obtained from Cancer Research Institute, Parel, by courtesy of Dr. C. V. Bapat and has been maintained here by serial transplantation in syngeneic Swiss mice. This tumour is very weakly immunogenic and never regresses spontaneously.

Neuraminidase

Vibrio cholerae neuraminidase (VCN) has been obtained from Behrinwerke A.G., Marburg, Lahn, West Germany. It contains 500 units of enzyme/ml. One unit of enzyme activity is equivalent to the release of 1 μg of N-acetyl neuraminic acid from a glycoprotein substrate at a pH of 5·5, at 37°C. This enzyme has been reported by the manufacturer to be free from aldolases, proteinases, and lecithinase C activity. The enzyme itself is not cytolytic [16, 17] and can release sialic acid from both normal and malignant cell surfaces at physiological pHs [18].

VCN treatment

Tumour cells were incubated with VCN (25 U/5 $\times 10^7$ cells) at 37°C for one hour. After incubation, cells were washed ($\times 3$) in excess of medium 199 (M 199) and finally suspended in M 199.

X-irradiation

In order to determine if VCN-inhibition of tumour growth was due specifically to VCN-treatments, additional experiments were done using X-irradiated tumour cells.

Measured numbers of tumour cells suspended in physiological saline in a petridish have been X-irradiated (100–2000 rads) using an X-ray machine working at a dose rate of 100 rad/min (250 kVp, 15/mA, 0·2 mm Cu, 54 cm target–object distance).

Tumour cell suspension

Tumours were collected in an asceptic way in a petri dish with media containing antibiotics The tumour mass was washed several times in media and then cut into very small pieces with scissors, then 0·1% trypsin (1:250, DifCo) was added and the tumour pieces were stirred for 10 min using a magnetic stirrer. Clumps were allowed to settle down and supernatant fluid was collected. A fresh volume of media was added and the process repeated several times. The cells were centrifuged (2000 rev/min, 10 min) and washed three times before finally being suspended in M 199 unless otherwise stated. Cell viability was determined by trypan blue exclusion [19].

RESULTS

Effect of VCN on the growth of chemically induced fibrosarcoma in syngeneic Swiss mice

Various doses of untreated and VCN treated tumour cells were inoculated subcutaneously in the adult male Swiss mice. Animals were checked every day to detect the tumour nodule and the survival times of the animals from the date of appearance of the tumour nodule were noted. Results are presented in Table 1. The formation of the tumour nodule is much delayed in animals which received VCN treated cells compared to animals which received sham-treated cells. It appears that transplantability of VCN treated cells is very much reduced. This observation confirms the previous findings [1–10] which note that at certain concentrations, VCN treated tumour cells cannot grow in an otherwise susceptible host.

In the present investigation, it has been established further that VCN-treated tumour cells did not develop tumours, while those animals receiving 1000 sham-treated tumour cells did develop tumours which ultimately killed the host. Although animals inoculated with higher doses of VCN treated tumour cells show tumour development, they survive longer than those receiving the corresponding number of sham-treated cells.

The above experiment suggests that though the transplantability of VCN treated tumour cells is very much reduced, their oncogenicity is still retained. VCN treated tumour can grow if inoculated at a high dose. Further, it has been shown that VCN treated tumour cells that do not develop tumours in normal recipients do develop tumours which ultimately kill the recipient when injected in immunosuppressed animals [4, 9]. Thus, the inhibition of growth of VCN treated tumour appears to be mediated through an active immune mechanism in the host. It is highly probable that tumour specific antigens are exposed after

Table 1. *Reduced transplantability of tumour cells treated with* Vibrio cholerae *neuraminidase (VCN)*

Number of cells in the initial inoculum	Fraction tumour appeared in animals receiving		Average incubation period (days)		Survival period (days ± S.E.)†	
	Untreated cells	VCN treated cells*	Untreated cells	VCN treated cells	Untreated cells (± S.E.)	VCN treated cells (± S.E.)
10^6	6/6	6/6	6	23	23 ± 2·5	25 ± 1·5
10^5	6/6	4/6	11	21	29 ± 1·5	36 ± 3·0‡
10^4	6/6	2/6	15	24	31 ± 2·0	38 ± 3·5‡
10^3	6/6	0/6	21	—	30 ± 2·5	Indefinite
10^2	5/6	0/6	23	—	35 ± 3·5	Indefinite

*Cells were viable by trypan blue exclusion test.
†Standard error of the mean (± S.E.).
‡Animals without tumours were not included.

VCN treatment. Recognizing them to be of foreign origin, the hosts' immune systems might become fully operative and ultimately reject the tumour cells.

Development of antitumour immunity by VCN treated tumour vaccine

In the above experiment, animals which received VCN treated tumour cells and did not develop any tumour up to 60 days were challenged with double the initial doses of untreated tumour cells. Results are presented in Table 2. These animals were found to be resistant against the challenging inoculum. It is quite significant that these immune animals can resist such high doses of tumour cells, while cohort mice all died. After 60 days, these resistant animals received a second challenge with 10 times the initial inoculum and could withstand even this high dose of viable tumour cells. The animals remained tumour-free in perfectly normal health up to 60 days.

Inhibition of the oncogenic potential of tumour cells by X-irradiation

Freshly prepared tumour cells were X-irradiated. The irradiated cells were then inoculated into animals and their oncogenic potential were studied (Table 3). A dose of 500 rad can inhibit completely the oncogenic potential of 50,000 tumour cells, while as high as 1500 rad is needed to suppress the tumourogenic ability of 10^6 tumour cells. It appears from the results that there is a direct relationship between the number of tumour cells in an inoculum and the amount of X-irradiation to be given to them to suppress their oncogenic potential. In some cases where tumours are seen following inoculation with X-irradiated cells, their appearances are much delayed and

Table 2. *Development of strong antitumour immunity by VCN-treated tumour vaccine*

Group of animals	Initial inoculum	Cell treatment	Fraction 1° tumour appearance	Challenging inoculum*		Fraction tumour appeared		Survival time (± S.E.)
				1st	2nd	1st	2nd	
I	10^5	VCN	4/6	2×10^5	10^6	0/2	0/2	Indefinite†
		None	6/6	—	—	—	—	29 ± 1·5
II	10^4	VCN	2/6	2×10^4	10^5	0/4	0/4	Indefinite
		None	6/6	—	—	—	—	31 ± 2·0
III	10^3	VCN	0/6	2×10^3	10^4	0/6	0/6	Indefinite
		None	6/6	—	—	—	—	30 ± 2·5
IV	10^2	VCN	0/6	2×10^2	10^3	0/6	0/6	Indefinite
		None	5/6	—	—	—	—	35 ± 3·5

*Animals which did not show primary tumours were only challenged with untreated viable tumour cells after 60 days. Second challenge was given 60 days after the first challenge. If the initial challenge is given with 10 times the initial inoculum, in many cases tumour developed in the beginning.
†Only the tumour free animals were considered.

they usually survive longer than the control groups of animals.

Development of antitumour immunity by X-irradiated tumour cells

Animals which did not develop any tumour following the inoculation of X-irradiated tumour cells in the above experiment (Table 3), were challenged with viable untreated tumour cells (double the initial inoculum). Results are presented in Table 4. Most of these animals were not resistant to the challenging inoculum indicating that the immunogenicity of the tumour cells was not increased with X-irradiation to prevent tumour growth. To examine the immunogenic potential of this combination the following experiments were performed.

Development of antitumour immunity by VCN treated and X-irradiated tumour vaccine

Three groups of animals were inoculated with either 10^5 VCN treated cells, X-irradiated (1500 rads) tumour cells, or VCN treated and X-irradiated (1500 rads) tumour cells. Eight out of ten animals receiving VCN treated cells developed tumours while none of the animals receiving irradiated or VCN treated and

Table 3. Reduced transplantability of X-irradiated tumour cells

Group of animals	Number of cells in the intial inoculum	Dose of irradiation (X-rays, rads)	Fraction tumour appearance	Average incubation period (days ± S.E.)	Average period of survival of tumour bearing animals*
I	2×10^6	500	6/6	18 ± 2·5	32‡2·5
	1×10^6	500	5/5	21 ± 3·0	33‡2·5
	5×10^5	500	1/6	19 ± 2·5	32 ± 3·0
	5×10^4	500	0/6	—	Indefinite
	5×10^3	500	0/6	—	Indefinite
	5×10^2	500	0/6	—	Indefinite
	5×10	500	0/6	—	Indefinite
II	1×10^6	100	4/4	11 ± 1·5	26 ± 1·5
		200	4/4	14 ± 1·5	27 ± 2·5
		300	6/6	14 ± 2·0	32 ± 3·0
		400	6/6	15 ± 1·5	34 ± 2·5
		500	4/6	30 ± 2·5	33 ± 3·0
		750	2/3	21 ± 3·5	36 ± 3·5
		1000	2/6	19 ± 2·5	43 ± 4·0
		1500	0/3	—	Indefinite
		2000	0/3	—	Indefinite

*Animals with tumours were only included.

following X-irradiation, at least to the extent that could inhibit the growth of the challenging inoculum.

Thus it appears that inoculation of X-irradiated tumour cells does not lead to the establishment of comparable antitumour immunity obtained with VCN treated tumour cells. Inhibition of oncogenicity of tumour cells by X-irradiation may be due to the killing of the tumour cells. On the other hand, the inhibition of the growth of VCN treated tumour cells, which are viable, appears to be due to the heightened tumour specific immunity developed in those animals (Table 2).

From the point of view of actual therapy using VCN treated tumour cells, which are otherwise viable and can show tumour development at higher doses (Table 1), it was necessary to combine VCN treatment of tumour cells

irradiated cells developed tumours (Table 5). Animals which remained tumour free were then challenged with untreated tumour cells (2×10^5). All animals which received irradiated tumour cells as a primary inoculum developed tumour without any change in their incubation time or in the period of survival of the animals (Table 5). Most of the animals which received VCN treated and X-irradiated cells as a primary inoculum were immune to the challenging inoculum. It is important to note that while X-irradiation inhibits the growth potential of 10^5 VCN treated tumour cells, it does not alter the immunogenic properties of VCN treated cells. From the therapeutic point of view, this method of preparation of tumour vaccine will have significance for a variety of reasons: (a) there will be no danger of inducing a fresh tumour growth by the inoculating cells,

(b) tumour vaccine containing large number of cells can be inoculated at a time (which is necessary for effective immunotherapy) to induce tumour specific immunity, and (c) a syngeneic tumour or even a portion of the tumour to be treated can serve as the source of cells for the preparation of the vaccine, thereby diminishing any chance of graft-versus-host reaction.

transplantability of tumour cells, it is not associated with the establishment of comparable antitumour immunity. In some cases, some degrees of immunity (lower than that given by VCN treated cells) could be established. But the degree of immunity is always lower compared to what has been observed with VCN treated tumour inoculation. However, VCN treated and X-irradiated tumour cells

Table 4. Weak antitumour immunity as developed by X-irradiated tumour cells

Initial inoculum	Cell treatment (X-rays, rads)	Fraction 1° tumour appearance	Challenging inoculum	Fraction tumour appeared	Mean day of death (± S.E.)*
1×10^6	2000	0/6	2×10^6	6/6	23 ± 2·0
1×10^6	1500	0/6	2×10^6	6/6	25 ± 2·5
1×10^6	1000	2/6	2×10^6	4/4	22 ± 1·5
1×10^6	500	5/6	2×10^6	0/1	Indefinite†
5×10^5	500	1/6	1×10^6	5/5	31 ± 3·0
1×10^5	500	0/6	2×10^5	6/6	29 ± 3·5
5×10^4	500	0/6	1×10^5	6/6	26 ± 2·5
5×10^3	500	0/6	1×10^4	3/6‡	27 ± 2·5

*Only those animals with tumours were included.
†This animal could resist a dose up to 4×10^6, but not 10^7 cells.
‡These animals could resist a dose up to 2×10^4, but not 10^5 cells.

Table 5. Development of antitumour immunity by VCN-treated and X-irradiated tumour vaccine

Initial inoculum	Cell treatment	Fraction 1° tumour appearance	Challenging inoculum*	Fraction tumour appearance	Mean day of death (± S.E.)†
1×10^5	VCN	8/10	2×10^5	0/2	Indefinite‡
	1500 rad	0/10	2×10^5	10/10	24 ± 3
	VCN + 1500 rad	0/10	2×10^5	2/10	Indefinite

*Animals which did not show primary tumour were only challenged with untreated viable tumour cells.
†Standard Error of the mean (S.E.).
‡Animals without tumours were included.

DISCUSSION

The above results suggest that VCN treatment of tumour cells reduces the transplantability of these cells, thus confirming the earlier observations from a number of laboratories [1–10]. Same doses of sham-treated tumour cells (10^3) as grown in 100% of animals, do not show any tumour at all if previously treated with VCN. At higher doses (10^4–10^5), a fraction of the animals receiving VCN treated tumour cells show tumour development. Animals which did not show any tumour following inoculation with VCN treated tumour cells, have been found to be refractory to normal tumour challenge. They could inhibit the growth of ten times the doses of initial inoculum. On the other hand, although X-irradiation of tumour cells could reduce the established comparable antitumour immunity as observed with VCN treated tumour cells with one extra benefit that higher doses can be inoculated. This 'tumour vaccine' perhaps can be successfully used for the immunotherapy of tumours.

It should be mentioned that different tumours and different laboratories yield different results [1–10, 20, 21], with VCN-induced modification of tumour cells. While most of the laboratories [1–10] confirmed the ability of VCN to increase the immunogenicity of tumour cells, contradictory reports [20, 21] are also available. However, it appears from the present report that VCN treated tumour cells, as also the VCN treated and X-irradiated tumour cells, lead to the establishment of strong antitumour immunity, unlike the irradiated tumour cells.

The above results point out that VCN perhaps acts in a specific way, increasing the availability of immunogens while the effect of irradiation appears to be non specific.

The exact mechanism as to how VCN treated cells help the host to establish anti-tumour immunity is not known. However, there are a number of possible explanations. Sanford [1], and Currie and Bagshawe [2] have suggested that the mechanism of increased immunogenicity of VCN treated tumour cells could be the result of "unmasking" of histo-compatibility antigens on the tumour cells. This has been contradicted by Ray and Simmons [16, 22] and subsequently by others [20, 23, 24]. However, while not unmasking H-2 antigens, VCN could unmask other antigens. Kassulke *et al.* [25] demonstrated an increased number of blood group isoantigens on VCN treated human leukemic cells. Ray *et al.* [26, 27] have demonstrated unmasking of xenogeneic neo-antigens on mouse lymphoid cells. Further, Ray and associates [16, 17, 26] have demonstrated that VCN treated normal lymph node cells become extremely susceptible to cytolysis by alloantibody and complement. Even autologous serum could show cytolysis of VCN treated autochthonous lymphoid cells [28]. Recently, Ray and Sundaram [29] have suggested the presence of autoantibody directed against VCN-exposed antigenic determinants on the autochthonous lymphoid cells. VCN treated autochthonous red blood cells have been found to form rosettes with autologous T cells [30]. In MLC, VCN treated stimulatory cells could give stimulation to the syngeneic human lymphocytes [31]. VCN treated tumour cells could stimulate the autologous human lymphocytes in culture [32]. Whatever may be the mechanism responsible for the suppression of growth of VCN treated tumour, it becomes apparent that VCN treated cells are more easily reacted upon by the immune components of the body.

We have reported earlier that VCN treated normal [33], and foetal cells [34] become increasingly immunogenic. It has also been observed that VCN treated sheep erythrocytes are more easily phagocytized [35], and phagocytosis is directly related to antigen handling and/or processing. Thus, the antigen processing may be facilitated by the above mechanisms. Further, VCN results in the decrease in the cell surface negative charges [36] and hence may facilitate the binding between a VCN treated cell and a negatively charged antigen responsive cell. VCN also results in an increased deformability of the cell membrane [36]. Increased deformability would facilitate a larger area of cell-to-cell contact and allow for an increased number of contacts between the antigenic determinants and the antigen-receptor sites on the antigen responsive cell.

However, it is difficult to establish precisely as to how the tumour specific immunity could be achieved following inoculations of VCN treated tumour vaccine. It is not known if either one or the other, or a summation of the events as discussed above might give rise to antitumour immunity.

Whatever the mechanism, the present method provides an effective way for producing a "tumour vaccine". The possible use of such a vaccine in the immunotherapy of cancer is obvious. Using tumour vaccine produced in this manner, experimental trials with Yoshida sarcoma in rat and a chemically induced fibrosarcoma in mice have achieved significant growth inhibition of these tumours resulting in the prolongation of life span of the treated animals (unpublished observation).

REFERENCES

1. B. H. Sanford, An alteration in tumour histocompatibility induced by neuraminidase. *Transplantation* **4,** 1273 (1967).
2. G. A. Currie and K. D. Bagshawe, Tumour specific immunogenicity of methyl cholanthrene-induced sarcoma cells after incubation in neuraminidase. *Brit. J. Cancer* **23,** 141 (1969).
3. R. L. Simmons, A. Rios, G. Lundgren, P. K. Ray, C. F. McKhann and G. R. Haywood, Immuno specific regression of methylcholanthrene fibrosarcoma using neuraminidase. *Surgery* **70,** 38 (1971).
4. R. L. Simmons, A. Rios, P. K. Ray and G. Lundgren, Effect of neuraminidase on the growth of a 3-methyl cholanthrene-induced fibrosarcoma in normal and immunosuppressed syngeneic mice. *J. nat. Cancer Inst.* **47,** 1087 (1971).
5. R. L. Simmons, A. Rios and P. K. Ray, Regression of established methyl cholanthrene Tumours by intratumour injections of *Vibrio cholerae* neuraminidase. *J. Surg. Oncol.* **4,** 298 (1972).

6. J. G. Bekesi, L. Walter and J. F. Holland, Immunogenicity of neuraminidase treated 6C3HED lymphoma in C3H/HeJ and C_3H/f mice. *Proc. Amer. Ass. Cancer Res.* **13,** 113 (1972).
7. J. G. Bekesi, G. St. Arneault, L. Walter and J. F. Holland, Immunogenicity of leukemia L1210 cells after neuraminidase treatment. *J. nat. Cancer Inst.* **49,** 107 (1972).
8. A. Rios and R. L. Simmons, Active specific immunotherapy of minimal residual tumour: Excision plus neuraminidase treated tumour cells. *Int. J. Cancer* **13,** 7 (1974).
9. K. K. Sethi and H. Brandis, Neuraminidase induced loss in the transplantability of Murine Leukaemia 1210, Induction of immunoprotection and the transfer of Induced Immunity to normal DBA/2 mice by serum and peritoneal cells. *Brit. J. Cancer* **27,** 106 (1973).
10. P. K. Ray, V. S. Thakur and K. Sundaram, Immunotherapy of Cancer. Inhibition of growth of an established tumour using neuraminidase treated tumour vaccine. *Proc. Symp. on Biological Approach to Problems of Medicine, Industry and Agriculture, Bombay*, p. 50, 1974.
11. L. Weiss, Neuraminidase, Sialic acids, and cell interactions. *J. nat. Cancer Inst.*. **50** (1), 3 (1973).
12. S. Oxley, W. O. Griffen, Immunogenic enhancement by neuraminidase. *Surg. Forum* **23,** 113 (1971).
13. H. Goldner, A. J. Girardi and V. M. Lanson, Interruption of SV40 virus tumourigenesis using irradiated homologous tumour antigen. *Proc. Soc. exp. Biol. (N.Y.)* **117,** 851 (1964).
14. H. R. Cox, H. S. Nozell and R. E. Walace, Immunologic studies with simian virus 40 (SV40) tumours in hamsters. *J. Med. (Basel)* **1,** 26 (1970).
15. G. L. Bartlett and B. Zbar, Tumour specific vaccine containing mycobacterium bovix and tumour cells: safety and efficacy. *J. nat. Cancer Inst.* **48,** 1709 (1972).
16. P. K. Ray, H. Gewurz and R. L. Simmons, The serologic behaviour of neuraminidase treated lymphoid cells: alloantigenicity and complement sensitivity. *Clin. exp. Immunol.* **11,** 441 (1972).
17. P. K. Ray and R. L. Simmons, Comparative effect of viral and bacterial neuraminidase on the complement sensitivity of lymphoid cells. *Clin. exp. Immunol.* **10,** 139 (1972).
18. P. K. Ray and R. L. Simmons, Differential release of sialic acid from normal and Malignant cells by *Vibrio cholerae* neuraminidase or Influenza virus neuraminidase. *Cancer Res.* **38,** 936 (1973).
19. E. A. Boyse, L. J. Old and I. Chouronlinkov, *Methods in Medical Research.* Vol. 10, p. 39. Yearbook Medical Publishers, Chicago (1964).
20. T. S. Hauschka, L. Weiss, B. A. Holdridge, Karyotypic and surface features of murine TA_3 carcinoma cells during immunoselection in mice and rats. *J. nat. Cancer Inst.* **47,** 343 (1971).
21. G. Ruhenstroth-Bauer, G. F. Fuhrmann, W. Kubler, Zur bedeutung der neuraminsauren in der zellmembran for das wachstum maligner zellen. *Z. Krebsforsch.* **65,** 37 (1962).
22. P. K. Ray and R. L. Simmons, Failure of neuraminidase to unmask allogeneic antigens on cell surface. *Proc. Soc. exp. Biol. (N.Y.)* **138,** 600 (1971).
23. B. H. Sanford and J. R. Codington, Further studies on the effect of neuraminidase on tumour cells transplantability. *Tissue Antigens* **1,** 153 (1971).
24. S. A. Rosenberg, B. A. Plocinik and J. R. Rogentine, Unmasking of human lymphoid cell heteroantigens by neuraminidase treatment. *J. nat. Cancer Inst.* **48,** 1271 (1972).
25. J. T. Kassulke, O. Stutman and E. J. Yunis, Blood group isoantigens in leukemic cells. Reversibility of isoantigenic changes by neuraminidase. *J. nat. Cancer Inst.* **46,** 1201 (1971).
26. P. K. Ray, H. Gewurz and R. L. Simmons, Mechanism of neuraminidase induced antigen unmasking. *Fed. Proc.* **29,** 573 (1970).
27. P. K. Ray and R. L. Simmons, Unmasking of xenogeneic neo-antigens on mouse lymphoid cell surface by *Vibrio cholerae* neuraminidase. *Proc. Soc. exp. Biol. (N.Y.)* **142,** 217 (1973).
28. P. K. Ray, H. Gewurz and R. L. Simmons, Complement sensitivity of neuraminidase treated lymphoid cells. *Transplantation* **12,** 327 (1971).

29. P. K. Ray and K. Sundaram, Naturally occuring autoantibody against hidden cellular antigens. *Clin. exp. Immunol.* in press (1975).
30. G. Baxley, G. B. Bishop, A. G. Cooper and H. H. Wortis, Rosetting of human red blood cells to thymocytes and thymus-derived cells. *Clin. exp. Immunol.* **15,** 385 (1973).
31. G. Lundgren and R. L. Simmons, Effect of neuraminidase on the stimulatory capacity of cells in human mixed lymphocyte cultures. *Clin. exp. Immunol.* **9,** 915 (1971).
32. E. Watkins, Y. Ogata and L. Anderson, Activation of host lymphocyte cultures with cancer cells treated with neuraminidase. *Nature (New Biol.)* **231,** 83 (1971).
33. R. L. Simmons, A. Rios and P. K. Ray, Immunogenicity and antigenicity of lymphoid cells treated with neuraminidase. *Nature (New Biol.)* **231,** 179 (1971).
34. R. L. Simmons, M. L. Lipschultz, A. Rios and P. K. Ray, Failure of neuraminidase to unmask histocompatibility antigens on trophoblast. *Nature (New Biol.)* **231,** 111 (1971).
35. J. R. Schmidtke, R. Hoffman, T. B. Grage, P. K. Ray and R. L. Simmons, Effect of neuraminidase on the phagocytosis of heterologous red cells. *J. reticuloendoth Soc.* **13,** 360 (1973).
36. L. Weiss, Studies on cell deformability. I. Effect of cell charge. *J. Cell Biol.* **26,** 735 (1975).

Europ. J. Cancer Vol. 11, pp. 9-16. Pergamon Press 1975. Printed in Great Britain

Differential Effects of Cytotoxic Concentrations of Chlorambucil on Drug-Resistant and Drug-Sensitive Cell Lines of the Yoshida Ascites Sarcoma in Culture

BRIDGET T. HILL*

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London SW3 6JB, U.K.

Abstract—*Two cell strains of the Yoshida sarcoma derived from ascites cells extracted from tumour-bearing animals were adapted to grow in primary suspension culture. Chlorambucil concentrations were selected which reduced the viability of both cell lines by* 90% (D_{90} *values), and established* in vitro *a 30-fold difference in sensitivity to the drug between these two cell lines* ($3{\cdot}3 \times 10^{-6}M$ *for the sensitive cells and* $10^{-4}M$ *for the resistant cells).*

The initial response to these D_{90} *concentrations of chlorambucil was quite different in the resistant cells compared with the sensitive cells. In the sensitive cells there was: (a) a reduction in cell number within* 12 *hr; and (b) an increase in cell volume, with elevated levels of DNA, RNA and protein. In contrast, in the resistant cells: (a) logarithmic growth continued for* 24 *hr after drug addition before the cell number began to fall; (b) no increase in cell volume or alteration in levels of DNA, RNA or protein per cell occurred; and (c) approximately four times as much* ^{3}*H-chlorambucil-derived radioactivity was associated with cellular nucleic acids and proteins as compared with the sensitive cells.*

These findings demonstrate that the response to lethal doses of chlorambucil (D_{90}) *values is quite different in the sensitive and resistant cell lines and suggest that: (i) the mechanism of action of the drug may be different in sensitive and resistant cells; and (ii) the nature of resistance to chlorambucil might be better understood if more studies were concentrated on resistant cell lines in approximation systems.*

INTRODUCTION

CHLORAMBUCIL has been used widely in the treatment of various malignant diseases, including chronic lymphocytic leukaemia [1], lymphomas [2], and ovarian carcinoma [3].

The reaction of alkylating agents of the mustard type with cellular DNA has been proposed as the basis for their cytotoxic action [4, 5]. However, the exact mode of action of chlorambucil is still unknown, and the explanation for the resistance to the drug remains to be determined. Earlier studies concerned with elucidating both the mode of action of chlorambucil and the mechanism of drug resistance were carried out using two strains of the Yoshida ascites sarcoma growing in rats [6, 7]. These experiments have shown that in the sensitive tumour cells, a dose of chloram-

Accepted 18 September 1974.

*Present address—where requests for reprints should be sent: Department of Cellular Pathology, Imperial Cancer Research Fund Laboratories, P.O. Box 123, Lincoln's Inn Fields, London, WC2A 3PX, U.K.

bucil of 8 mg/kg (1/2 LD_{50}) produced a condition of "unbalanced growth" as originally described in *E. coli* by Cohen and Barner [8], and subsequently verified for eukaryotic cells [9]. In this condition there was an increase in cell volume and an accumulation of DNA, RNA and protein before the tumour cell number was reduced. In the drug-resistant cells, however, these changes could not be achieved by chlorambucil treatment. The malignant cells were not killed and the host animals died at the same time as untreated tumour-bearing rats [6, 7].

Subsequently, an attempt was made to explain this lack of response in drug-resistant cells by comparing the uptake, the macromolecular binding, and the intracellular metabolism of chlorambucil in the sensitive and resistant cell lines. In contrast to the sensitive cells, the resistant cells showed a reduced uptake of chlorambucil (approx. 50%) [10], a greater degree of intracellular metabolism of the drug (approx. 2-fold) [11, 12], and a reduced amount of drug bound to macromolecules (approx. 50%) [12]. These differences, however, are not by themselves sufficient to account for the 30-fold order of resistance encountered in this experimental system [13].

Accordingly the present studies were carried out in an attempt to obtain more information on the following question: if a lethal dose of chlorambucil could be determined and achieved for both sensitive and resistant cells, are the events leading up to and associated with cell death similar in the two lines?

To answer this question, an experimental system was devised in which sensitive and resistant Yoshida ascites sarcoma cells were adapted to grow in suspension culture. Drug concentrations were then selected, *in vitro* which reduced cell viability in both cell lines to less than 10%. Using this system the events associated with the lethal effects of chlorambucil on these cells were studied, and evidence of further differences in the handling of the drug by the resistant cells was obtained. This paper presents the results of these experiments, and discusses their possible relationship to the mechanism(s) of drug resistance.

MATERIAL AND METHODS

Drugs

Chlorambucil (Leukeran). $(ClCH_2CH_2)_2NC_6H_4(CH_2)_3COOH$ was prepared and tritium-labeled chlorambucil 4-(4-di(2-chloroethyl)amino-3,5-(^{3}H)phenyl) butyric acid (specific activity 118 mCi/m mole) was synthesised by the reductive tritiation of the iodinated derivative in the Chester Beatty Research Institute, London, U.K., and checked for radiochemical purity [11]. Ninety-six per cent of the total radioactivity was associated with the authentic material.

Chemical

Thymidine-2-^{14}C (Specific activity 43·7 mCi/m mole) was obtained from the Radiochemical Center, Amersham, U.K. Thymidine was supplied by Sigma London Chemical Co. Ltd., and other chemicals were purchased from Hopkin and Williams Ltd., London, U.K., or British Drug Houses Ltd., London, U.K., AnalaR grades being used where available. Foetal bovine serum and Fisher's medium for leukaemic cells of mice were supplied by Flow Laboratories Ltd., Glasgow, U.K., and Grand Island Biological Co., California, U.S.A., respectively.

Cell cultures

For full details of tumour origin, transplantation and animal experimentation, see techniques previously used [6, 7]. The resistant tumour line was induced originally by repeated treatment of rats with low doses of drug over 30 tumour passages. The Yoshida cells were grown as short-term primary cultures under prior established conditions [14]. The sensitivity of the cells to chlorambucil was determined from dose response curves and in part by colony-forming assays, according to the method of Chu and Fisher [15]. The low cloning efficiencies of these cells, only approximately 6–10% and 20–28% for sensitive and resistant cell lines respectively, under the experimental conditions used, restricted the usefulness of this latter technique.

The cellular DNA, RNA and protein levels were measured in aliquots of cell suspension containing 5×10^6–10^7 cells, determined by haemocytometer counts, using the Scott extraction procedure [16]. DNA was estimated according to Burton [17], RNA by the orcinol procedure [18] and protein by the method of Lowry *et al.* [19]. In determining the extent of ^{3}H-drug-derived radioactivity associated with these macromolecules, aliquots of these extracts were assayed for radioactivity using a Toluene–Phosphor scintillant in a Packard Tri-Carb Liquid Scintillation Counter Model 3375 with 36% efficiency for tritium.

Cell size

An estimation of the mean cell diameter was obtained after exposure of the cells to either

chlorambucil or thymidine, from 1 μm toluidine blue sections by conventional light microscopy. Any size changes due to fixation and handling were likely to be comparable for all cell types. The diameters were measured from enlarged photographic prints and the mean value calculated from 100 measurements per culture.

Enzyme assay

Thymidine kinase activity was determined according to a modification of the method of Voytek *et al.* [20]. Cells were removed from suspension by centrifugation at $350 \times g$ for 20 min, washed in isotonic NaCl and adjusted to a concentration of approximately 10^8 cells per ml and recentrifuged. The cells were then resuspended in 0·05 M Tris–HCl buffer pH 7·8 prior to disruption by sonic oscillation at 20 kc/sec on an M.S.E. ultrasonicator at 4° for 90 sec. The cellular debris was removed by centrifugation at 20,000 *g* for 20 min at 4° and the enzyme activity of the supernatant determined. The ^{14}C-labelled components of the reaction mixture were separated, at the end of the reaction, by absorbing the nucleoside polyphosphate on PEI-cellulose thin layer chromatography plates and eluting the non-absorbed nucleoside with 0·005M Tris–HCl buffer pH 8·0.

RESULTS

Studies on growth rate and colony forming ability

The effect of chlorambucil on the growth rate of sensitive and resistant Yoshida sarcoma cells growing in suspension culture is shown in Fig. 1. Significant suppression of cell growth occurred at chlorambucil concentrations of $3{\cdot}3 \times 10^{-6}$M for sensitive cells and 10^{-4}M for resistant cells. This 30-fold difference in sensitivity to the drug was confirmed by colony-forming assays following a 24 hr exposure to the drug, where these concentrations reduced the surviving fraction of cells to less than 10% (i.e., D_{90} values). These results are shown in Table 1 and confirm those previously obtained using an *in vivo–in vitro* bioassay technique [13]. When both cell lines were exposed to D_{90} drug concentrations, the sensitive cells continued in logarithmic growth for 12 hr before the number of cells per culture began to fall. In resistant cells, no reduction in cell number occurred before 24 hr. This delayed effect of the drug has also been demonstrated after the administration of chlorambucil to tumour-bearing animals [6, 7].

Exposure in culture of the sensitive cells to concentrations of chlorambucil greater than 10^{-5}M caused *immediate* and extensive cell lysis and disintegration. However, these changes were not seen either by light or electron-microscopy, when resistant cells were exposed to concentrations of chlorambucil up to 2×10^{-4}M.

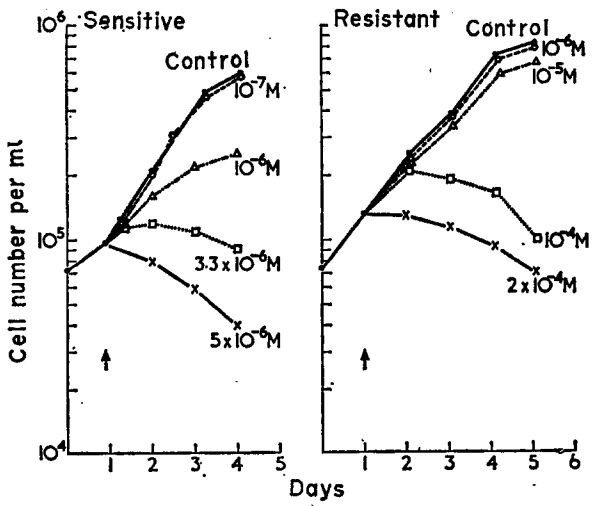


Fig. 1. Growth curves of sensitive and resistant Yoshida sarcoma cell lines exposed continuously to various concentrations of chlorambucil.

*Table 1. Effects of 24 hr exposure to a range of chlorambucil concentrations on the colony-forming ability of drug-sensitive and drug-resistant Yoshida cells growing in suspension culture**

Chlorambucil concentration	Percentage surviving fraction† Sensitive cells	Resistant cells
Zero	100	100
10^{-7}	100	—‡
10^{-6}	52	100
$3{\cdot}3 \times 10^{-6}$	7	98
5×10^{-6}	1	—
10^{-5}	—	93
10^{-4}	—	9
2×10^{-4}	—	1

*Conditions of cell culture were as described in MATERIAL AND METHODS section.

†The surviving fraction was determined from colony-forming assays carried out as described in MATERIAL AND METHODS section using the technique of Chu and Fisher [15]. Sufficient cells were plated out to result in approx. 100 colonies/tube in the control cells after allowing for the different plating efficiencies of each cell line (*i.e.*, 1000 and 400 cells/tube for sensitive and resistant cells respectively).

‡Not estimated.

Each value represents the mean of 8 determinations and the overall scatter never exceeded 30%.

Biochemical studies of the effects on cell size, nucleic acid and protein metabolism

(i) *Estimation of DNA, RNA and protein following exposure to chorambucil.* The variation in content of DNA, RNA and protein in sensitive and resistant tumour cells following exposure to the D_{90} drug concentrations is shown in Fig. 2. No significant change occurred in any of these measurements in the resistant cells. In the sensitive cells, however, the cellular RNA and protein levels doubled and their DNA content increased by 50% 24 hr after the drug was added to the cultures. Further increases occurred in all these parameters by 48 hr.

(ii) *Estimations of cell size and protein content following exposure to D_{90} chlorambucil concentrations or 1 mM thymidine.* The results in Table 2 show that chlorambucil had no effect on the cell size or protein content of resistant cells, but there was a concomitant increase in mean cell diameter of the sensitive cells as the protein content per cell increased. However, the exposure of sensitive and resistant cells to these cytotoxic concentrations of thymidine which result in cell death, was accompanied by

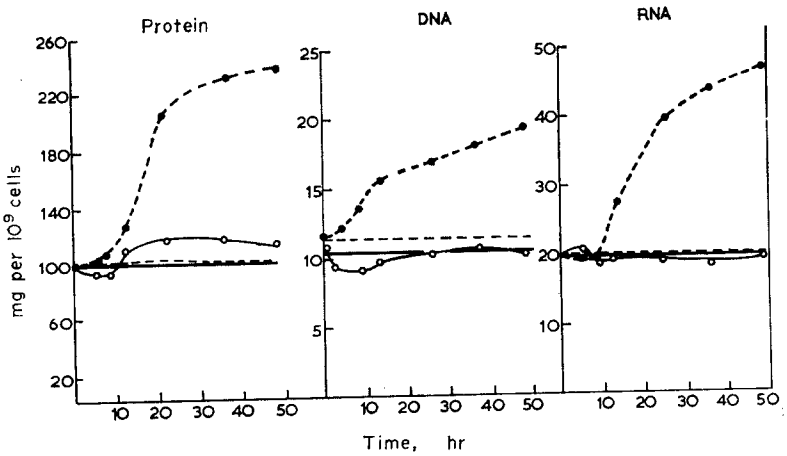


Fig. 2. Effects of chlorambucil treatment (D_{90} concentrations) on the levels of DNA, RNA and protein in sensitive and resistant cell lines of the Yoshida sarcoma growing in suspension culture. D_{90} concentrations:
for sensitive cells $3{\cdot}3 \times 10^{-6}$M
for resistant cells 10^{-4}M.
●– – –● *sensitive chlorambucil-treated cells*
– – – – – *sensitive untreated cells*
○———○ *resistant chlorambucil-treated cells*
——————— *resistant untreated cells*
Each point represents the mean of at least 6 determinations. Overall scatter ≯ 10%.

Table 2. Estimation of the mean cell diameter (MCD) and the levels of protein of sensitive and resistant lines of Yoshida sarcoma cells after exposure in culture to D_{90} chlorambucil concentrations or thymidine (10^{-3}M)

Time of exposure in culture (hr)	Sensitive cells				Resistant cells			
	chlorambucil ($3{\cdot}3 \times 10^{-6}$M)		thymidine (10^{-3}M)		chlorambucil (10^{-4}M)		thymidine (10^{-3}M)	
	MCD (μm)	Protein level/ 10^9 cells (mg)	MCD (μm)	Protein level/ 10^9 cells (mg)	MCD (μm)	Protein level/ 10^9 cells (mg)	MCD (μm)	Protein level 10^9 cells (mg)
0	9·3 ± 0·6	108 ± 6	9 3 ± 0·6	108 ± 6	9·1 ± 0·7	102 ± 6	9·1 ± 0·7	102 ± 6
24	11·8 ± 0 5	188 ± 5	13 2 ± 0·3	193 ± 3	9·7 ± 0·3	107 ± 8	14·1 ± 0·5	210 ± 8
48	13·5 ± 0·5	228 ± 5	12·2 ± 0·4	218 ± 4	10·1 ± 0·4	113 ± 5	13·7 ± 0·3	241 ± 5

The standard deviations are evaluated over 6 values per point.
For each culture 100 diameters were measured and the MCD calculated.

giant cell formation and elevated levels of protein per cell in both cell lines, consistent with the phenomenon of "unbalanced growth" [8, 9].

(iii) *Thymidine kinase estimations.* Figure 3 shows the effect on thymidine kinase activity following exposure of both cell lines to chlorambucil. Again, a difference in response to D_{90} drug concentrations was observed between the two cell lines. In the sensitive cells, after treatment with $3{\cdot}3 \times 10^{-6}$M drug there was an increased activity of thymidine kinase, reaching a level twice that of the control value by 24 hr. The resistant cells, in contrast, showed an almost linear fall in thymidine kinase activity which decreased to 40% of the control values 24 hr after the addition of 10^{-4}M drug. A similar fall occurred when resistant cells were exposed to a chlorambucil concentration of $3{\cdot}3 \times 10^{-6}$M (D_{10} value). In this experiment, however, the reduction in enzyme activity was transient, being most marked between 4–8 hr after the drug was added to the cultures (only 40% of control values) and then returning to control levels. This finding is consistent with the failure of this drug concentration to reduce significantly either the viability or growth rate of resistant cells.

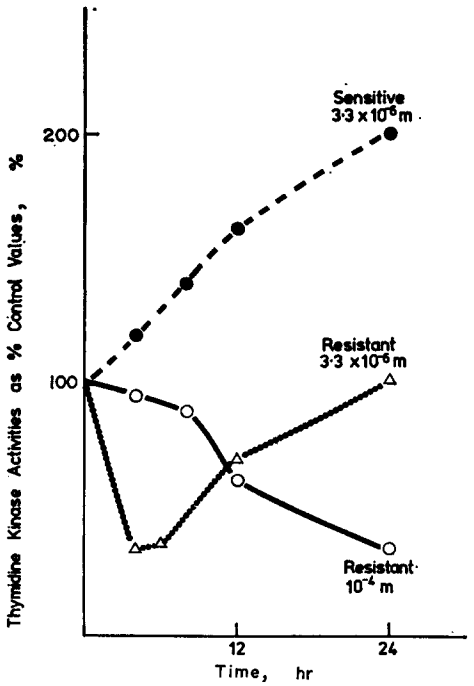


Fig. 3. Effects of chlorambucil treatment on the levels of thymidine kinase activity of sensitive and resistant cell lines of the Yoshida sarcoma growing in suspension culture. Sensitive cells (● – – – ●) were exposed to $3{\cdot}3 \times 10^{-6}$M *chlorambucil (D_{90} concentration). Resistant cells were exposed to* $3{\cdot}3 \times 10^{-6}$M *(D_{90} concentration) (△ . . . △) and* 10^{-4}M *(D_{10} concentration) (○———○) concentrations of chlorambucil. The activity of thymidine kinase as a % control values is plotted as a function of time in hours after exposure of the cells to the drug. Control values of enzyme activity, expressed as n moles thymidine converted/mg protein/minute, are 2·9 and 2·3 for sensitive and resistant cells respectively. Each point represents the mean of 6 determinations. Overall scatter at each point* ≯ 15%.

Studies of cellular uptake and binding of ^{3}H-*chlorambucil*

Figure 4 shows the extent of binding of ^{3}H-chlorambucil-derived radioactivity to the nucleic acids and proteins of the tumour cells following exposure to D_{90} chlorambucil concentrations. For equivalent cell kill, the initial rate and final extent of ^{3}H-labelling of these macromolecules was higher in resistant cells compared with the sensitive cells. This finding was consistent with the previous observation that at a concentration of 10^5 cells per ml, uptake and binding of chlorambucil to macromolecules increased linearly with drug concentration for each cell line [11]. In the sensitive cells, peak binding of ^{3}H-chlorambucil-derived radioactivity to proteins and nucleic acids occurred 12 hr after drug addition as opposed to 24 hr in the resistant cells. This period of peak binding coincided with the time at which logarithmic growth of the cells ceased and the cell number began to fall (see Fig. 1).

Table 3 shows the extent of binding of ^{3}H-chlorambucil-derived radioactivity to DNA, RNA and protein following a 12 hr exposure of the cells to various concentrations of the drug. These illustrate the following points: (a) to achieve a similar reduction in cell viability, more drug-derived radioactivity is associated with DNA (7 times), RNA (3 times) and protein (2·5 times) in the resistant cells compared with the sensitive cells; (b) when exposed to the same drug concentrations ($3{\cdot}3 \times 10^{-6}$M), the sensitive cells accumulated more ^{3}H-labelled material (approximately 2–3 times) into these macromolecules than the resistant cells. This confirms earlier work which showed a reduced uptake of chlorambucil by resistant cells both *in vivo* and *in vitro* [10, 12, 21]; and (c) an equivalent degree of binding of ^{3}H-chlorambucil-derived radioactivity to cellular nucleic acids and proteins may be achieved using concentrations of $3{\cdot}3 \times 10^{-6}$M for sensitive cells and 10^{-5}M for resistant cells. However, this extent of binding is associated with the death of the sensitive cells, whereas the viability of the resistant cells is only slightly reduced (by less than 10%).

DISCUSSION

The data presented above are concerned only with measurements of tritium concentra-

tions in the cells and no attempt has been made in these experiments to identify any of the chlorambucil metabolites with which the tritium label may be associated. A knowledge of the complete metabolism of chlorambucil is lacking, but a study concerned with identifying these metabolites is under consideration at this Institute.

These results show that chlorambucil-resistant Yoshida sarcoma cells growing in primary suspension culture can be killed provided they are exposed to concentrations of chlorambucil 30 times greater than the dose which reduces viability of drug-sensitive cells by 90%. This order of resistance is similar to that observed previously using the *in vivo–in vitro* bioassay technique for these ascites cells growing in tumour-bearing animals [13].

However, there are marked differences in the responses of the resistant cells compared to the sensitive cells when both lines are exposed in culture to D_{90} chlorambucil concentrations. The sensitive cells, before they die, go through an orderly sequence of events involving increased levels and rates of synthesis of various proteins and nucleic acids. They show an increase in cell volume and elevated levels of DNA, RNA and protein. These latter changes have been attributed to an enhanced biosynthesis of DNA together with a stabilization or reduced turnover of RNA and protein [22]. The activity of thymidine kinase is also elevated in these cells (Fig. 3). The activity of this enzyme has been linked both to the extent of DNA synthesis and also to the rate of cell division [23]. These results are compatible

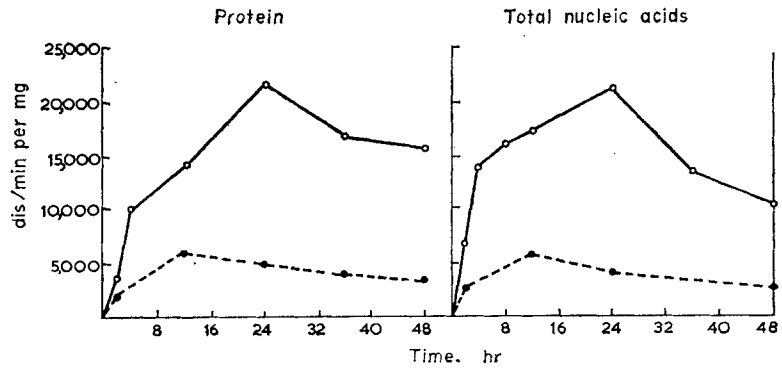


Fig. 4. *Distribution of radioactivity between nucleic acids and protein (isolated by the Scott procedure) from sensitive and resistant Yoshida sarcoma cells growing in suspension culture, after exposure to* D_{90} *concentrations of* ^{3}*H-chlorambucil.* D_{90} *concentrations:*
for sensitive cells $3{\cdot}3 \times 10^{-6}$M
for resistant cells 10^{-4}M
○———○, *resistant cells;* ●– – –●, *sensitive cells. Each point represents the mean of* 6 *determinations. Overall scatter at each point* ≯ 10%.

Table 3. *Extent of binding of* ^{3}*H-chlorambucil-derived radioactivity to molecules extracted from sensitive and resistant cell lines of the Yoshida sarcoma, after* 12 *hr exposure to various concentrations of chlorambucil*

Cell line	Drug concentration (M)	Protein	DNA dis/min mg^{-1}	RNA	Percentage cell survival*
Sensitive	10^{-6}	2102	79	1900	40
Sensitive	$3{\cdot}3 \times 10^{-6}$	6000	230	5680	10
Resistant	10^{-4}	1400	1820	15175	10
Resistant	10^{-5}	8796	722	8700	90
Resistant	$3{\cdot}3 \times 10^{-6}$	1466	116	3035	100

DNA, RNA and protein were extracted by the Scott procedure [16] and assayed as described in the MATERIAL AND METHODS section. Each value represents the mean of 4 determinations. Overall scatter ≯ 15%.
*Percentage survival was determined from dose response curves and in part by colony-forming assays, after 24 hr exposure of the cells to the drug, as described in the MATERIAL AND METHODS section.

with previous studies following administration of chlorambucil to rats bearing the drug-sensitive tumour, which have also shown certain elevated enzyme activities and rates of nucleic acid biosynthesis, together with alterations in cell size and chromatin structure [6, 7, 22, 24–27].

In contrast, however, in drug-resistant cells the same sequence of biochemical changes between drug addition (D_{90} concentrations) and cell death, did not occur. There was no significant alteration in DNA, RNA or protein levels (Fig. 2 and control Table 2). This raised the possibility that resistant cells might be incapable of exhibiting this phenomenon of "unbalanced growth" (involving the continued enlargement of the cell due to accumulation of RNA and protein, in the absence of DNA synthesis, prior to cell death), shown by the sensitive cells. However, it has been demonstrated that this characteristic condition can be induced in both cell types following prolonged exposure of the cells in culture to 1 mM thymidine (Table 2).

Therefore, these results suggest that the way in which chlorambucil kills resistant cells may be quite different from the way in which the drug kills sensitive cells.

Another difference observed between the two cell lines was that for a dose of drug which produced a 2 log cell kill in each cell line, approximately four times more ^{3}H-chlorambucil-derived radioactivity was bound to the nucleic acids and proteins of the resistant cells compared with the sensitive cells. There is, therefore, no evidence to suggest that a critical amount of drug-derived radioactivity has to be bound intracellularly to kill both sensitive and resistant cells. The fact that resistant cell bind 4 times more ^{3}H-chlorambucil-derived radioactivity when the input was 33 times that for the sensitive cells emphasizes the previously reported reduced ability of these resistant cells to transport the drug [10, 12, 21]. Furthermore, chlorambucil appeared to have an earlier, more rapid effect on the sensitive than on the resistant cells, with peak binding of the ^{3}H-drug-derived radioactivity to macromolecules occurring 12 hr earlier in the sensitive than in the resistant cell (Fig. 4) coinciding with the time at which logarithmic growth ceased in either cell line (Fig. 1).

It is concluded, therefore, that responses to lethal doses of chlorambucil are quite different in the sensitive and resistant cell lines used in this study, since the sequence of biochemical events occurring after drug addition and leading up to cell death is markedly different. This difference in the initial responses of these two cell lines to chlorambucil may account, in part, for the resistance experienced. Therefore, in attempting to design effective methods for killing alkylating resistant cells, it may be important to bear in mind that the resistant cells handle the drug in a different manner from the sensitive cells. Perhaps the nature of this resistance would become clearer if more studies were concentrated on resistant cell in approximation systems.

Acknowledgement—The author wishes to thank Drs. L. A. Price and J. H. Goldie for much helpful advice and discussion in the preparation of the manuscript, Professor A. B. Foster and Dr. M. Jarman for the supply of ^{3}H-labelled chlorambucil, and Mr. M. S. C. Birbeck, Mr. D. Robertson and other members of the Electron-microscopy Department for their help in the preparation of light micrographs. This work was supported by a Wellcome Foundation Fellowship.

REFERENCES

1. D. T. Kaung, R. M. Whittington and M. E. Patno, Treatment of chronic lymphocytic leukaemia with chlorambucil (NSC 3088) and cyclophosphamide (NSC 26271). *Cancer Chemother. Rep.* **39,** 41 (1964).
2. D. T. Kaung, R. M. Whittington, H. Spencer and M. E. Patno, Comparison of chlorambucil and streptonigrin (NSC 45383) in the treatment of malignant lymphomas. *Cancer* **23,** 1280 (1969).
3. E. Wiltshaw, Chlorambucil in the treatment of primary adenocarcinoma of the ovary. *J. Obstet. Gynaec. Brit. Cwlth.* **72,** 596 (1965).
4. W. C. J. Ross, The chemistry of cytotoxic alkylating agents. *Advanc. Cancer Res.* **1,** 397 (1953).
5. P. Brookes and P. D. Lawley, The action of mono- and difunctional alkylating agents with nucleic acids. *Biochem. J.* **80,** 496 (1961).
6. K. R. Harrap and B. T. Hill, The selectivity of action of alkylating agents and drug resistance. Part I: Biochemical changes occurring in sensitive and resistant strains of the Yoshida ascites sarcoma following chemotherapy. *Brit. J. Cancer* **23,** 210 (1969).

7. K. R. HARRAP and B. T. HILL, The selectivity of action of alkylating agents and drug resistance. Part II: A comparison of the effects of alkylating drugs on growth inhibition and cell size in sensitive and resistant strains of the Yoshida ascites sarcoma. *Brit. J. Cancer* **23,** 227 (1969).
8. S. S. COHEN and H. D. BARNER, Studies on unbalanced growth in *E. coli*. *Proc. nat. Acad. Sci.* **40,** 885 (1954).
9. R. R. RUEKERT and G. C. MUELLER, Studies on unbalanced growth in tissue culture. I. Induction and consequence of thymidine deficiency. *Cancer Res.* **20,** 1584 (1960).
10. K. R. HARRAP and B. T. HILL, The selectivity of action of alkylating agents and drug resistance—III: The uptake and degradation of alkylating drugs by Yoshida ascites sarcoma cells *in vitro*. *Biochem. Pharmacol.* **19,** 209 (1970).
11. B. T. HILL, M. JARMAN and K. R. HARRAP, The selectivity of action of alkylating agents and drug resistance. Part IV: The synthesis of tritium-labelled chlorambucil and a study of its cellular uptake by drug-sensitive and drug-resistant strains of the Yoshida ascites sarcoma cells *in vitro*. *J. Med. Chem.* **14,** 614 (1970).
12. B. T. HILL, Studies on the transport and cellular distribution of chlorambucil in the Yoshida ascites sarcoma. *Biochem. Pharmacol.* **21,** 495 (1972).
13. K. R. HARRAP and M. E. FURNESS, The cytotoxicity of chlorambucil and its associated effects on NAD metabolism. *Europ. J. Cancer* **9,** 343 (1973).
14. B. T. HILL, I. D. C. DOUGLAS and P. L. GROVER. The increased antitumour activity of chlorambucil following pre-treatment with inducers of drug-metabolising enzymes. *Biochem. Pharmacol.* **22,** 1083 (1973).
15. M. CHU and G. A. FISCHER, The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukaemic cells (L5178Y). *Biochem. Pharmacol.* **17,** 753 (1968).
16. J. P. SCOTT, A. P. FRASCASTORO and E. B. TAFT, Studies in histochemistry: I Determination of nucleic acids in microgram amounts of tissue. *J. Histochem. Cytochem.* **4,** 1 (1956).
17. K. BURTON, A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315 (1956).
18. A. M. BROWN. Determination of pentose in the presence of large quantities of glucose. *Arch. Biochem.* **11,** 269 (1946).
19. O. H. LOWRY, N. J. ROSEBOROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin Phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
20. P. VOYTEK, P. K. CHANG and W. M. PRUSOFF, Purification of deoxythymidine kinase by preparative disc gel electrophoresis and the effects of various halogenated nucleoside triphosphates on its enzymatic activity. *J. biol. Chem.* **246,** 1432 (1971).
21. B. T. HILL and P. G. RICHES, The absorption, distribution and excretion of ^{3}H-chlorambucil in rats bearing the Yoshida ascites sarcoma. *Brit. J. Cancer* **25,** 831 (1971).
22. B. T. HILL and K. R. HARRAP, The effect of chlorambucil on the incorporation of tritiated precursors into the nucleic acids and proteins of Yoshida ascites sarcoma cells. *Neoplasma (Bratisl.)* **17,** 485 (1970).
23. T. HASHIMOTO, T. ARIMA, H. OKUDA and S. FUJII, Purification and properties of deoxythymidine kinases from the Yoshida sarcoma. *Cancer Res.* **32,** 67 (1972).
24. K. R. HARRAP, R. C. JACKSON and B. T. HILL, Some effects of chlorambucil on enzymes of glutathione metabolism in drug-sensitive and -resistant strains of the Yoshida ascites sarcoma. *Biochem. J.* **111,** 603 (1969).
25. B. T. HILL, P. G. RICHES and M. E. FURNESS, Effects of chlorambucil treatment on the rates of respiration and glycolysis in drug- sensitive and -resistant strains of the Yoshida ascites sarcoma. *Biochem. Pharmacol.* **19,** 2973 (1970).
26. B. T. HILL and M. H. N. TATTERSALL, The effect of chlorambucil on thymidine utilization by Yoshida ascites tumour cells. *J. nat. Cancer Inst.* **51,** 1387 (1973).
27. P. G. RICHES and K. R. HARRAP, Some effects of chlorambucil on the chromatin of Yoshida ascites sarcoma cells. *Cancer Res.* **33,** 389 (1973).

Europ. J. Cancer Vol. 11, pp. 17–22. Pergamon Press 1975 Printed in Great Britain

Carbamylhydrazine Hydrochloride as a Lung and Blood Vessel Tumour Inducer in Swiss Mice

BELA TOTH†, HIDESUKE SHIMIZU‡ and JAMES ERICKSON†

† *The Eppley Institute for Research in Cancer, University of Nebraska Medical Center, 42nd Street and Dewey Avenue, Omaha, Nebraska* 68105, *U.S.A.*

‡ *Jikei University School of Medicine, Department of Public Health, 3-chome, Nishi-shinbashi Minato-Ku, Tokyo, Japan*

Abstract—*Administration of* 0·0625% *carbamylhydrazine hydrochloride in the drinking water of* 6*-week-old, randomly bred Swiss albino mice for the remainder of their lifetimes, enhanced the development of tumors of the lungs and blood vessels. As compared with the untreated controls, the incidence of lung tumors rose from* 21 *to* 50% *in the females and from* 23 *to* 30% *in the males, while the incidence of blood vessel tumors increased from* 5 *to* 18% *in females, but not in the males.*

The study thus proves for the first time the tumorigenicity of this important industrial-laboratory chemical. The tumor-inducing capabilities of substituted hydrazines as a class and their possible environmental significance are discussed.

INTRODUCTION

TUMORIGENESIS studies with substituted hydrazines are aimed at revealing the environmental significance of these chemicals in cancer causation and at the possibility of discovering their mechanisms of action. The studies have been systematically pursued since 1968. The first stage of each experiment has consisted of separate toxicity studies in mice and hamsters [1, 2]. Afterwards, the compounds were given *ad libitum* to both species at the maximum tolerated dose in drinking water daily for life.

So far the following hydrazine derivatives were found to be tumorigenic in mice in this laboratory: methyl- [3], 1,2-dimethyl- [4], 1,1-dimethyl- [5], benzoyl- [6], ethyl- [7], *n* butyl- [8], *n* amyl- [9], 1-carbamyl-2-phenyl- [10], and 1-acetyl-2-isonicotinoyl- [11] hydrazines.

Accepted 18 September 1974.

*This study was supported by Public Health Service contract PH43–NCI–E–68–959 from the National Cancer Institute NIH, USA. Dr. B. Toth is recipient of Public Health Service Research Career Development Award KO5–42, 552 from the National Cancer Institute.

The present investigation is part of the broad program in this field and records for the first time the tumorigenicity in randomly bred Swiss mice of carbamylhydrazine hydrochloride administered orally in drinking water for life.

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 Wayne lab-blox diet in regular pellets (Allied Mills, Inc., Chicago, Illinois) and tap water or the test solution *ad libitum* as described below.

The chemical used was carbamylhydrazine hydrochloride, certified, (CH, semi-carbazide hydrochloride), Fig. 1., MW: 111·53, MP: 175–177°, obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

Toxicity study was carried out prior to the chronic experiment. Five dose levels of CH, namely, 1, 0·5, 0·25, 0·125, and 0·0625% were administered in the drinking water daily for 35 days to Swiss mice. By taking into account

four parameters; survival rates, body weights, chemical consumption figures and histological changes, the 0·0625% was found to be suitable for the lifelong treatment. This toxicity technique was developed in this laboratory and published recently [1].

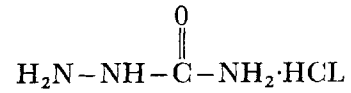


Fig. 1. *Chemical structure of carbamylhydrazine hydrochloride.*

The solutions were prepared thrice weekly, and the total consumption of water containing CH 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 chronic experimental groups and the controls as follows:

Group 1

CH was dissolved in the drinking water as a 0·0625% solution and was given for the life span of 50 female and 50 male mice which were 6 weeks old (47 days) at the beginning of the experiment. The average daily consumption per animal of water containing CH was 7·8 ml for the females and 5·3 ml for the males. Therefore, the average daily intake of CH was 3·3 mg for a female and 4·8 mg for a male.

Group 2

As an untreated control, 100 female and 100 male mice were kept and observed from weaning time (5 weeks of age).

The experimental and control animals were checked carefully and weighed at weekly intervals and the gross pathological changes were recorded. The animals were either allowed to die or were killed with ether when found in poor condition. Complete necropsies were performed on all animals. All organs were examined macroscopically and were fixed in 10% buffered formalin. Histological studies were done on the liver, spleen, kidney, bladder, thyroid, heart, pancreas, testis, brain, nasal turbinale and at least four lobes of the lungs of each mouse as well as on those organs showing gross pathological changes. Sections from these tissues were stained routinely with hematoxylin and eosin.

In tabulating the results, the determination of the latent period was made from the date of birth of animal to the time of death.

RESULTS

The survival rates after weaning are recorded in Table 1. It appears that the treatment shortened somewhat the survival time when compared with the untreated controls.

The number, percentage of animals with tumors and their age at death (latent periods) are summarized in Table 2. In the following, the two most important neoplasms are described in detail:

Lung tumors

Of the treated females, 25 (50%) developed 37 such neoplasms. Of these, 20 mice had 31 adenomas, and 5 had 6 adenocarcinomas. The average age at death was 89 weeks; the first was found at the 50th week, and the last at the 111th week of age. In the treated males, 15 (30%) developed 21 lung tumors. Of these, 11 mice had 12 adenomas, 3 had 5 adenocarcinomas and 1 had an adenoma and 3 adenocarcinomas. The average age at death was 75 weeks; the first was observed at the 20th week and the last at the 111th week of age.

Grossly and histologically, these tumors appeared similar to those described earlier in this mouse strain in this laboratory [10, 12]. The diagnostic criteria of an adenoma were the well demarcated, nodular lesion composed of proliferating neoplastic, alveolar cells type B, exhibiting adenoid structures. The cells were cuboidal or columnar with dark nuclei and acidophilic cytoplasms. They were supported by thin fibrous stroma with small blood vessels in it. In case of adenocarcinoma, the composing cells showed irregular growth pattern, they usually invaded the surrounding tissues including blood vessels and bronchi. The sizes and shapes of the cells varied, often many mitoses were seen.

Table 1. *Treatment and survival rate in carbamylhydrazine HCL (CH)-treated and control Swiss mice*

Group	Treatment	Initial no. and sex	No. of survivors (age in weeks)													
			10	20	30	40	50	60	70	80	90	100	110	120	130	140
1	0·0625% CH in drinking water daily for life	50 ♀	50	50	49	48	47	42	31	20	17	10	3	2	—	
		50 ♂	49	48	43	37	32	25	14	7	4	2	1	1	—	
2	Untreated Control	100 ♀	100	100	97	95	91	88	81	71	57	36	14	5	1	—
		100 ♂	100	100	96	93	88	83	74	65	48	27	12	7	1	—

Table 2. Tumor distribution in carbamylhydrazine HCl (CH)-treated and control Swiss mice

Group	Treatment	Effective No. and Sex	Animals with: Lung tumors No.	%	Latent periods*	Blood vessel tumors No.	%	Latent periods*	Other Tumors+
1	0·0625% CH in drinking water daily for life	50 ♀	25	50	89 (50–111)	9	18	92 (74–107)	5 Malignant lymphomas (54, 73, 75, 91, 107) 5 Malignant histiocytoma (104) 1 Sarcoma of glandular stomach (52) 1 Papilloma of forestomach (105)
		50 ♂	15	30	75 (20–111)	3	6	81 (64–93)	2 Hepatomas (57, 71) 1 Hibernoma (111) 1 Malignant lymphoma (79)
2	Untreated Control	99 ♀	21	21	95 (60–122)	5	5	113 (97–130)	24 Malignant lymphomas (31, 36, 68, 69, 71, 79, 80, 80, 90, 94, 96, 97, 101, 102, 103, 105, 106, 109, 115, 118, 122, 123, 130) 2 Adenocarcinomas of breast (73, 93) 2 Adenocarcinomas of ovaries (104, 106) 1 Adenoma of thyroid (116) 1 Papilloma of skin (109) 1 Malignant histiocytoma (58) 1 Papillary adenoma of ovary (103) 1 Fibrosarcoma, subcutaneous (44) 1 Papilloma of esophagus (130) 1 Leiomyosarcoma of uterus (81) 1 Adenoma of glandular stomach (115)
		99 ♂	23	23	92 (53–125)	6	6	88 (65–105)	12 Malignant lymphomas (40, 57, 76, 88, 93, 94, 95, 97, 102, 107, 116, 126) 1 Fibrosarcoma, subcutaneous (82) 1 Adenocarcinoma of duodenum (113) 1 Malignant histiocytoma (101) 1 Carcinoma of skin (122) 1 Adrenocortical adenoma (130) 1 Papilloma of esophagus (63) 1 Papilloma of forestomach (63)

* Age in weeks (average and range)
+ Latent period given in parentheses.

Blood vessel tumors

Of the treated females, 9 (18%) developed blood vessel tumors. Of these, 3 had angiosarcomas in livers, 2 had angiosarcomas in ovaries, 2 had angiomas in livers, 1 had an angioma in ovary and 1 had an angioma in lymph node. The average age at death was 92 weeks; the first was found at the 74th week and the last at the 107th week of age. In the treated males, 3 (6%) developed such lesions. Of these, 2 had angiosarcomas in livers at the 64th and 87th weeks and 1 had angiosarcomas in liver and lungs at the 93rd week of age.

Macroscopically and histologically, the observed blood vessel lesions were similar to those found earlier in the variously treated animals in this laboratory and described in detail [4, 5]. Our diagnostic criterion of angioma was the development of several layers of proliferating endothelial cells. A lesion to be considered an angiosarcoma was composed of highly anaplastic endothelial cells forming vascular spaces and invading the surrounding tissues and sometimes giving metastases to other organs.

Other tumors

A few other types of tumors were also observed which are shown in Table 2. Because they occurred in low incidences their appearances can not be attributed to the treatment.

Tumors in untreated controls

The incidences and the detailed descriptions of lesions which appeared spontaneously in the controls were published recently [10]. Their incidences, however, are included in Table 2 for comparison. The incidences of malignant lymphomas were significantly lower in treated mice than in the untreated controls. This was due apparently to the fact that the treatment shortened the survival time, and the malignant lymphomas usually develop in older ages.

DISCUSSION

The present investigation showed that the continuous lifetime administration to Swiss mice of 0·0625% carbamylhydrazine hydrochloride in drinking water significantly enhanced tumor development in the lungs and blood vessels. The incidence of lung neoplasms rose from 22 to 40% while the incidence of blood vessel tumors increased from 5 to 12% as compared with the controls. Histopathologic examination of the induced lesions revealed that they were adenomas and adenocarcinomas of lungs and angiomas and angiosarcomas of blood vessels. Thus this study demonstrates for the first time, the tumorigenicity of carbamylhydrazine hydrochloride in mice.

Studies on the tumorigenic potentialities of substituted hydrazines began in 1962, when it was shown that the base compound hydrazine sulfate induced lung neoplasms in mice [13]. Subsequently, a series of hydrazine derivatives were investigated in the various laboratories for tumor-inducing capabilities. These works clearly demonstrated that these chemicals are indeed powerful tumor-producing substances in the intestines, brain, lungs, blood vessels, liver, breasts, kidneys, etc. of mice, hamsters, and rats. As of today, we know 19 derivatives of hydrazine which have proved tumorigenic in animals [14–22]. These investigations were especially encouraged because these synthetic chemicals were extensively present in the environment and used in industry, agriculture, and medicine. The most notable, which were or are in use include: hydrazine, monomethylhydrazine and 1,1-dimethylhydrazine in rocket fuels [23], 2-hydroxyethylhydrazine for the ripening of pineapples and other plants [24, 25], phenylhydrazine for treatment of policythemia vera [23], 1-isonicotinyl-2-isopropylhydrazine as both an antidepressant and antituberculous drug [26, 27], and N-isopropyl-α-(2-methylhydrazino)-*p*-toluamide as an anti-neoplastic agent [28]. The estimated annual carbamylhydrazine production in the USA alone is 50 tons [29], and is or can be used in tobacco smoke filters [30], polymer stabilization [31], rubber vulcanization [32] and as a flame retardant [33], depressant in propellants [34], and laboratory reagent [23].

Finally, it may be of interest to note the tissue specific tumorigenicity of the substituted hydrazines all administered orally to mice. So far, five out of ten have induced tumors of the lungs and blood vessels. These are the symmetrical and unsymmetrical dimethyl-, ethyl-, *n* amyl-, and carbamyl- hydrazines. Also, the unsymmetrical dimethyl-hydrazine induced tumors in kidney and liver [4, 5, 7 9]. Four of them inducing only lung tumors included hydrazine monomethyl-, *n* butyl-, and 1-carbamyl-2-phenyl-hydrazines [3, 8, 10]. Furthermore, benzoylhydrazine produced tumors of the lungs and lymphoreticular tissue [6]. Therefore, it seems that these compounds exert some sort of organ- and tissue-specific neoplastic action. Additional studies now in progress may clarify this particular aspect of our objectives.

Acknowledgment—The authors are thankful to Miss Nancy Marren for her technical assistance in the experiment.

REFERENCES

1. B. Toth, A toxicity method with calcium cyclamate for chronic carcinogenesis experiments. *Tumori* **58,** 137 (1972).
2. H. Shimizu and B. Toth, Species dependent toxicity of six substituted hydrazines. *Res. Comm. chem. Pathol. Pharm.* **7,** 671 (1974).
3. B. Toth, Hydrazine, methylhydrazine and methylhydrazine sulfate carcinogenesis in Swiss mice. Failure of ammonium hydroxide to interfere in the development of tumors. *Int. J. Cancer* **9,** 109 (1972).
4. B. Toth and R. B. Wilson, Blood vessel tumorigenesis by 1,2-dimethylhydrazine dihydrochloride (symmetrical). Gross, light and electron microscopic descriptions. I. *Amer. J. Path.* **64,** 585 (1971).
5. B. Toth, 1,1-Dimethylhydrazine (unsymmetrical) carcinogenesis in mice. Light microscopic and ultrastructural studies on neoplastic blood vessels. *J. nat. Cancer Inst.* **50,** 181 (1973).
6. B. Toth, Benzoylhydrazine carcinogenesis in lungs and lymphoreticular tissues of Swiss mice. *Europ. J. Cancer* **8,** 341 (1972).
7. H. Shimizu, D. Nagel and B. Toth, Ethylhydrazine hydrochloride as a tumor inducer in mice. *Int. J. Cancer* **13,** 500 (1974).
8. B. Toth, H. Shimizu and D. Nagel, Tumor induction studies with ethyl-, *n* butyl- and 1-carbamyl-2-phenylhydrazines. Abstract. *Proc. Amer. Ass. Cancer Res.* **15,** 23 (1974).
9. H. Shimizu, D. Nagel and B. Toth, Tumor induction study with *n* amylhydrazine hydrochloride in Swiss mice. *Brit. J. Cancer* (Submitted for publication).
10. B. Toth and H. Shimizu, 1-Carbamyl-2-phenylhydrazine tumorigenesis in Swiss mice. Morphology of lung adenomas. *J. nat. Cancer Inst.* **52,** 241 (1974).
11. B. Toth and H. Shimizu, Lung carcinogenesis with 1-acetyl-2-isonicotinoylhydrazine, the major metabolite of isoniazid. *Europ. J. Cancer* **9,** 285 (1973).
12. B. Toth, P. N. Magee and P. Shubik, Carcinogenesis study with dimethylnitrosamine in orally treated adult and subcutaneously injected newborn BALB/c mice. *Cancer Res.* **24,** 1712 (1964).
13. C. Biancifiori and R. Ribacchi, Pulmonary tumours in mice induced by oral isoniazid and its metabolites. *Nature (Lond).* **194,** 488 (1962).
14. D. B. Clayson, C. Biancifiori, U. Milia and F. E. Giornelli-Santilli, The induction of pulmonary tumours in Balb/Cb/Se mice by derivatives of hydrazine. In *Lung Tumors in Animals.* Edited by L. Severi, p. 869. Proc. Quadrenn. Conf. on Cancer, Perugia, Italy (1966).
15. H. Druckrey, R. Preussman, F. Matzkies, and S. Ivankovic, Selective Erzeugung von Darmkrebs bei Ratten durch 1,2-Dimethylhydrazine. *Naturwissenschaften* **54,** 285 (1967).
16. F. J. C. Roe, G. A. Grant and D. M. Millican. Carcinogenicity of hydrazine and 1,1-dimethylhydrazine for mouse lung. *Nature (Lond).* **216,** 375 (1967).
17. M. G. Kelly, R. W. O'Gara, S. T. Yancey, K. Gadekar, C. Botkin and V. T. Oliverio, Comparative carcinogenecity of *N*-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide HCl (Procarbazine Hydrochloride): Its degradation products, other hydrazines and isonicotinic acid hydrazide. *J. nat. Cancer Inst.* **42,** 337 (1969).
18. H. Osswald and F. W. Krüger, Die carcinogene Wirkung von 1,2-Dimethylhydrazin beim Goldhamster. *Arzneimittel-Forsch.* **19,** 1891 (1969).
19. B. Wiebecke, U. Löhrs, J. Gimmy and M. Eder, Erzeugung von Darmtumoren bei Mäusen durch 1,2-Dimethylhydrazin. *Z. ges. exp. Med.* **149,** 277 (1969).
20. J. R. M. Innes, B. M. Ulland, M. G. Valerio, L. Petrucelli, L. Fishbein, E. R. Hart, A. J. Pallotta, R. R. Bates, H. L. Falk, J. J. Gart, M. Klein, I. Michell and J. Peters, Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: a preliminary note. *J. nat. Cancer Inst.* **42,** 1101 (1969).
21. A. Schauer, T. Völlnagel and F. Wildanger, Cancerisierung des Rattendarmes durch 1,2-Dimethylhydrazin. *Z. ges. exp. Med.* **150,** 87 (1969).
22. H. Druckrey, Production of colonic carcinomas by 1,2-dialkylhydrazines and azoxyalkanes. In *Carcinomas of the Colon and Antecedent Epithelium.* Edited by W. J. Burdette, Charles C. Thomas, p. 269. Springfield, Ill. (1970).

23. The Merck Index, **8,** Merck, Rahway, N.J. (1968).
24. D. P. Gowing and R. W. Leeper, Induction of flowering in pineapple by beta-hydroxyethylhydrazine. *Science* **122,** 1267 (1955).
25. R. W. Leeper and D. P. Gowing, Studies on the relation of chemical structure to plant growth-regulatory activity in the pineapple plant. IV. Hydrazine derivatives, compounds with an unsaturated aliphatic moiety and miscellaneous chemicals. *Bot. Gaz.* **123,** 34 (1961).
26. E. A. Zeller, J. Barsky, J. R. Fouts, W. F. Kirchheimer and L. S. van Orden, Influence of isonicotinic acid hydrazide (INH) and 1-isonicotinyl-2-isopropyl hydrazine (IIH) on bacterial and mammalian enzymes. *Experientia (Basel)* **8,** 349 (1952).
27. I. J. Selikoff, E. H. Robitzek and G. G. Ornstein, Toxicity of hydrazine derivatives of isonicotinic acid in the chemotherapy of human tuberculosis. *Quart. Bull. Sea View Hosp.* **13,** 17 (1952).
28. G. Mathé, O. Schweisguth, M. Schneider, J. L. Amiel, L. Berumen, G. Brule, A. Cattan and L. Schwarzenberg, Methylhydrazine in treatment of Hodgkin's disease. *Lancet* **2,** 1077 (1963).
29. Fairmont Chemical Co. Inc., Newark N.J. 07105.
30. H. T. Schreus and F. Schönhöfer, Tobacco-smoke filter. *Chem. Abstr.* **52,** 7627 (1958).
31. E. R. Meincke, Color-stabilized copolymers of ethylene and vinyl acetate. *Chem. Abstr.* **73,** 5682 (1970).
32. B. A. Hunter, Foamed, vulcanized polysulfide rubber. *Chem. Abstr.* **67,** 12366 (1967).
33. R. Aarons, W. H. Baumgartner and D. R. English, Flame retardant compositions. *Chem. Abstr.* **54,** 18978 (1960).
34. G. D. Sammons, Composite propellants containing a burning-rate depressant. *Chem. Abstr.* **56,** 14522 (1962).

Europ. J. Cancer Vol. 11, pp. 23–31. Pergamon Press 1975. Printed in Great Britain

Enhancement of Azo Dye Carcinogenesis by Dietary Sodium Sulphate*

JILL M. BLUNCK and CAROL E. CROWTHER†
Department of Pathology, University of Melbourne, Parkville, Victoria, Australia

Abstract—*Male Sprague–Dawley rats were fed diets containing* 0·06% *3'methyl-4-dimethylaminoazobenzene* (*3'MeDAB*), 0·06% *3'MeDAB plus* 0·84% Na_2SO_4 *and* 0·06% *3'MeDAB plus* 0·8% *acetanilide; further groups were fed a control diet and diets containing either* 0·84% Na_2SO_4 *or* 0·8% *acetanilide. Addition of* Na_2SO_4 *to the 3'MeDAB diet increased the probability of developing liver tumours* 1 *cm or more in diameter, multiple tumours, and metastic spread. These increases seemed to be a result of a shorter latent period rather than an increase either in the rate of tumour development or in the number of transformed foci per liver. In contrast, addition of acetanilide to the 3'MeDAB diet completely inhibited tumour induction and prevented the development of cirrhosis and hyperplastic changes in the liver which have been regarded as preneoplastic.*

INTRODUCTION

IT HAS been established that aromatic amine and amide carcinogens require metabolic activation in order to produce the ultimate carcinogenic metabolites. In many instances, an essential intermediate step involves the production of an N-hydroxy metabolite [1]. The consensus of opinion in recent reports is that N-hydroxylation is probably followed either by esterification of the N-hydroxy compound by such processes as acetylation, transacetylation and conjugation (with glucuronic acid, sulphuric acid or phosphoric acid) or by conversion to an arylhydroxylamine by deacetylation [2]. There is evidence that derivatives of these carcinogens interact with various biological macromolecules and the ultimate carcinogenic metabolites are presumed to be highly reactive ions or radicals [1, 2]. Reactions with tissue nucleophiles may be of more than one type, for there is evidence that the carcinogen N-acetoxy-2-acetylaminofluorene both arylamidates and acetylates ribonuclease [3] and histones [4] *in vitro*; it is therefore quite possible that a combination of activation mechanisms is operating in any one instance and presumably the reaction products play an important but as yet unidentified role in tumour induction. However, it would be difficult to obtain direct evidence for the role of an N-hydroxy ester such as acetylaminofluorene-N-sulphate in arylamidation reactions, for according to De Baun *et al.* [5], loss of sulphate ion on reaction with nucleophiles makes it impossible to infer its presence from the structure of reaction products.

The activation steps subsequent to N-hydroxylation are possibly best understood in hepatocarcinogenesis by 2-acetylaminofluorene (AAF) in the rat, where there is evidence that sulphate conjugation may be of critical importance in the activation. Thus sulphotransferases present in rat liver 100,000 × *g* supernatant catalyze

Accepted 25 July 1974.

*This work was supported by grants from the National Health and Medical Research Council and from University of Melbourne Medical Research funds.
†Commonwealth Postgraduate Award recipient.

the production of macromolecular-bound forms of N-hydroxyacetylaminofluorene (N-hydroxy-AAF) *in vitro* [6, 7] and these adducts are identical with those isolated from the livers of rats given AAF *in vivo* [8]. If sulphate conjugation is necessary for the activation of N-hydroxy-AAF, manipulation of the amount of sulphate available for this process should alter macromolecular binding and tumour induction, as the liver sulphate pool is limited [9]. In agreement with this hypothesis, injections of Na_2SO_4 enhance both the binding by liver protein and the toxicity of intraperitoneally administered N-hydroxy-AAF [5]. In addition, the partial restoration of the tumour yield by increasing the level of dietary Na_2SO_4 following inhibition of liver carcinogenesis in the rat by acetanilide has been interpreted as evidence that sulphate conjugation plays an important role in AAF carcinogenesis [10]. It was postulated that acetanilide depleted the liver sulphate pool and that when this effect was overcome by dietary Na_2SO_4, sulphate again became available for conjugation with the carcinogen.

The situation regarding the possible ultimate carcinogenic metabolite(s) of azo dyes in the liver is less well documented, as detailed study of the metabolism of carcinogenic azo dyes has been hampered by the unavailability of N-hydroxy-4-monomethylaminoazobenzene (N-hydroxy-MAB), a postulated intermediate metabolite which has not yet been synthesized [11]. However, the products obtained when an ester of N-hydroxy-MAB was allowed to react with macromolecules *in vitro* [11] were identical with products formed *in vivo* following the administration of 4-monomethylaminoazobenzene (MAB) to the rat [12,13], and these products were similar to those obtained when the *in vitro* binding of N-hydroxy-AAF to identical macromolecules was catalyzed by sulphotransferases. It therefore seems likely that sulphate conjugation of an N-hydroxy metabolite may be involved in the activation process of azo dyes, and if so, the amount of sulphate in the diet might be expected to influence azo dye carcinogenesis. The present report describes the effects of supplementation of 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB)-containing diets with either Na_2SO_4 or acetanilide on tumour induction in rat liver and it was found that dietary Na_2SO_4 enhanced hepatocarcinogenesis by 3′MeDAB in the rat.

MATERIAL AND METHODS

Animals

Ninety male Sprague–Dawley rats (mean weight 211·6 g) obtained from random-bred departmental stock were housed 5 to a cage and allowed free access to tap water. They were pair-fed by a modified method whereby the total food consumption of each cage of rats was restricted to that of the cage of rats consuming the least. Rats from each experimental group were started on the test diets at two different times (Series A and Series B). The rats were pair-fed one of the test diets for 16 consecutive weeks, then returned to the basal diet for 8 weeks. At this time, 2 rats from each of the groups on the 3′MeDAB-containing diets and one rat from each of the control, acetanilide or Na_2SO_4-containing diets in Series A were killed and the livers examined. The remaining rats were then returned to the various experimental diets for several 4-week periods, with a week between each period during which they were fed basal diet. Rats were killed when they became moribund or when there was gross abdominal distension and any found dead during the course of the experiment were autopsied whenever possible. Some rats from each group in Series A were killed at 33 weeks; the numbers involved were: 3′MeDAB-fed group, 4 rats; 3′MeDAB plus acetanilide-fed group, 4 rats; 3′MeDAB plus Na_2SO_4-fed group, 3 rats; control, acetanilide and Na_2SO_4-fed groups, one rat from each. The remaining rats in both series were killed when there were 3 or less survivors in any group receiving a 3′MeDAB-containing diet. Series A was concluded after 41 weeks and Series B after 27 weeks. Rats were killed by exsanguination under light ether anaesthesia and samples of all liver lesions greater than 0·2 cm in diameter were fixed in acetic acid: formalin: ethanol (5:10:85) for 24 hr, then transferred to 70% (v/v) ethanol prior to processing to paraffin. Liver and spleen weights were recorded, in some instances haemoglobin estimations were performed, and in addition, samples of the livers and any macroscopic lesions in other organs were fixed and processed. Liver and lung sections were routinely stained with haematoxylin and eosin and by the periodic acid–Schiff (PAS) method; in some instances alcian blue or toluidine blue [14] stains were also used.

Diets

The basal diet (IRM; Victorian Wheatgrowers Corporation Ltd., North Melbourne) was supplied as pellets which were crushed prior to mixing the diets; the composition being: protein 15–16%; fat 6–8%; and fibre 8–9%. The test diets for the various groups

contained the following additives:— control diet: 30 ml maize oil/kg; 0·8% acetanilide diet: 30 ml maize oil plus 8 g acetanilide/kg; 0·84% Na_2SO_4 diet: 30 ml maize oil plus 8·4 g Na_2SO_4/kg; 0·06% 3′MeDAB diet: 30 ml of a 2% (w/v) solution of 3′MeDAB in maize oil/kg; 0·06% 3′MeDAB plus 0·8% acetanilide diet: 30 ml of a 2% (w/v) solution of 3′MeDAB in maize oil plus 8 g acetanilide/kg; and 0·06% 3′MeDAB plus 0·84% Na_2SO_4 diet: 30 ml of a 2% (w/v) solution of 3′MeDAB in maize oil plus 8·4 g Na_2SO_4/kg. 3′MeDAB (m.p. 119°C) was prepared by the method of Giese *et al.* [15] and Na_2SO_4 and acetanilide were supplied by Ajax Chemicals Ltd.

Diets were freshly prepared every 3–4weeks and were stored in polythene containers with lids. In Series A, the approximate rate of diet consumption was 10 g/rat per day in all experimental groups during the first 2 weeks; this was then gradually increased to 17 g/rat per day by week 27 and remained at this level for the duration of the experiment. In Series B, rats received 14 g/rat per day of diet initially; this was increased to 17 g/rat per day after 15 weeks and continued at this level until the experiment was concluded.

Statistical analyses

The progression with time of the cumulative probability of observing liver tumours of 1 cm or more in diameter, evidence of metastatic spread or multiple tumours at death was calculated by an actuarial method [16] similar to that of Pilgrim and Dowd [17]. The significance of differences between the numbers of rats bearing tumours of at least 1 cm dia or with metastases in the groups fed 3′MeDAB-containing diets was compared by Fisher's exact test as the expected frequencies in any one cell were less than 5 in each instance [18]. The correlation coefficient was computed in the standard manner [19].

Table 1. *Groups used, initial body weights, and body weights and liver weights of rats killed at the conclusion of the experiment*

Dietary Group	Series*	No. of rats/group	Effective no. of rats/group†	Starting weight (g) Mean ± S.D.	No. of rats surviving the duration of the experiment	Terminal body weight (g) Mean ± S.D. or range‡	Terminal liver weight (g) Mean ± S.D. or range‡
Control	A	5	5	230·8 ± 18·6	2	358·5(345–372)	10·11(9·94–10·27)
	B	5	5	193·4 ± 33·6	5	358·4 ± 16·0	10·20 ± 0·49
Acetanilide	A	5	4	207·0 ± 21·5	2	340·5(323–358)	10·55(9·90–11·20)
	B	5	5	188·4 ± 35·5	5	332·6 ± 35·4	10·64 ± 1·56
Na_2SO_4	A	5	5	252·0 ± 30·2	3	414·7 ± 39·5	14·30 ± 1·42
	B	5	5	194·2 ± 35·7	5	332·0 ± 53·7	10·03 ± 0·99
3′MeDAB	A	15	14	234·0 ± 29·4	6	351·3 ± 46·1	29·55 ± 21·17
	B	5	5	200·8 ± 20·7	2	334·5(328–341)	40·97(20·76–61·18)
3′MeDAB and Acetanilide	A	15	15	229·3 ± 24·3	6	351·0 ± 16·8	13·35 ± 1·83
	B	5	5	195·9 ± 27·0	5	313·6 ± 39·3	11·54 ± 1·38
3′MeDAB and Na_2SO_4	A	15	14	231·0 ± 32·7	3	346·3 ± 37·5	26·06 ± 1·87
	B	5	5	181·8 ± 26·6	1	361·0	28·23

*Rats in Series A and Series B were started on the dietary regimens at different times; all rats remaining in Series A were killed after 41 weeks and those remaining in Series B were killed after 27 weeks.

†Animals dying during the experiment which could not be autopsied because of cannibalism or advanced autolysis were excluded from the effective number of rats/group.

‡These data were derived from rats which survived for the duration of the experiment (either 27 weeks for Series B or 41 weeks for Series A).

RESULTS

No significant differences in overall body weight gain or in liver weight were noted between the various experimental groups at the conclusion of the experiment (Table 1), with the exception of livers bearing tumours which on occasion weighed as much as 82·1 g. Rats receiving the acetanilide-containing diets showed splenomegaly and were anaemic, probably due to the effects of metabolites of acetanilide on the haemopoietic system [20]. A significant correlation was demonstrated between the spleen size and the haemoglobin value ($r = -0{\cdot}833$, $P < 0{\cdot}01$).

The livers of many rats which survived at least 16 weeks on diets containing either 3′MeDAB or 3′MeDAB plus Na_2SO_4 were finely granular in appearance and showed

*Table 2. Showing incidence of hyperplastic and/or dysplastic change, cholangiofibrosis and mild cirrhosis in livers of rats surviving at least 16 weeks on the various regimens and either found dead or killed at subsequent stages of the experiment**

Dietary group	No. of rats surviving for 16 weeks	No change	Mild cirrhosis†	Areas of hyperplasia‡ and/or dysplasia	Areas of cholangiofibrosis§
Control	10	10	—	—	—
Acetanilide	10	10	—	—	—
Na_2SO_4	10	10	—	—	—
3′MeDAB	16	—	10 (62·5%)	15 (93·8%)	2 (12·5%)
3′MeDAB and acetanilide	20	20	—	—	—
3′MeDAB and Na_2SO_4	18	—	17 (94·4%)	17 (94·4%)	5 (27·8%)

*Some rats receiving diets containing 3′MeDAB or 3′MeDAB and Na_2SO_4 which died in the first weeks of the experiment showed a periportal round cell infiltrate and in a few instances, fatty change. The livers of rats fed acetanilide-containing diets were smooth, dark reddish-brown in colour and contained prominent Kupffer cells.

†The external surface of these livers was observed to be finely granular and an increase in periportal connective tissue was observed microscopically; this occasionally connected adjacent portal triads.

‡Areas of basiphilic cells, discrete collections of vacuolated cells or areas of large cells with large nuclei.

§Based on microscopic findings.

microscopic evidence of mild cirrhosis; local areas of hyperplasia or dyplasia were also frequently observed (Table 2). These comprised discrete localized alterations in the cells and could take the form of groups of deeply basiphilic cells which sometimes showed a tubular appearance, cells with vacuolated cytoplasm, large cells with a normal nucleocytoplasmic ratio and hyperchromatic pleomorphic nuclei (the dysplasia of Anthony *et al.* [21]) and cells which showed pleomorphism and variations in the nucleocytoplasmic ratio. These areas often contained less PAS positive material than the surrounding liver, but in some instances, PAS staining was increased; these findings are in agreement with data of Forget and Daoust [22]. Areas of cells which were basiphilic when stained with haematoxylin and eosin were hyperbasiphilic when stained with toluidine blue.

The relative risk of developing liver tumours of 1 cm or more in diameter in rats fed diets containing either 3′MeDAB or 3′MeDAB plus Na_2SO_4 is shown in Fig. 1. It can be seen that the addition of Na_2SO_4 to the diet shortened the latent period but did not affect the rate of tumour development, which is indicated by the slope of the lines in Fig. 1. The relative risks of developing multiple tumours (Fig. 2) and metastatic tumours (Fig. 3) respectively

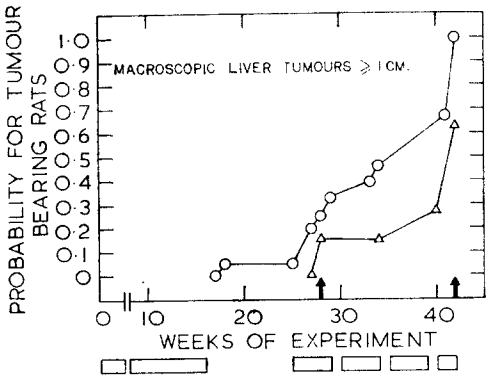


Fig. 1. Probabilities for the observation of a macroscopic liver tumour of at least 1 cm dia at death in rats pair-fed diets containing 0·06% 3′MeDAB, △———△ or 0·06% 3′MeDAB plus 0·84% Na_2SO_4, ○———○, calculated by the method of Saffiotti et al. [16]. *The bars represent the periods when the special diets were given and all rats consumed basal diet for the remainder of the experiment. The arrows indicate the times when Series B and Series A respectively, were concluded. Each point above the abscissa represents a time subsequent to the discovery of the first tumour when rats died or were sacrificed.*

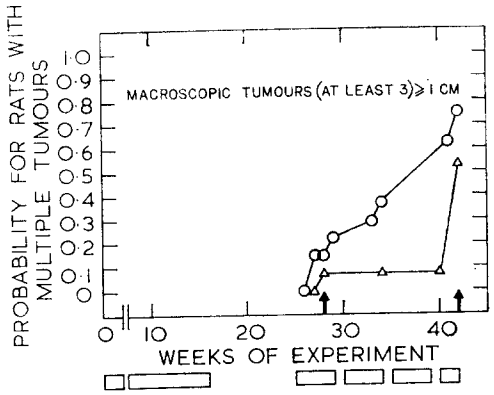


Fig. 2. Probabilities for the observation of at least 3 macroscopic liver tumours which had attained 1 cm dia at death in rats pair-fed diets containing 0·06% 3′MeDAB, △———△ or 0·06% 3′MeDAB plus 0·84% Na_2SO_4, ○———○, calculated by the method of Saffiotti et al. [16]. *The bars represent the periods when the special diets were given and all rats consumed basal diet for the remainder of the experiment. The arrows indicate the times when Series B and Series A respectively, were concluded. Each point above the abscissa represents a time subsequent to the discovery of the first tumour when rats died or were sacrificed.*

were also increased if the 3'MeDAB diet was supplemented with Na_2SO_4. In contrast, supplementation of the 3'MeDAB diet with acetanilide completely prevented the induction of liver tumours (Table 3). Tumours attaining 1 cm or more in diameter were usually either hepatocarcinomas or cholangiocarcinomas with some tumours showing areas of both hepatocarcinoma and cholangiocarcinoma within the one nodule. In only one instance was a nodule of 1 cm dia or larger shown to be a hyperplastic liver nodule of the type described by Farber [23].

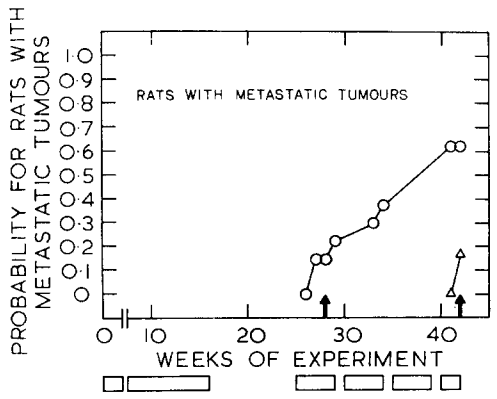


Fig. 3. Probabilities for the observation of liver tumours with evidence of metastic spread in rats pair-fed diets containing 0·06% *3'MeDAB* △———△ *or* 0·06% *3'MeDAB plus* 0·84% Na_2SO_4, ○———○, *calculated by the method of Saffiotti* et al. [16]. *The bars represent the periods when the special diets were given and all rats consumed basal diet for the remainder of the experiment. The arrows indicate the times when Series B and Series A respectively, were concluded. Each point above the abscissa represents a time subsequent to the discovery of the first tumour when rats died or were sacrificed.*

Because of variations in the microscopic appearance in sections made from different areas of the same tumour, no attempt was made to classify tumours exclusively as either hepatocarcinoma or cholangiocarcinoma. Hepatocarcinomas frequently show areas with either a trabecular or an acinar pattern [24] and if this observation is taken into account, the majority of liver tumours induced in this experiment should probably be regarded as being in this category. This inference is in agreement with the relatively low incidence of cholangiofibrosis noted in this study (Table 2), for cholangiofibrosis has been implicated as a precursor of cholangiocarcinoma [25]. Tumours were found in all lobes of the liver, but a detailed analysis of the relative frequency of tumours in the various lobes was not undertaken. All the carcinomas observed contained much less PAS positive material than the surrounding liver, and were hyperbasiphilic when stained with toluidine blue.

Metastases within the peritoneal cavity were observed in 7 of the 11 rats fed 3'MeDAB plus Na_2SO_4 which had liver carcinomas 1 cm or more in diameter, and in 4 of these rats, multiple foci of secondary tumour were present in the lungs (Table 3). In one rat fed 3'MeDAB plus Na_2SO_4, the entire contents of the peritoneal cavity had to be removed *en bloc* because of diffuse infiltration by tumour tissue. In contrast, peritoneal metastasis was only observed in one of 6 rats fed 3'MeDAB which had liver carcinomas of this size, and pulmonary metastases were not present.

Many nodules smaller than 1 cm dia were observed in the livers of rats fed either the 3'MeDAB or the 3'MeDAB plus Na_2SO_4 diets, whereas the livers of rats receiving the 3'-MeDAB plus acetanilide diet were in every instance smooth and slightly darker than the livers in control rats. No abnormalities were noted in the livers of rats on the control or Na_2SO_4 diets, but the livers from rats which had received acetanilide were darker than usual.

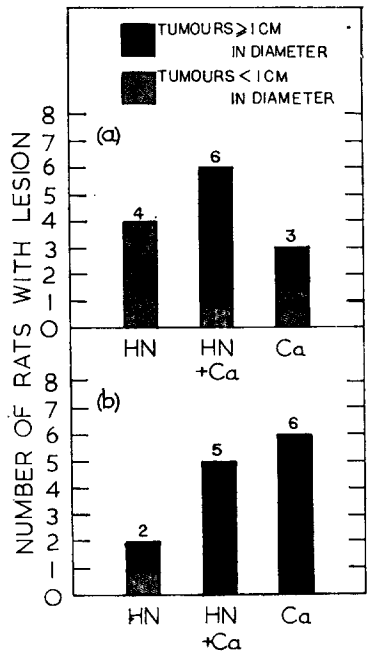


Fig. 4. Frequency distribution in livers of rats surviving at least 16 *weeks on (a) the 3'MeDAB diet or (b) the 3'MeDAB plus Na_2SO_4 diet of histologically confirmed hyperplastic nodules (HN), carcinoma (Ca) or both lesions (HN & Ca) occurring simultaneously in the same animal. The most advanced lesions found in each rat were scored except where both hyperplastic nodules and carcinoma were found in the one liver, when the rat was scored as HN & Ca. The results were derived from pooled data from both Series. Tumour nodules smaller than 1 cm dia were found in the livers of* 13 *rats fed the diet containing 3'MeDAB and* 15 *rats fed the diet containing 3'MeDAB plus Na_2SO_4. The black bars represent the numbers of rats in which macroscopic tumours ≥ 1 cm dia were present in the liver and the cross hatched bars represent smaller tumours where the diagnosis was confirmed by microscopy.*

*Table 3. Effect of dietary supplementation with either sodium sulphate or acetanilide on tumour induction by 3'MeDAB in the liver of male Sprague–Dawley rats**

Dietary group	No. of rats surviving at least 16 weeks	Weeks to first tumour†	Other rats alive at time first tumour found	No. of rats developing tumours by conclusion of experiment	Mean no. of tumours/tumour-bearing rat(± S.D.)	Total tumours in tumour-bearing rats	Microscopic findings in tumour-bearing rats‡		Rats with metastases¶
							Hyperplastic nodule	Carcinoma	
Control	10	—	—	—	—	—	—	—	—
Acetanilide	10	—	—	—	—	—	—	—	—
Na_2SO_4	10	—	—	—	—	—	—	—	—
3'MeDAB	16	27	11	6	3·83 ± 2·14	23	—	6	1
3'MeDAB and acetanilide§	20	—	—	—	—	—	—	—	—
3'MeDAB and Na_2SO_4	18	17	17	12	3·25 ± 2·05	39	1	11	7

*The data presented represent pooled results from Series A and Series B and includes data from all rats either found dead or killed subsequent to the first 16 weeks of the experiment.

†The term *tumour* refers to a macroscopic nodule $\geqslant$ 1 cm dia.

‡The results represent the most advanced lesion present.

§A subcutaneous fibrosarcoma was found in a rat in this group which was killed at 33 weeks. The inhibition of liver tumour induction when acetanilide was added to the 3'MeDAB-containing diet was significant ($P < 0{\cdot}01$, Fisher's exact test).

¶There were metastases present in the peritoneal cavity in all of these rats and in addition there were metastases present in the lungs in 4 rats in the group receiving 3'MeDAB and Na_2SO_4. There was a significant increase in the number of rats with metastases when the diet containing both 3'MeDAB and Na_2SO_4 was fed rather than the 3'MeDAB diet ($P < 0{\cdot}05$, Fisher's exact test).

The data shown in Fig. 4 represent the results of microscopic analysis of nodules equal to or greater than 0·2 cm dia that were present in livers. It can be seen that many of the lesions less than 1 cm dia, particularly in the group receiving 3'MeDAB alone, were hyperplastic liver nodules. More nodules were seen macroscopically than could be accounted for by microscopic examination. Although an attempt was made to identify microscopically all lesions greater than 0·2 cm dia, in some instances this was not practicable as it would have involved cutting serial sections from a large number of blocks. In many instances, both hyperplastic nodules and carcinoma were present in the same liver (Fig. 4) but there were relatively more rats with carcinoma and no hyperplastic nodules in the group which had been fed 3'MeDAB plus Na_2SO_4. In contrast, there were more rats in which hyperplastic liver nodules represented the most advanced lesion in the group fed 3'MeDAB. This overlap in the incidence of hyperplastic nodules and carcinoma and the lower frequency of hyperplastic nodules relative to carcinoma in rats receiving 3'MeDAB plus Na_2SO_4, which had a significantly greater risk of developing metastasizing carcinomas, supports Farber's contention [23] that hyperplastic liver nodules are precursors of carcinoma. There was no significant difference in the mean number of tumours/tumour bearing rat between groups which were fed either 3'MeDAB alone or 3'MeDAB plus Na_2SO_4. This observation was true regardless of whether all those tumours $\geqslant$ 1 cm (Table 3) or $\geqslant$ 0·2 cm (3'MeDAB-fed group: $6{\cdot}077 \pm 3{\cdot}904$ (S.D.); 3'MeDAB plus Na_2SO_4-fed group: $7{\cdot}133 \pm 4{\cdot}470$) in diameter were considered. It therefore seems that the enhancement of 3'MeDAB carcinogenesis by dietary Na_2SO_4 is due to a decrease in the latent period rather than to an increase in the number of transformed foci.

DISCUSSION

The significant enhancement of 3'MeDAB hepatocarcinogenesis by dietary Na_2SO_4 supplementation that has been demonstrated in the present study suggest that sulphate conjugation, possibly of an N-hydroxy metabolite such as N-hydroxy-MAB, might play a role in the activation of carcinogenic azo dyes. There is evidence that N-hydroxy derivatives of the azo dyes are produced in the rat, for N-hydroxy-N-acetyl-4-aminoazobenzene has been identified in the urine following the administration of 4-dimethylaminoazobenzene, MAB or 4-aminoazobenzene [26]. This urinary metabolite does not appear to be carcinogenic in the rat [26], but its presence suggests that other reactive N-hydroxy derivatives might be produced *in vivo* and then undergo subsequent conjugation to form the ultimate carcinogenic metabolite(s). An observation of possible relevance in this context is that enzymic esterification of N-hydroxy-AAF was significantly decreased by administration of 3'MeDAB [27] as well as by the administration of AAF [28] and N-hydroxy-AAF [8]. These findings seem to support the suggestion of De Baun *et al.* [7] that sulphotransferases might be inactivated by reaction with the ultimate carcinogenic metabolites as soon as they are produced, but it could also be a result of a generalized toxic effect of these carcinogens or their metabolites on a variety of intracellular processes. If the former possibility is true, the evidence implies that the sulphotransferases, which catalyze the production of sulphate-conjugated metabolites, might be necessary for the formation of highly reactive metabolites of the azo dyes.

The finding that acetanilide inhibits azo dye carcinogenesis in the liver of the rat is in agreement with unpublished data of Weisburger's group [2]. Acetanilide administration apparently interferes with both N-hydroxylation [29] and sulphate conjugation [10] of AAF, therefore it is quite possible that either or both of these processes might also be inhibited when acetanilide and 3'MeDAB are administered in the diet. If this is shown to be so, it would provide additional evidence in support of a significant role for both N-hydroxylation and sulphate conjugation in the metabolic activation of the carcinogenic azo dyes.

It was of interest to note that in the present study, in which there was a significant enhancement of 3'MeDAB hepatocarcinogenesis by dietary Na_2SO_4 supplementation, there was no evidence of increased toxic effects, as manifested by a decreased percentage of survivors, in rats receiving the 3'MeDAB plus Na_2SO_4 diet as compared with the 3'MeDAB diet. The administration of 0·032% N-hydroxy-AAF in the diet together with 0·84% Na_2SO_4 to rats for a period of 16 weeks also apparently did not decrease the percentage of survivors after 32 weeks [10]. In contrast, injections of Na_2SO_4 increased the toxicity of intraperitoneally administered N-hydroxy-AAF and this increase was correlated with increased macromolecular binding of AAF derivatives

[5]. The failure of Na_2SO_4 supplementation of the diet to increase the toxic effects of dietary 3′MeDAB and N-hydroxy-AAF is probably a consequence of the differences in the dose and route of administration of these carcinogens, for De Baun *et al.* [5] reported that intraperitoneal N-hydroxy-AAF (5 mg/100 g body wt) induced massive periportal necrosis, while in previous studies [30], there was no evidence of this following dietary administration of 0·06% (w/v) 3′MeDAB.

The findings of the present study suggested that supplementation of the 3′MeDAB-containing diet with Na_2SO_4 decreased the latent interval, but did not significantly increase either the number of transformed foci per liver or the rate of tumour development. It would seem that the addition of Na_2SO_4 to the 3′MeDAB diet influences the process of carcinogenesis in a manner similar to that of promoting agents, for in the example of two-stage carcinogenesis in mouse skin, the effects of promoters such as croton oil include a reduction in the latent period and an increase in the tumour response if the first stage is brought about by a carcinogenic dose of the initiator [31, 32]. The "initiation" step probably represents alterations either in individual cells or areas (fields) of cells which arise either as a result of somatic mutations and/or instability of gene expression [33]. These altered cells are in many instances undetectable by presently available techniques, but when the tissue is subjected to some appropriate environmental stimulus, macroscopic tumours eventually develop. For many years, evidence for promotion was confined to data derived from experiments which utilized the mouse skin system, but more recent reports suggest that promoting agents also decrease the latent interval in hepatocarcinogenesis [34] and in a tissue culture transformation system [35]. In addition, Peraino *et al.* [36] found that when phenobarbital was administered to rats after they had been fed for 18 days only on a diet containing 0·02% AAF, there was a decrease in the latent interval between the end of carcinogen treatment and the appearance of tumours; this finding suggests that phenobarbital may be acting like a promoter in the liver. The mechanism of action of promoting agents is as yet unclear, but phorbol esters, which are the active components of croton oil, have been shown to react with soluble cytoplasmic proteins [37] and cell membranes [38] and these types of reaction could conceivably affect gene regulatory mechanisms. Barbiturates are reversibly bound by components of liver homogenates [39] and there is evidence that the carcinogen 20-methylcholanthrene competes with a promoting agent for binding sites on mouse epidermal cytoplasmic proteins [37]; protein binding of carcinogens might therefore influence promotion. If this is so, the correlation noted by De Baun *et al.* [8] between sulphotransferase activity and protein binding of AAF derivatives in the liver assumes increased significance in the light of the present report, in which dietary Na_2SO_4 supplementation, which would be expected to increase sulphate conjugation and therefore protein binding, has been demonstrated to decrease the latent interval in hepatocarcinogenesis.

Although administration of Na_2SO_4 to rats has previously been shown to increase the toxicity and protein binding of metabolites of AAF [5] and there is evidence that manipulation of the liver sulphate pool affects the process of AAF hepatocarcinogenesis [10], the present report seems to be the first instance in which it has been demonstrated that Na_2SO_4 administration accelerates hepatocarcinogenesis due to aromatic amine and amide carcinogens. In an earlier study [10], supplementation of a diet containing N-hydroxy-AAF with Na_2SO_4 did not increase the tumour yield, but under these conditions, the incidence of tumours was 100% in a control group receiving only N-hydroxy-AAF. It is important that where evidence for an increase in tumour yield is being sought, the control group receiving carcinogen only should have a relatively low tumour incidence. In the present study, only 6 of 16 (37·5%) rats receiving the 3′MeDAB-containing diet and surviving for at least 16 weeks had developed tumours 1 cm or more in diameter at the conclusion of the experiment, and under these conditions an enhancement of carcinogenesis by Na_2SO_4 supplementation of the 3′MeDAB diet was readily detected.

REFERENCES

1. J. A. Miller, Carcinogenesis by chemicals: an overview—G. H. A. Clowes Memorial Lecture. *Cancer Res.* **30,** 559 (1970).
2. J. H. Weisburger and E. K. Weisburger, Biochemical formation and pharmacological, toxicological, and pathological properties of hydroxylamines and hydroxamic acids. *Pharmacol. Rev.* **25,** 1 (1973).

3. E. J. Barry and H. R. Gutmann, Protein modifications by activated carcinogens. I. The acetylation of ribonuclease by N-acetoxy-2-fluorenylacetamide. *J. biol. Chem.* **248,** 2730 (1973).
4. E. J. Barry and H. R. Gutmann, Acetylation and arylamidation of histones by the carcinogen, N-acetoxy-2-fluorenylacetamide (N-acetoxy-2-FAA). *Fed. Proc.* **33,** (Abstract 1211), 1438 (1974).
5. J. R. de Baun, J. Y. R. Smith, E. C. Miller and J. A. Miller, Reactivity *in vivo* of the carcinogen N-hydroxy-2-acetylaminofluorene: increase by sulfate ion. *Science* **167,** 184 (1970).
6. C. M. King and B. Phillips, Enzyme-catalyzed reactions of the carcinogen N-hydroxy-2-fluorenylacetamide with nucleic acid. *Science* **159,** 1351 (1968).
7. J. R. de Baun, J. Y. Rowley, E. C. Miller and J. A. Miller, Sulfotransferase activation of N-hydroxy-2-acetylaminofluorene in rodent livers susceptible and resistant to this carcinogen. *Proc. Soc. exp. Biol. (N.Y.)* **129,** 268 (1968).
8. J. R. de Baun, E. C. Miller and J. A. Miller, N-hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein-(methion-S-yl) binding in rat liver. *Cancer Res.* **30,** 577 (1970).
9. H. G. Bray, B. G. Humphris, W. V. Thorpe, K. White and P. B. Wood, Kinetic studies of the metabolism of foreign organic compounds 4. The conjugation of phenols with sulphuric acid. *Biochem. J.* **52,** 419 (1952).
10. J. H. Weisburger, R. S. Yamamoto, G. M. Williams, P. H. Grantham, T. Matsushima and E. K. Weisburger, On the sulfate ester of N-hydroxy-N-2-fluorenylacetamide as a key ultimate hepatocarcinogen in the rat. *Cancer Res.* **32,** 491 (1972).
11. L. A. Poirier, J. A. Miller, E. C. Miller and K. Sato, N-Benzoyloxy-N-methyl-4-aminoazobenzene: its carcinogenic activity in the rat and its reactions with proteins and nucleic acids and their constituents *in vitro*. *Cancer Res.* **27,** 1600 (1967).
12. J. A. Miller, J. K. Lin and E. C. Miller, N-(Guanosin-8-yl) and N-(deoxyguanosin-8-yl)-N-methyl-4-aminoazobenzene: degradation products of hepatic RNA and DNA from rats administered N-methyl-4-aminoazobenzene (MAB). *Proc. Amer. Ass. Cancer Res.* **11,** (Abstract 221), 56 (1970).
13. J. D. Scribner, J. A. Miller and E. C. Miller, 3-Methylmercapto-*N*-methyl-4-aminoazobenzene: an alkaline-degradation product of a labile protein-bound dye in the livers of rats fed *N,N*-dimethyl-4-aminoazobenzene. *Biochem. biophys. Res. Commun.* **20,** 560 (1965).
14. R. Daoust and F. Molnar, Cellular populations and mitotic activity in rat liver parenchyma during azo dye carcinogenesis. *Cancer Res.* **24,** 1898 (1964).
15. J. E. Giese, J. A. Miller and C. A. Baumann, The carcinogenicity of *m'*-methyl-*p*-dimethylaminoazobenzene and of *p*-monomethylaminoazobenzene. *Cancer Res.* **5,** 337 (1945).
16. U. Saffiotti, R. Montesano, A. R. Sellakumar, F. Cefis and D. G. Kaufman, Respiratory tract carcinogenesis in hamsters induced by different numbers of administrations of benzo(a)pyrene and ferric oxide. *Cancer Res.* **32,** 1073 (1972).
17. H. I. Pilgrim and J. E. Dowd, Correcting for extraneous death in the evaluation of morbidity or mortality from tumor. *Cancer Res.* **23,** 45 (1963).
18. A. E. Maxwell, *Analysing Qualitative Data*, p. 23. Methuen, London (1961).
19. W. C. Schefler, *Statistics for the Biological Sciences*, p. 140. Addison-Wesley, Massachusetts (1969).
20. G. Hustedt and M. Kiese, Umsetzungen von acetanilid und acetylphenylhydroxylamin im organismus. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **236,** 435 (1959).
21. P. P. Anthony, C. L. Vogel and L. F. Barker, Liver cell dysplasia: a premalignant condition. *J. clin. Path.* **26,** 217 (1973).
22. A. Forget and R. Daoust, Histochemical study on rat liver glycogen during DAB carcinogenesis. *Int. J. Cancer* **5,** 404 (1970).
23. E. Farber, Hyperplastic liver nodules, In *Methods in Cancer Research.* Edited by H. Busch, Vol. 7, p. 345. Academic Press, New York (1973).
24. H. L. Stewart and K. C. Snell, The histopathology of experimental tumors of the liver of the rat, In *The Physiopathology of Cancer.* Edited by F. Homburger, 2nd Edition, p. 85. Cassell, London (1958).

25. E. L. Opie, The pathogenesis of tumors of the liver produced by butter yellow. *J. exp. Med.* **80,** 231 (1944).
26. K. Sato, L. A. Poirier, J. A. Miller and E. C. Miller, Studies on the N-hydroxylation and carcinogenicity of 4-aminoazobenzene and related compounds. *Cancer Res.* **26,** 1678 (1966).
27. J. M. Blunck and B. J. Lees, Depression of enzymic esterification of N-hydroxy-AAF in the liver during aminoazo dye carcinogenesis in the rat. *Res. Commun. chem. Path. Pharmacol.* 5, 421 (1973).
28. C. C. Irving, C. D. Jackson and L. T. Russell, Changes in liver sulfotransferase activities during hepatocarcinogenesis by 2-acetylaminofluorene (AAF). *Fed. Proc.* **30,** (Abstract 1176), 1254 (1971).
29. P. H. Grantham, L. Mohan, R. S. Yamamoto, E. K. Weisburger and J. H. Weisburger, Alteration of the metabolism of the carcinogen N-2-fluorenylacetamide by acetanilide. *Toxicol. appl. Pharmacol.* **13,** 118 (1968).
30. J. M. Blunck, Inhibition by chloramphenicol of aminoazo dye carcinogenesis in rat liver: morphological studies. *Pathology* **3,** 99 (1971).
31. I. Berenblum and P. Shubik, The role of croton oil applications, associated with a single painting of a carcinogen, in tumour induction of the mouse's skin. *Brit. J. Cancer* **1,** 379 (1947).
32. I. Berenblum and P. Shubik, A new, quantitative, approach to the study of the stages of chemical carcinogenesis in the mouse's skin. *Brit. J. Cancer* **1,** 383 (1947).
33. R. T. Prehn, Neoplasia, In *Principles of Pathobiology.* Edited by M. F. La Via and R. B. Hill, Jr. p. 191. Oxford University Press, New York (1971).
34. V. Armuth and I. Berenblum, Systemic promoting action of phorbol in liver and lung carcinogenesis in AKR mice. *Cancer Res.* **32,** 2259 (1972).
35. C. Lasne, A. Gentil and I. Chouroulinkov, Two-stage malignant transformation of rat fibroblasts in tissue culture. *Nature (Lond.)* **247,** 490 (1974).
36. C. Peraino, R. J. M. Fry, E. Staffeldt and W. E. Kisieleski, Effects of varying the exposure to phenobarbital on its enhancement of 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. *Cancer Res.* **33,** 2701 (1973).
37. T. J. Slaga, J. D. Scribner and J. M. Rice, *In vitro* binding of labeled 20-methylcholanthrene to a specific cytosol-protein. *Chem.-Biol. Interactions* **7,** 51 (1973).
38. A. Sivak and B. L. van Duuren, Cellular interactions of phorbol myristate acetate in tumor promotion. *Chem.-Biol. Interactions* **3,** 401 (1971).
39. L. R. Goldbaum and P. K. Smith, The interaction of barbiturates with serum albumin and its possible relation to their disposition and pharmacological actions. *J. Pharmacol. exp. Ther.* **111,** 197 (1954).

Europ. J. Cancer Vol. 11, pp. 33–37 Pergamon Press 1975. Printed in Great Britain

EORTC Meetings: Intravascular Events in Cancer Dissemination

Abstract—*This subject was the topic for discussion at the first Meeting of the E.O.R.T.C. Metastasis Club held on the 15th March 1974 at the Imperial Cancer Research Fund Laboratories in London. Some thirty members gathered from a number of European countries for the meeting which was chaired by the President, Professor K. Hellmann of the Imperial Cancer Research Fund.*

Members came from France, Germany, Italy, The Netherlands, Switzerland and the U.K. They resolved to meet twice a year and if possible to engage in co-ordinated research on substances of common interest.

Seven papers were given, the abstracts of which follow.

Anne Atherton (Chemotherapy Department, Imperial Cancer Research Fund, London W.C.2, U.K.)

THE ADHESION OF CELLS TO VASCULAR ENDOTHELIUM

BEFORE the inflammatory response can take place, leucocytes flowing freely in the blood must become attached to the vascular endothelium. This process, about which we know so little, is probably necessary for the attachment of tumour cells to the endothelium. The nature of the subtle changes causing adherence, and the factors influencing them remain unknown. We can hypothesise that cells in the blood hit the vascular endothelium in a series of random collisions. In a normal vessel these are elastic, but in an inflamed vessel they become inelastic, and the cells, instead of returning to the bloodstream, tend to stick and roll along the vessel walls. Tumour cells may only stick to damaged endothelium, or may be pecular in that they also adhere to intact vessel walls. Intravascular adhesion is a reaction between cells in an environment, the bloodstream. Changes in all of these may affect subsequent adhesion. The possible list of factors which may affect this process is long, but includes bloodflow, pH, the availability of divalent cations, the molecular configuration of the cell surfaces, and structure of the endothelial cells. In addition, adherent tumour cells are thought to precipitate the formation of a fibrin clot, which surrounds the tumour cell until it leaves the vessel. Thus platelet and leucocyte adhesion may affect this process. Electron microscopic evidence substantiates this theory, but close inspection of the adherent cell surfaces reveals no obvious structural change.

Using the hamster cheek pouch preparation, the number of white cells seen adhering to the vessel walls could be increased by injury of the presence of endogemous inflammatory agents. We can postulate that, like white cells, circulating tumour cells are affected by pathological changes in the vessel wall. These changes could range from a tendency to attachment, to entrapment within preformed platelet thrombi. It is an established experimental fact that metastatic nodules appear more frequently at the site of trauma.

Studies on the relationship between platelet and leucocyte adhesion and bloodflow in this preparation have shown that leucocytes roll along the venule wall at a speed which is proportional to the shear force of the bloodflow, presuming the adhesive force between endothelium and leucocyte to be constant. This latter force was calculated to be in the order of $1{\cdot}5 \times 15^{-5}$ dynes. These *in vivo* measurements correspond well with *in vitro* values for this force determined by other workers. It may be possible to change this relationship by the use of agents which alter the detailed structure of the cell surface.

Accepted 18 September 1974.

Blood velocity also affects the rate at formation of platelet thrombi produced by the iontophoresis of adenosine diphosphate into venules in the hamster cheek pouch. The effect of bloodflow on tumour cell adherence is unknown, but tumour cells are usually bigger or in small emboli, giving a larger area over which the shear force of the blood may act. Their attachment may also be affected by the ease with which thrombi can form.

P. Hilgard (Klinikum Essen, 43 Essen F.R.G.)

COAGULATION FACTORS

Before their penetration into vessel walls, embolic tumour cells are enmeshed in a fibrin–platelet thrombus. Agents interfering with the activation of the clotting mechanism, such as heparin and warfarin, have been shown to have anti-metastatic action in various experimental tumour models [1]. The i.v. injection of $2{\cdot}5 \times 10^5$ V2 carcinoma cells into rabbits which were rendered afibrinogenaemic by a single injection of ancrod (Arvin) prior to the tumour cells injection, resulted in significant decrease of lung and liver metastases, indicating that fibrin formation around embolic tumour cells is of pathogenic significance in the early phase of metastasis formation. However, all anticoagulants have a variety of pharmacological actions, some of which could also account for their antimetastatic effects.

Some interest has focussed on a possible interaction between blood platelets and tumour cells [2]. Thrombocytopenia as well as altered platelet function were shown to decrease metastasis formation in some mouse tumours and tumour cells may even aggregate platelets *in vitro* [3]. Using the V2 carcinoma system, acetylsalicylic acid (Aspirin) was tested for its possible antimetastatic effect in rabbits. The animals were pretreated with a dose of 50 mg/kg/24hr. At the time of tumour cell injection the collagen induced platelet aggregation was reduced by 70% in the treated animals. Although the platelet function obviously was altered no effect upon the number and distribution of metastases could be demonstrated.

The injection of tumour cell suspensions into rats and mice resulted in a thrombocytopenia which was directly dependent upon the number of tumour cells injected. Studies with ^{51}Cr labelled platelets and ^{125}I-fibrinogen suggested that the disappearance of platelets from the circulation was due to thrombus formation around tumour cell emboli in the lungs of the animals [4]. Electron microscopy studies showed that tumour cells adherent to the vessel wall were surrounded rapidly by irreversible platelet aggregates. Only minor amounts of fibrin could be detected, however, if the fibrinolytic response was inhibited with epsilon-aminocaproic acid pretreatment, the association of these blood platelets with polymerized fibrin became evident.

The extent of the thrombocytopenia after tumour cell injection is closely related to the route of injection (femoral vein, portal vein or arterial side). This observation led to the assumption that local factors are involved in the pathogenesis of the thrombocytopenia. The behavior of blood platelets after i.v. tumour cell injection into animals which were pretreats with different anticoagulants, showed a specific pattern [5]. Based on these experiments, we believe that tumour cells adhere to intact vessel walls and induce a minimal endothelial damage leading to the exposure of cytoplasmic contents to the plasma and subsequent activation of the clotting mechanism with the formation of thrombin around the adherent tumour cell. When the tumour cell embolus is established fibrin is dissolved by secondary fibrinolysis.

Although the local activation of blood coagulation during the initial phase of haematogenous metastasis formation seems to be established, the pathogenic significance of this event is still not fully understood. Fibrin formation, platelet aggregation and fibrinolysis are just the visible steps of multiple enzymatic processes with the generation of many pharmacologically active intermediate products which, for their part, may be of some importance in the formation of metastases from blood borne cancer cells.

REFERENCES

1. S. Wood, Jr., Mechanisms of establishment of tumor metastases. In *Pathobiology Annual*. Edited by H. L. Ioachim, New York (1971).
2. B. A. Warren, Environment of the blood-borne tumor embolus adherent to vessel wall. *J. Med.* **4,** 150 (1973).
3. G. J. Gasic, T. B. Gasic, N. Galanti, T. Johnson and S. Murphy, Platelet-tumor-cell interactions in mice. The role of platelets in the spread of malignant disease. *Int. J. Cancer* **11,** 704 (1973).
4. P. Hilgard and E. C. Gordon-Smith, Microangiopathic haemolytic anaemia and experimental tumour-cell emboli. *Brit. J. Haemat.* **26,** 651 (1974).
5. P. Hilgard, The role of blood platelets in experimental metastases. *Brit. J. Cancer* **28,** 429 (1973).

G. Easty (Chester Beatty Research Institute, London S.W.7, U.K.)

BIOCHEMICAL FACTORS

The bone damage inflicted by invading secondaries with associated hypercalcaemia could be due to pressure of expansive growth or to a liberation of substances bringing about lysis of the bone and subsequent release of calcium. In order to investigate the production of osteolytic substances a model system was set up. The bones of mice were labelled with $^{45}Ca\ Cl_2$ and the calvarium dissected out. It was then divided into two parts for a test and a control group. The bones were cultured on grids and tumour cells were placed at a distance from the bones so that any factors released from the cells would diffuse through the medium and the resulting release of ^{45}Ca from the bone could be measured. The system responded well to known osteolytic substances (e.g. Prostaglandin E_2), and the degree of osteolysis was measured by estimating the release of calcium into the medium.

Primary breast cancers of women were tested and the results showed a reasonable correlation between the presence of bone metastases or hypercalcaemia, and the ability of the tumour to cause calcium release in the test system. The effect of aspirin (because of its effectiveness in osteoarthritis) was tested on the release of calcium and a significant depression found. This was not due to a direct cytotoxic effect on the tumour cells, but metabolic experiments indicated that perhaps the aspirin was inhibiting the synthesis of the factor which caused the osteolysis. *In vitro* studies with the Walker tumour (previously shown to possess osteolytic activity) indicated that acid production by the tumour was not the factor involved, because addition of aspirin abolished the osteolysis without affecting the pH of the medium. *In vivo* studies with the same tumour system, showed that aspirin and indomethacin given orally for 7 days after intra-aortic injection of tumour cells, completely abolished bone deposits, although soft tissue secondaries were not affected. Associated with this inhibition of bone secondaries was a reduction of hypercalcaemia in the treated rate.

Clinical trials are now planned with both aspirin and indomethacin to establish whether these agents can retard the development of bone metastases in patients with carcinoma of the breast. Although they are unlikely to inhibit the growth of the tumour, it is hoped that they might alleviate some of the unpleasant effects which accompany it.

L. Owen (Dept. of Animal Pathology, School of Veterinary Medicine, Cambridge CB3 0ES, U.K.)

INFLUENCE OF RADIOTHERAPY

A transplantable osteosarcoma model system has been set up in dogs, by injecting tumour cells intrafoetally at the 6th or 7th week of pregnancy or by injecting them i.v. at birth followed by antilymphocyte serum (ALS) immuno-suppression. Tumours grow rapidly and have a marked affinity for the bone, although some deposits occur in the lungs and soft tissues.

The effect of lung X-irradiation was tested [1]. Radiation given to the right lung (one dose of 600 R given 24 hr after i.v. injection of tumour cells) in ALS treated dogs reduced the amount of tumour. If radiation was given 24 hr before tumour cells an increase in the amount of tumour growing in the irradiated lobe was seen. In cases of spontaneous osteosarcomas in dogs, the primary tumours were either X-irradiated with 4–5000 R or the limb amputated. The right lung (radiographically free of tumour) was X-irradiated using a linear accelerator, 2 doses of 600 R being given with 1 week interval. After periods ranging from 2 months to 1 year, animals were destroyed. Post-mortem examination revealed a difference in the number of metastases between the irradiated and control lungs, although this was only statistically significant in one dog in which more tumours appeared on the X-irradiated side. In this dog the irradiated primary tumour was still *in situ*.

In melanoma of the oropharynx (which is a more radio-sensitive tumour), the primary was excised, and the site was irradiated with 3000 R, with no apparent recurrence at post-mortem. In the 2 dogs where one lung was irradiated (2×600 R), post-mortem examination 4–5 months later revealed metastases in both lungs although they were reduced considerably on the irradiated side.

In a case of squamous carcinoma of the tonsil, the primary tumour together with the submandibular lymph node was excised, and both sites irradiated. One lung was irradiated with 2×600 R. Three months later there was recurrence of the primary tumour and by 5 months euthanasia was necessary. At necropsy there were 57 metastases (measuring 2–3 mm or more) in the irradiated lung and none in the control side. These tumours were associated with post-irradiation pneumonitis. From these results, it can be concluded that irradiation of the lungs from the metastatic point of view is wrong, if there is an active primary tumour

still growing. Post-irradiation infection can result, which in turn can lead to pneumonitis. In the example quoted above, tumour cells developed in the damaged, pneumonic lung, whereas secondaries did not develop in the normal lung, malignant cells presumably passing through it. Experiments are planned where ^{51}Cr labelled cells are injected into dogs with one irradiated and one control lung. Assessment of tumour cells lodgement in both lungs should clarify the influence of irradiation on the development of these tumour deposits.

REFERENCE

1. L. N. Owen and D. E. Bostock, Prophylactic X-irradiation of the Lung in canine tumours with particular reference to Osteosarcoma. *Europ. J. Cancer* **9,** 747 (1973).

K. Hellmann (Cancer Chemotherapy Dept., Imperial Cancer Research Fund, London W.C.2, U.K.)

SIGNIFICANCE OF VASCULAR DEVELOPMENT

The Lewis lung carcinoma (3LL) in mice metastasises spontaneously to the lungs and 21 days after s.c. implantation, macroscopic metastasis can be seen. Histological examination of the primary tumour shows that the invading margin consists of large areas of haemorrhage with blood flowing through very ill-defined vascular sinusoids lined in places by tumour cells and elsewhere by endothelium. The lining of tumour cells can presumably detach themselves easily and enter the blood stream. Treatment with the anti-mitotic/antimetastatic drug ICRF 159 changes the vascular architecture at the periphery of the primary tumour so that well-formed and well-endothelialized vessels develop.

Evidence suggests that this normalization of the tumour blood vessels by ICRF 159 inhibits the formation of lung secondaries in the 3LL system by preventing the release into the circulation of tumour cells with the potential to form metastases. Although selective inhibition of metastases is important experimentally, clinically the limitations of such inhibition is obvious since the majority of patients probably have metastases at the time of diagnosis.

It is possible however, that the change in vascular architecture by treatment with ICRF 159 could improve the blood flow through the tumour and this in turn could result in a higher drug concentration reaching the tumour tissue. It might also provide improved oxygenation of the tumour so that it would respond more readily to irradiation.

Treatment with ICRF 159 not only changes the vessels morphologically, but also functionally. Experiments with i.v. injected "Pelikan" ink have shown that the trapping of carbon particles between the basement membrane and the endothelium seen in control tumour vessels, is abolished following ICRF 159 treatment. X-ray angiographic studies have confirmed that ICRF 159 treatment normalizes the vascular arrangement of blood vessels, not only in the 3LL tumour, but also in the Walker tumour, where the vessels become arranged in a more regular fashion following ICRF 159 treatment.

D. J. Griffiths (Royal Marsden Hospital, London S.W3, U.K.)

PERFUSION OF ISOLATED TUMOURS

Interest in the blood supply of tumours led to the establishment of a perfusion technique for human tumours. This involved removal of the tumour at operation, cannulation and connection to a heart lung type machine and kidney-like unit for extracting metabolites. Although the system is not biologically accurate, it has proved to be very satisfactory for studying the factors which influence the dissemination of tumour cells.

Simple perfusion of the tumour for 15 min and estimation of circulating malignant cell numbers gave a figure comparable to that seen in the periphery before operation. Tumour handling or the addition of streptokinase liberated large numbers of malignant cells. Duke's classification of the tumour correlated very well with the percentage of cancer cells obtained in the perfusate, although the degree of differentiation of the tumours had no significance.

An important fact that emerged from these experiments was the correlation between lack of venous invasion and a high number of circulating tumour cells and this in turn was associated with an increase in survival rate. Furthermore, the increase in tumour cells was associated with a significant fibrinolytic activity, suggesting that generalised fibrinolysis would be beneficial for the patient.

A patient demonstrating thrombus growth in a vein has a poor prognosis. If there is no venous invasion, the prognosis is better even though circulating cells are present. This

suggests the presence of a factor responsible for the difference in prognosis between these patients and its identification and/or manipulation would be of great importance in improving survival.

A. J. Salsbury (Department of Haematology, Brompton Hospital, London S.W.3, U.K.)

THE SIGNIFICANCE OF CIRCULATING CANCER CELLS

The first problem is the identification of malignant cells in the blood and their distinction from the profusion of non-malignant cells in blood concentrates. Having identified the circulating malignant cells, the question must be posed of their significance in the prognosis of the cancer patient. A twelve year follow-up [1] of a group of patients (over 200) who had several samples for blood cell analysis taken from various venous sites, before, during and after operation showed very little difference in survival of those patients who had circulating malignant cells as against those who did not. The results suggested that the prognosis was slightly better if circulating malignant cells were present. In relation to venous invasion, the figures were a little more significant. The prognosis was definitely better in patients who had circulating malignant cells and no venous invasion than in those patients who had circulating cells with venous invasion. These results raise a number of questions: are cancer cells found in the blood viable? what role does host resistance play? and finally, are circulating malignant cells a bad thing? Do they, in fact, represent circulating cells that are unable to implant and will eventually be killed off in the blood, never producing distant metastases?

A study of 50 patients with malignant and 30 with non-malignant disease [2] where fibrinolytic activity (as indicated by several parameters including the euglobulin lysis time) was determined before, during and after operation showed that there was a high incidence of fibrinolytic episodes during operation.

In the tumour group, 77% of patients who developed fibrinolytic episodes had circulating malignant cells whereas only one patient of those with no fibrinolysis showed circulating malignant cells. In a high proportion of the former cases, the tumour itself was fibrinolytic whereas with non-fibrinolytic tumours circulating malignant cells were not found. A follow-up of these patients will be necessary to appraise the significance of the circulating cancer cells.

REFERENCES

1. J. D. Griffiths, J. A. McKinna, H. D. Rowbotham, P. Tsolakidis and A. J. Salsbury, Carcinoma of the colon and rectum: Circulating malignant cells and five-year survival. *Cancer* **31,** 226 (1973).
2. A. J. Salsbury, C. White, P. Tsolakidis, J. A. McKinna and J. D. Griffiths, Fibrinolysis and circulating malignant cells. *Surg. Gynec. Obstet.* **136,** 733 (1973).

Europ. J. Cancer Vol. 11, pp. 39-41. Pergamon Press 1975. Printed in Great Britain

Mammary Cancer Produced in Mice with Estriol*

G. RUDALI, F. APIOU and B. MUEL
Laboratoire de Génétique, Fondation Curie, Institut du Radium, Paris

Abstract—*Estriol is considered by some to be a non-carcinogenic estrogen. It is shown that estriol pellets implanted under the skin produce, after short latencies, a high frequency of mammary tumours in castrated RIII males and castrated (C3H × RIII) F1 males and females. The latencies were the same as those observed in the positive-controls implanted with estradiol pellets.*

INTRODUCTION

ESTRIOL is an estrogen produced by the ovaries. In comparison to estrone and estradiol, the ovaries contain only small amounts of estriol. In pregnant women, however, its extracted level in the urine is very high [1]. In fact, estriol is a metabolite of estrone which is converted by 16-a-hydroxylase to 16-a-hydroxy-estrone and finally to estriol [2].

Compared to the two other major natural estrogens, little research has been devoted to estriol, particularly with regard to its carcinogenic activity for the mammary gland. It is well known that all the natural or synthetic estrogens which have been tested in a suitable animal material have shown a more or less pronounced carcinogenic activity [3–8]. Estriol seemed to be an exception.

Pullinger [9] obtained only few cancers in virgin C3H/f females and Lemon [10] did not observe mammary tumors in DMBA treated Sprague–Dawley female rats which received estriol. He has even stated that estriol should be considered a possible inhibitor for mammary carcinogenesis and that there is competition at the level of the cellular receptors between estriol and estradiol. Huggins and Jensen [11] proposed consideration of estriol as an "impeded estrogen".

It interested us to test the eventual carcinogenic activity of estriol for the mammary gland in castrated (C3H × RIII)F1 male and female mice, highly susceptible to the induction of mammary cancers with hormones [8, 12, 13], and castrated RIII males known for many decades as good animal material for such research [3].

MATERIAL AND METHODS

Mice

(C3H × RIII)F1 mice of both sexes were castrated at the age of 22–24 days: 78 females and 107 males; 46 RIII males were castrated at the same age.

Hormones

On the day of the operation, the animals had implanted under the skin, either a paraffin pellet (negative controls), or 10% estradiol in paraffin (positive controls), or a pellet

Accepted 14 August 1974.

*This paper was ready for publication one year ago. As is customary, we submitted the manuscript to several biologists competent in problems of cancer and endocrinology. One of these reviewers suggested the possibility that our estriol sample contained a high proportion of impurities, such as estradiol. Unfortunately, this original sample was lost and we could not analyse it. Accordingly, we decided to repeat our experiments with a highly purified estriol purchased from another laboratory: the Fluka Cie. These experiments are still in progress, but the available results are the same as those obtained previously.

containing 10% estriol in paraffin (Chemically pure estriol and estradiol were purchased at the Th. Schuchardt–France Cie.).

The pellets were prepared by suspending in the most homogeneous way the hormone in melted cytoparaffin (60°C). A small drop of the mixture was deposited on an ice-cooled Petri dish for instantaneous solidification. The average size of the pellets was 1·5 mm. The weights of the pellets varied between 6·4 and 8·5 mg.

RESULTS

Spectroscopic examination of control has shown that the estrogen content of the pellets before implantation varied between 6 and 9%. The pellets were recovered in extremis in some mice at their sacrifice. It appeared that their pellets contained only 2–5% estrogen. Several hundred microgrammes of estrogens were absorbed in 4–5 months.

Table 1. *Mammary carcinogenesis in (C3H × RIII) F1 castrated female mice implanted with a pellet containing estrogens*

Treatment	Number of mice	Number with MT	Frequencies MT p. 100	Latencies in days
None	34	28	82,3*	446 ± 28
Estradiol	16	15	93,7*	176 ± 31
Estriol	18	18	100	133 ± 10

*The difference between the two frequencies is not significant.

As can be seen in Table 1 and appreciated by the latencies, estriol is at least as active a carcinogen as estradiol for the castrated female. Similar results were obtained with castrated

Table 2. *Mammary carcinogenesis in (C3H × RIII) F1 castrated male mice implanted with a pellet containing estrogens*

Treatment	Number of mice	Number with MT	Frequencies MT p. 100	Latencies in days
None	61	10	16,4	576 ± 54
Estradiol	16	15	93,7	137 ± 15
Estriol	30	25	83,3	135 ± 44

male (C3H × RIII)F1 mice. Table 3 indicates that estriol is also a potent carcinogenic agent for the castrated RIII male.

Table 3. *Mammary carcinogenesis of castrated RIII males implanted with a pellet containing estriol*

Treatment	Number of mice	Number with MT	Frequencies MT p. 100	Latencies in days
None	22	0	0	—
Estriol	24	24	100	137 ± 6

DISCUSSION

In three different experiments, it was demonstrated that contrary to usual statements, estriol seems to be, like all the other estrogens, a potent carcinogenic agent for the mammary gland of some mice.

These results were expected. There was no reason to suppose that estriol should be deprived of such activity. It is well known that natural or synthetic estrogens induce mammary tumors not only by a direct action on the mammary cells, but also through their influence on the pituitary and the modification of the feed-back balance of the endocrine system. It has been established that the FSH-inhibiting activity of estriol and of estrone are identical [1].

One of the interesting points of these results is their contradiction of those obtained in previous research on the same problem but with animals of different genetical constitution and not harbouring the mammary tumor virus [9, 10]. Muhlbock and Boot have shown that in one of their mouse strains, the 020, prolactin is a highly active carcinogenic agent for the mammary gland. Contrarily, estrogens seem to be inactive [14]. In several experiments in progress in our laboratory, similar results were observed in C3H/f and RIII/f mice. Prolactinemia induced by pituitary grafting in the kidney capsule, induce mammary cancers in over 90% of the females. Estriol or estradiol pellets implanted under the skin did not produce tumors.

During a recent meeting of a Working Group of the International Agency for Research on Cancer, no information could be found in the literature on long-term carcinogenicity tests with estriol [15].

REFERENCES

1. G. F. Marrian and J. A. Loraine, *The Clinical Application of Hormone Assay.* p. 169. Livingstone, London (1958).

2. PH. GENET, R. HENRY, S. VANDEL, B. DESFOSSES et M. F. JAYLE, Comparaison de l'activité biologique de l'oestrone, de la 16 α-hydroxy-oestrone et de l'oestriol. *Ann. Endocrinol.* **23,** 693 (1962).
3. A. LACASSAGNE, Apparition de cancers de la mamelle chez la souris mâle soumise à des injections de folliculine. *C.R. Acad. Sci. (Paris)* **195,** 630 (1932).
4. A. LACASSAGNE, A comparative study of the carcinogenic action of certain oestrogenic hormones. *Amer. J. Cancer* **28,** 735 (1936).
5. A. LACASSAGNE, Apparition d'adénocarcinomes mammaires chez des souris mâles traitées avec une substance oestrogène synthétique. *C.R. Soc. Biol. (Paris)* **129,** 641 (1938).
6. J. M. ROBSON and G. M. BONSER, Production of mammary carcinomas in mice of a susceptible strain by a synthetic oestrogen, triphenylene. *Nature (Lond.)* **142,** 836 (1938).
7. G. RUDALI, Production de tumeurs mammaires à l'aide d'un dérivé de l'acide allénolique chez des souris mâles. *C.R. Soc. Biol. (Paris)* **146,** 916 (1952).
8. G. RUDALI, E. COEZY, F. FREDERIC and F. APIOU, Susceptibility of mice of different strains to the mammary carcinogenic action of natural and synthetic oestrogens. *Rev. Europ. Etudes clin. Biol.* **16,** 425 (1971).
9. B. D. PULLINGER, Increase of mammary carcinoma and adenoma and incidences of other tumors in C3Hf virgin females after ovariectomy and high dosage with some estrogens. *Brit. J. Cancer* **15,** 574 (1961).
10. H. M. LEMON, Abnormal estrogen metabolism and tissue estrogen receptor proteins in breast cancer. *Cancer* **25,** 423 (1970).
11. CH. HUGGINS and E. V. JENSEN, The depression of estrone induced uterine growth by phenolic estrogens with oxygenated functions at position 6 or 16: the impeded estrogens. *J. exp. Med.* **102,** 335 (1955).
12. G. RUDALI, E. COEZY and R. CHEMAMA, Mammary carcinognesis in female and male receiving contraceptives or gestagens. *J. nat. Cancer Inst.* **49,** 813 (1972).
13. G. RUDALI, Induction of tumors in mice with synthetic sex hormones. *Gann Monogr.* In press 1975.
14. O. MUHLBOCK and L. M. BOOT, Induction of mammary cancer in mice without the mammary tumor agent by isografts of hypohyses. *Cancer Res.* **19,** 402 (1959).
15. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Sex Hormones. Vol. 6, p. 117 (1974).

Europ. J. Cancer Vol. 11, pp. 43–49. Pergamon Press 1975. Printed in Great Britain

Plasma Half-Life of Cytosine Arabinoside in Patients with Leukaemia—the Effect of Uridine*

BRUCE C. BAGULEY and ELLEN-MARIE FALKENHAUG
Department of Cell Biology, University of Auckland, Private Bag, Auckland, New Zealand

Abstract—*Cytosine arabinoside, used in the treatment of acute granulocytic leukaemia, is not equally effective in all patients, a result which may be related to variability in the rate of its elimination from the body. A sensitive and accurate method, providing results in about 5 hr, has been devised to measure the concentration of the drug in plasma. The method utilizes the inhibition of ^{3}H-thymidine incorporation into the DNA of murine leukaemia L1210 cells* in vitro *by nanogram quantities of cytosine arabinoside. The plasma half-life of cytosine arabinoside in 15 patients studied, all of whom had acute granulocytic leukaemia, varied from 3 to 14 min. Patients obtaining haematological remissions had plasma half-lives of 8–14 min. An attempt was made in 6 patients to inhibit the enzymatic deamination of cytosine arabinoside with uridine and thus increase its plasma half-life. The prior oral administration of uridine (15 g) produced plasma concentrations of uridine of 2–8 μg/ml for several hours, but did not increase the plasma half-life to any practicable extent.*

INTRODUCTION

CYTOSINE ARABINOSIDE (NSC-63878) is now commonly used in the treatment of malignancy especially acute granulocytic leukaemia [1]. Following injection, it is known to be rapidly metabolised by the enzyme cytidine deaminase to an inactive compound, uracil arabinoside [2]. The rate of deamination varies from species to species [3], and in humans, from individual to individual [4–6]. Early estimates of the plasma half-life of cytosine arabinoside [4, 7] were probably low because of further degradation during sample processing [8]. Subsequent studies on 10 patients by Baguley and Falkenhaug [5] provided estimates of half-lives between 3 and 9 min, and those on 8 patients by Ho and Frei [6] showed a variation in the initial plasma half-life of between 7 and 17 min. Of the many methods available for the assay of cytosine arabinoside [4–10], few have been used for human studies. The present communication describes improvements in a biological assay technique [5] which has been used for a reasonably large group of patients, and presents plasma half-life data for 15 patients.

The variation in individual rates of degradation of cytosine arabinoside raises the possibility that some patients may be receiving inadequate doses of this drug. Our previous data [5], together with that presented here, suggest that patients in which the initial half-life for the disappearance of cytosine arabinoside is less than 6 min, have a very low chance of obtaining a remission. Treatment of these patients could be well modified either by

Accepted 31 July 1974.

*Supported by the Auckland Division, Cancer Society of New Zealand, Inc., and in part by grants from the Auckland Hospital Board and the Medical Research Council of New Zealand.

altering the rate of deamination of the drug, or by increasing the dose of drug to compensate for the excessive rate of degradation. We have considered here the possibility of inhibiting the enzyme cytidine deaminase. Representative types of inhibitors of this enzyme [3, 11–13] are shown in Table 1. Tetrahydrouridine [13] is the most potent inhibitor, but was not available for trial. However, uridine itself is a competitive inhibitor of the enzyme [3] and is available clinically for the treatment of *orotic aciduria* [14]. The effect of prior administration of uridine on the plasma half-life of cytosine arabinoside was examined, therefore, in a total of 6 patients.

Table 1. *Inhibitors of the deamination of cytosine arabinoside**

Inhibitor	Concentration	% Inhibition	Reference
Uridine	2×10^{-3}M	86	3
Uracil arabinoside	2×10^{-3}M	80	3
N^4-hydroxy-5-methyl-2′-deoxycytidine	$2{\cdot}8 \times 10^{-4}$M	93	11
9-[*p*-(2-amino-1-hydroxyethyl)-anilino]-6-chloro-2-methoxy-acridine	$3{\cdot}3 \times 10^{-4}$M	88	12
Tetrahydrouridine	$1{\cdot}7 \times 10^{-7}$M	90	13

*Results are taken from data of Camiener and co-workers, who used a preparation of cytidine deaminase from human liver for *in vitro* studies. The concentration of cytosine arabinoside taken for the above data is 10^{-4}M.

MATERIAL AND METHODS

Processing of blood sampless

Cytosine arabinoside ('Cytosar', the Upjohn Co., Kalamazoo, Mich.) was infused i.v. at a dose of 2·5 mg/kg over a period of 30 min. Blood samples (5 ml) were taken from a vein in the arm not used for the infusion of cytosine arabinoside. The samples were collected in heparinised tubes containing tetrahydrouridine (50 μg) and immediately cooled in ice to prevent enzymatic degradation of cytosine arabinoside [8]. Tetrahydrouridine was generously provided by Dr. G. L. Neil, the Upjohn Co., Kalamazoo, Mich.

The samples were centrifuged (2000 ***g***, 10 min) to remove blood cells, and the plasma was centrifuged through a conical "Centriflo" ultrafilter (Type CF50A, Amicon Corp., Lexington, Mass.) for 40 min at 800 ***g*** to remove the majority of plasma proteins. About 1·5 ml of processed plasma was obtained.

Assay method

Murine L1210 leukaemia cells were obtained from Dr. I. Wodinsky, Arthur D. Little Laboratories, and were passaged i.p. in DBA/2 mice or in DBA/2 × C3H hybrid mice. Buffered balanced salt solution (3 ml) as used by Shortman [15] was injected i.p. into a mouse (following cervical dislocation) and aspirated through a 20-gauge needle to remove the L1210 leukaemia cells. These cells were dispersed at a density of 10^6 cells/ml in buffered balanced salt solution containing ^{3}H-thymidine (0·5 μCi/ml, Radiochemical Centre, Amersham, England, made up to 10^{-6} M with non-radioactive thymidine). Duplicate samples (50μl or 200 μl) of the processed plasma samples were added at 4°C to 1 ml portions of the cell suspension. The cell suspensions were incubated in McCartney bottles at 37°C for 2 hr with gentle swirling in a water bath (Metabolyte shaker, New Brunswick Scientific Co., New Brunswick, N.J.). After incubation, the bottles were cooled in iced water and the samples were mixed with an equal volume of 0·9% saline containing 0·3% Nonidet P-40 (Shell Chemical Co.) to lyse any erythrocytes. The contents of each tube were filtered on a glass fibre disc (24 mm, Whatman GF/C) and washed with two portions (10 ml) of ice cold water followed by 1% trichloroacetic acid (10 ml) ahd 5% trichloroacetic acid (10 ml) to remove acid soluble radioactivity.

The filters were dried at 100°C for 2 hr. A shorter drying time (20 min) was possible if the filters were washed by two additional portions of ethanol (10 ml) in the above procedure. The filters were immersed in scintillation fluid [5 ml of a solution of 0·01% 1,4-bis-2-2-(4-methyl-5-phenyloxalolyl)benzene and 0·3% 2,5-diphenyloxazole in toluene].

Assay of uridine

The conditions used were the same as for the assay of cytosine arabinoside, except that

^{14}C-uridine (0·2 μCi/ml, 20 mCi/mM, Radiochemical Centre, Amersham, England) was used in place of ^{3}H-thymidine. The Nonidet P-40 detergent was omitted from the saline in the filtering procedure. A standard series of samples containing known amounts of uridine was included in each set of assays. The decrease in incorporation resulting from dilution of the labelled uridine was compared with that obtained by addition of the processed plasma sample.

RESULTS

Assay of cytosine arabinoside in human plasma samples

The assay system relies on the inhibition by cytosine arabinoside of the incorporation of ^{3}H-thymidine into the DNA of cultured L1210 cells. These cells were removed from leukaemic mice directly before use, and an incubation time of 2 hr was found to be satisfactory. In principle, the inhibition caused by a plasma sample containing an unknown concentration of cytosine arabinoside was compared to that caused by known concentrations. However, plasma samples containing no drug inhibited the incorporation strongly, and the inhibitors had to be removed from the plasma samples by ultrafiltration. Control experiments showed that such ultrafiltration did not alter the concentration of cytosine arabinoside in the filtrate.

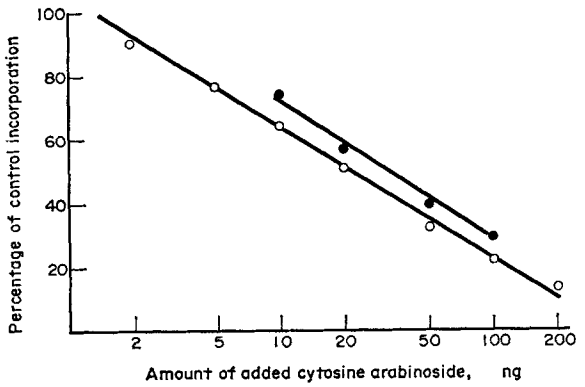


Fig. 1. Effect of addition of different amounts of cytosine arabinoside on the incorporation of ^{3}H-thymidine into DNA of L-1210 leukaemia cells. Cells were incubated in buffered balanced salt solution (1 ml) plus ultrafiltered plasma (50 μl). Results are expressed as a percentage of the incorporation by cells in the absence of cytosine arabinoside (2 × 10^{4} c/m). Open circles and closed circles represent two examples from assays made on different days. In general, points usually fell on the lower line for each set of assays, but sometimes the inhibition was slightly less, as shown by the closed circles.

To compensate for any other stimulatory or inhibitory activity left in the ultrafiltered plasma, blood was taken from the patient before, as well as during and after drug treatment. Plasma from this blood sample was subjected to ultrafiltration and added to assay tubes containing the known concentrations of cytosine arabinoside. In this way, the calibration curve (Fig. 1) could be used confidently to determine unknown drug concentrations from the same patient.

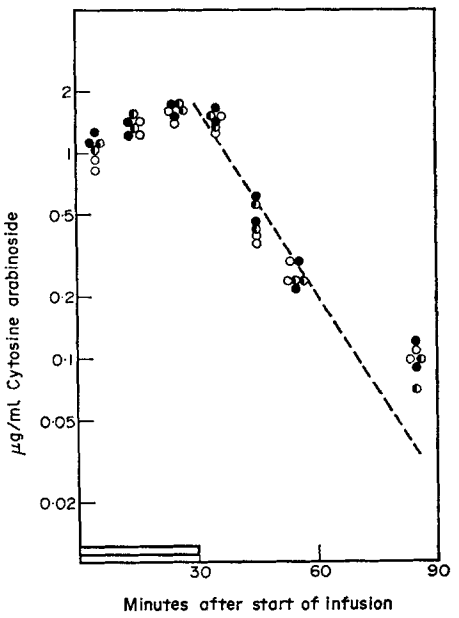


Fig. 2. Results of assay of multiple plasma samples taken during and after a 30 min infusion of cytosine arabinoside (2·5 mg/kg). Plasma samples were analysed in duplicate on three separate occasions, as shown by the different symbols. There was no consistent difference between the three sets of assays. Dashed line shows the calculated decay rate for a plasma half-life of 10 min.

The limit of detection of the method was about 2 ng/ml of cytosine arabinoside. Control experiments showed that changes in the concentration of uridine, tetrahydrouridine, and uracil arabinoside had no effect on the assay system. Results were reproducible from day to day (Fig. 2) with an average error of ± 15%.

Calculation of the plasma half-life of cytosine arabinoside

Each dose of cytosine arabinoside (2·5 mg/kg body weight) was infused over a period of 30 min [16] at a rate of 2 ml/min. The time of the beginning of the infusion was corrected for the time required for the drug to travel

from the reservoir to the patient (5 min). Assuming that body fluid comprised 60% of the body weight [17], the maximum predicted concentration from dosage alone (Co) would be 4·2 μg/ml. Allowing for first-order decay in concentration (decay constant k, where the half-life = 0·693/k), the concentration at the end of the infusion is given by $C = Co/kT\,(1-e^{-kT})$ where T is the infusion time. The concentration at a time t after the end of the infusion is given by $C = Co/kT\,(1-e^{-kT})\,e^{-kt}$. The experimental data were fitted to theoretical curves derived from this equation, in order to estimate the initial half-life of the drug.

Plasma half-life of cytosine arabinoside in a series of patients

All patients had acute granulocytic leukaemia. Treatment varied for different groups of patients as described below. Measurements were made following a dose of cytosine arabinoside (2·5 mg/kg) administered as a 30 min constant infusion (infusion rate monitored manually) at 9:00–9:30 in the morning. Blood samples were taken before, during and after the infusion, and the plasma concentrations were measured. Values obtained during the infusion were dependent on the infusion rate, since the distribution of drug through the circulatory system during this time was non-uniform (i.e. the path from the infusion point to the collection point did not include the liver, the main organ of deamination). Therefore, only samples taken after the completion of the infusion were used for calculation of half-life data. Representative results from 6 of the 15 patients are shown in Figs. 2, 3 and 4. The

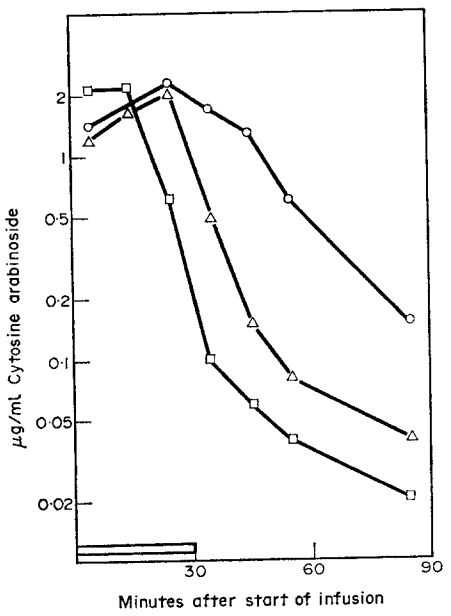


Fig. 3. Assay of plasma samples taken during and after 30 min infusions of cytosine arabinoside (2·5 mg/kg). Results from three patients are indicated as open circles, squares and triangles. The calculated initial plasma half-lives were, respectively, 14, 5 and 3 min.

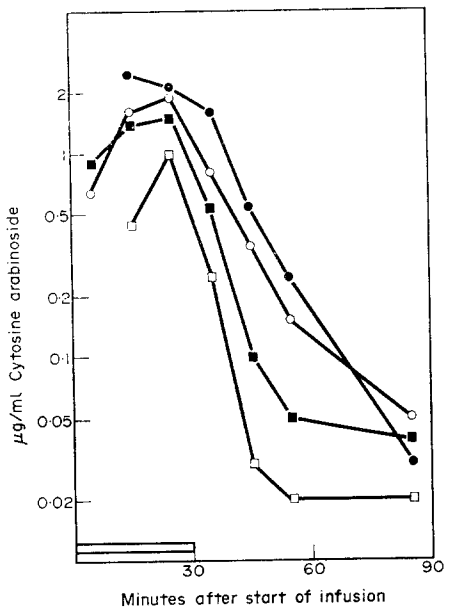


Fig. 4. Assay of plasma samples taken during and after a 30 min infusion of cytosine arabinoside (2·5 mg/kg) with or without prior administration of uridine (15 g). Open circles (no uridine) and closed circles (uridine plasma concentration of 4–6 μg/ml) are for a patient (no remission, died after two courses of treatment) with a plasma half-life of 9 min. Open squares (no uridine) and closed squares (uridine plasma concentration of 8–10 μg/ml) are for a patient (no remission on this therapy, but a subsequent partial remission following therapy with twice the dose of cytosine arabinoside) with a plasma half-life of 3 min.

half-life of cytosine arabinoside appeared to change in most patients when the concentration dropped below 0·1 μg/ml. The initial half-life values were calculated therefore for plasma concentrations of 0·1 μg/ml or more. The results for groups of patients with different histories of treatment can be summarised as follows:

(i) Eight patients were treated with cytosine arabinoside alone (2·5 mg/kg per 12 hr × 8) as described by Buchanan [16]. Only one patient had a remission while on this therapy according to the strict criteria used [18]. This patient had a half-life for cytosine arabinoside of 10 min. The other patients, (half-lives 9, 5, 5, 5, 4, 4, 3 and 3 min) did not obtain a remission while on this treatment, although some did respond subsequently to modified therapy. This group was an atypical one in terms of the general response to therapy [16].

(ii) Two patients were treated with cytosine arabinoside and mercaptopurine as described by Buchanan *et al.* [19]. One, (complete remission) had a plasma half-life of 8 min, and the other, (partial remission) had one of 14 min.

(iii) Five patients had relapsed from previous therapy with cytosine arabinoside and 6-mercaptopurine [19], in which they had obtained remissions (4 complete, 1 partial). During relapse they were treated with cytosine arabinoside alone, and none obtained remissions. The half-lives were 7, 6, 5, 5 and 4 min. In the latter case, plasma samples had been taken during previous courses of treatment and analysed according to our previously described method [5]. The results of the first three courses of treatment indicated a plasma half-life of 7–8 min (treatment at monthly intervals) and those of the following three courses of treatment, 4–5 min.

Effect of uridine on plasma half-life of cytosine arabinoside

Preliminary experiments established that the plasma half-life in (DBA/2 × C3H) mice increased from 17 min to 22 min when the cytosine arabinoside (15 mg/kg) was injected i.p. with a 20-fold molar excess of uridine (Results not shown). Since a pyrogen-free preparation of uridine was not available for human use, uridine (15 g) was given orally to patients. A simple assay system was developed for the assay of uridine, based on dilution of ^{3}H-uridine by non-labelled uridine. The assay system was unaffected by cytosine arabinoside, uracil arabinoside or deoxycytosine. It was found that the plasma uridine concentration increased over the first hour and then remained approximately constant for 90 min. The plasma concentrations obtained varied between 2 and 8 μg/ml. Six patients were treated with uridine (15 g) 60 min before a standard infusion of cytosine arabinoside. Plasma cytosine arabinoside concentrations were determined, and compared to those obtained with infusion of cytosine arabinoside alone, measured during one of the other infusions in the four-day course of treatment. The results of two such comparisons are shown in Fig. 4. Those of the other four patients were similar. Although a slightly greater persistence of the drug was seen following uridine pre-treatment in five of of the six patients, a useful increase in plasma half-life was not obtained.

DISCUSSION

We have described here what we consider a practical method for the measurement of plasma concentrations of cytosine arabinoside during human therapy. The assay system as described here is capable of detecting a plasma concentration of 20 ng/ml of cytosine arabinoside and the sensitivity could be increased by the use of larger plasma samples (500 μl) in the assay tubes. No trouble with interfering substances has been experienced, and because mammalian cells are used in the assay, inhibition by antibiotics is unlikely. The L_{1210} leukaemia cells used for the assay can be routinely passaged twice weekly in the appropriate mice to provide a reserve of cells, and only a small colony of mice is required. If necessary, cultured cells or even suspended mouse spleen cells may be used [5]. We have employed the method for a large number of assays and have found it rapid and reproducible.

The analysis of multiple plasma samples taken during and after infusion of cytosine arabinoside shows that the initial plasma half-life of the drug is in the range of 3–14 min. When the concentration of cytosine arabinoside fell below 0·1 μg/ml, the half-life appeared to increase, as reported by others [6]. It was not possible to correlate the plasma half-life values with the clinical results in this study because of the different types of chemotherapy used. However, it is noteworthy that those patients who responded to therapy had plasma half-lives for cytosine arabinoside of 5–14 min. In our previous study, all patients who failed to respond to therapy had plasma half-lives of 3–4 min. These results taken together suggest that an excessive rate of degradation of the drug contributes to its lack of effectiveness. It is worth noting that all patients in this study showed depression of the number of blood leukocytes following chemotherapy. Burke *et al.* [20] showed that leukocyte depression is not a good indication that a maximum dose of cytosine arabinoside has been administered, since a wide range of doses gave almost the same degree of depression.

Results in one patient, studied over a six month period, suggest that the plasma half-life of cytosine arabinoside may shorten with repeated therapy. This could be a result either of changes in the activity of cytidine deaminase in the liver, or of an increase in the activity of cytidine deaminase in the leukaemic cells themselves [21]. A large number, (10^{13}) of tumour cells containing this enzyme may act

as an effective organ of deamination of the drug.

The study of the effect of administration of uridine on the half-life of cytosine arabinoside was based on the known inhibition of human liver cytidine deaminase by uridine [3], and on the prolongation by uridine of the lives of leukaemic mice treated with suboptimal quantities of cytosine arabinoside [22]. However, the results both with mice and with humans show that uridine did not cause a practical increase in the half-life of cytosine arabinoside. The effect of uridine in mice given cytosine arabinoside i.p. was similar to that reported for tetrahydrouridine [23]. On the other hand, the mouse may not be a good model for studies on cytidine deaminase [24]. The lack of effect of uridine may indicate that, even though plasma concentrations of uridine are sufficiently high, the concentration within cells containing cytidine deaminase remains low. If cytidine deaminase had been inhibited by uridine treatment, it is possible that the plasma concentration of deoxycytidine would have increased. This would interfere with the assay system, since deoxycytidine is about one fifth as effective as cytosine arabinoside in inhibiting thymidine uptake by L_{1210} cells. However, other studies have shown that the concentration of deoxycytidine in plasma is less than 0·3 μg/ml for up to 2·5 hr after administration of uridine (Baguley, unpublished results). An increase of this magnitude would not materially change the results presented here. Nevertheless, more potent inhibitors of cytidine deaminase could allow plasma deoxycytidine concentrations to increase to the point where they not only interfere with the assay system, but also reverse the effect of cytosine arabinoside, as discussed previously [5].

It is concluded that uridine, at the concentrations obtained here, cannot be used to control the degradation of cytosine arabinoside. In that group of patients with short plasma half-lives, however, some modified form of therapy should be investigated. Two possibilities are to increase the amount of drug administered, or to administer the drug by continuous infusion, at a rate which gives a standard plasma concentration of cytosine arabinoside. Measurements of plasma drug concentrations, in either single drug, or combination-chemotherapy, would provide a basis for treatment more in keeping with the biochemical characteristics of the individual patient.

Acknowedgement—The authors wish to thank Associate Professor J. G. Buchanan, Department of Pathology, University of Auckland Medical School, and Dr. J. R. D. Matthews, Auckland Hospital, for arranging the collection of blood samples, for providing clinical information about the patients and for advice and encouragement. The cooperation of Sister S. Gulbis and other members of the staff at Auckland Hospital is also gratefully acknowledged.

REFERENCES

1. R. R. Ellison, J. F. Holland, M. Weil, C. Jacquillat, M, Boiron, J. Bernard, A. Sawitsky, F. Rosner, B. Gussoff, R. T. Silver, A. Karanas, S. Cuttner, C. L. Spurr, D. M. Hayes, J. Blom, L. A. Leone, F. Haurani, R. Kyle, J. L. Hutchinson, R. J. Forcier and J. H. Moon, Arabinosyl cytosine: a useful agent in the treatment of acute leukaemia in adults. *Blood* **32,** 507 (1968).
2. M. Y. Chu and G. A. Fischer, A proposed mechanism of action of 1-β-D-arabinofuranosyl–cytosine as an inhibitor of the growth of leukaemic cells. *Biochem. Pharmacol.* **11,** 423 (1962).
3. G. W. Camiener and C. G. Smith, Studies of the enzymatic deamination of cytosine arabinoside—I. Enzyme distribution and species specificity. *Biochem.* Pharmacol. **14,** 1405 (1965).
4. W. A. Creasey, R. J. Papac. M. E. Markiw, P. Calabresi and A. D. Welch, Biochemical and pharmacological studies with 1-β-D-arabinofuranosylcytosine in man. *Biochem. Pharmacol.* **15,** 1417 (1966).
5. B. C. Baguley and E-M. Falkenhaug, Plasma half-life of cytosine arabinoside (NSC-63878) in patients treated for acute myeloblastic leukaemia. *Cancer Chemother. Rep.* **55,** 291 (1971).
6. D. H. W. Ho and E. Frei, III, Clinical pharmacology of 1-β-D-arabinofuranosyl cytosine. *Clin. Pharmacol. Ther.* **12,** 944 (1971).
7. R. W. Talley, R. M. O'Bryan, W. G. Tucker and R. V. Loo, Clinical pharmacological and human antitumour activity of cytosine arabinoside. *Cancer (Philad.)* **20,** 809 (1967).

8. L. J. HANKA, S. L. KUENTZEL and G. L. NEIL, Improved microbiological assay for cytosine arabinoside (NSC-63878). *Cancer Chemother. Rep.* **54,** 393 (1970).
9. H. H. BUSKIRK, Assay of cytotoxic agents with L-1210 cells. *Proc. Tis. Culture Ass.* **20,** 23 (1968).
10. R. L. MOMPARLER, A. LABITAN and M. ROSSI, Enzymatic estimation and metabolism of 1-β-D-arabinofuranosyl cytosine in man. *Cancer Res.* **32,** 408 (1972).
11. G. W. CAMIENER, Studies on the enzymatic deamination of aracytidine—III. Substrate requirements and inhibitors of the deaminase of human liver. *Biochem. Pharmacol* **16,** 1691 (1967).
12. G. W. CAMIENER and R. V. TAO, Studies on the enzymatic deamination of ara-cytidine—IV Inhibition by an acridine analogue and organic solvents. *Biochem. Pharmacol.* **17,** 1411 (1968).
13. G. W. CAMIENER, Studies on the enzymatic deamination of aracytidine–V. Inhibition *in vitro* and *in vivo* by tetrahydrouridine and other reduced pyrimidine nucleosides. *Biochem. Pharmacol.* **17,** 1981. (1968).
14. D. M. O. BECROFT and L. I. PHILLIPS, Hereditary orotic aciduria and megaloblastic anaemia: a second case, with response to uridine. *Brit. med. J.* **1,** 547 (1965).
15. K. SHORTMAN, N. WILLIAMS and P. ADAMS, The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell debris, damaged cells and erythroid cells from lymphoid cell suspensions. *J. Immunol. Meth.* **1,** 273 (1972).
16. J. G. BUCHANAN, Cytosine arabinoside therapy for acute myeloid leukaemia in adults. *N.Z. med. J.* **74,** 153 (1971).
17. W. S. SPECTOR, *Handbook of Biologic Data*, p. 340. W. B. Saunders, Philadelphia (1956).
18. T. S. GEE, K. P. YU and B. D. CLARKSON, Treatment of adult acute leukaemia with arabinosyl cytosine and thioguanine. *Cancer* **23,** 1019 (1969).
19. J. G. BUCHANAN, J. R. D. MATTHEWS, J. G. DAVISON, R. R. GRIGOR and T. P. CASEY, Treatment of acute myeloid leukaemia of adults with cytosine arabinoside in combination with 6-mercaptopurine. *Cancer* **32,** 789 (1973).
20. P. J. BURKE, A. A. SERPICK, P. P. CARBONE and N. TARR, A clinical evaluation of dose and schedule of administration of cytosine arabinoside (NSC-63878). *Cancer Res.* **28,** 274 (1968).
21. C. D. STEUART and P. J. BURKE, Cytidine deaminase and the development of resistance to arabinosyl cytosine. *Nature New Biol.* **233,** 109 (1971).
22. L. D. SASLAW, G. E. GRINDEY, I. KLINE and V. S. WARAVDEKAR, Sparing effect of uridine on the activity of arabinosylcytosine with normal and leukaemic mice. *Cancer Res.* **28,** 11 (1968).
23. G. L. NEIL, T. E. MOXLEY and R. C. MANAK, Enhancement by tetrahydrouridine of 1-β-D-arabinofuranosyl cytosine (cytarabine) oral activity in L1210 leukaemic mice. *Cancer Res.* **30,** 2166 (1970).
24. D. H. W. Ho, Inhibition of 1-β-D-arabinofuranosyl cytosine phosphorylation in human liver by tetrahydrouridine. *Biochem. Pharmacol.* **21,** 2936 (1972).

Europ. J. Cancer Vol. 11, pp. 51–57. Pergamon Press 1975. Printed in Britain

Herpes Zoster-Varicella Infection in Malignant Lymphomas. Influence of Splenectomy and Intensive Treatment

SILVIO MONFARDINI, EMILIO BAJETTA, CHARLES A. ARNOLD*, RADO KENDA and GIANNI BONADONNA

Instituto Nazionale Tumori, Milan, Italy

Abstract—*The medical records of* 407 *consecutive patients with malignant lymphoma (Hodgkin's disease* 184, *non-Hodgkin's lymphomas* 223) *admitted to the Istituto Nazionale Tumori of Milan from August* 1970 *to April* 1973 *were analyzed for the occurrence of varicella-zoster (V-Z). Two hundred and thirty-two patients underwent laparotomy with splenectomy as part of their diagnostic work up. This procedure was not carried out in* 175 *either because of extralymphatic involvement before surgery or because they were high risk patients. V-Z occurred in a total of* 17% *of patients with Hodgkin's disease and in* 9% *of those with non-Hodgkin's lymphomas, respectively. In Hodgkin's disease the infection was more often encountered in the lymphocytic depletion type* (26%), *while no statistical difference was observed among lymphocytic, histiocytic and mixed type lymphomas. The incidence of infection during the first* 12 *months from staging was* 58% *for Hodgkin's disease and* 71% *for non-Hodgkin's lymphomas. The incidence of V-Z was higher* (16%) *in splenectomized compared to non-splenectomized* (9%) *patients. The difference was statistically significant* ($P < 0{\cdot}05$). *The incidence of zoster was found to be particularly high in splenectomized patients both with Hodgkin's* (27%) *and non-Hodgkin's* (15%) *lymphomas treated with cyclic combination chemotherapy.*

INTRODUCTION

IT IS WELL known that among malignancies, herpes zoster is more common in lymphoma patients, especially in those with Hodgkin's disease [1, 2]. Both varicella and herpes zoster are each generally recognized as a distinct clinical manifestation of infection produced by varicella-zoster (V-Z) virus. While varicella is considered an exogenous infection whose epidemiology is well established, herpes zoster, on the contrary, is suggested to represent a reactivation of latent virus which has remained dormant in the spinal root ganglia [3, 4]. Although the reactivation would be caused in neoplastic disease mainly by irradiation and immunosuppressive therapy, the epidemiology of V-Z is still not well defined. The problem of reinfection versus activation of latent virus is also debated [2, 3].

It is recognized that herpes zoster initially localized to a single dermatome may become generalized [5]. However, the impaired defense mechanisms of patients with lymphoma to V-Z virus results in disease patterns which may differ from those in the general population [6]. The prognosis for lymphoma patients with localized infection has been considered generally to be unchanged by the localized herpes complication, but disseminated V-Z was always associated in the past with a poor prognosis

Accepted 10 July 1974.

*Present address: Palo Alto Medical Clinic, Palo Alto, California, U.S.A.

Reprint requests to Dr. Silvio Monfardini, Via Venezian, 1, 20133 Milan, Italy.

[6, 7]. The incidence of V-Z in patients with Hodgkin's disease was reported to range from 8 to 25% [2, 5, 6, 8]. The infection in non-Hodgkin's lymphoma occurs in about 7–9% [2, 5]. The incidence of V-Z in a total of 726 consecutive patients observed in our Institute from January 1962 to July 1970 was 5% (Hodgkin's disease 21/364, 6%, and non-Hodgkin's lymphomas 15/362, 4%). This low occurrence of V-Z infection can be explained only in part by lack of prolonged follow up information in some patients with advanced disease observed in the Institute from 1962 to 1965. In 1972, Goffinet *et al.*, [5] reported an increased prevalence of V-Z in lymphomas subjected to laparotomy with splenectomy (24%). In their series, the incidence of V-Z was particularly high in splenectomy and non splenectomy patients treated with combination chemotherapy (29%).

This report intends to analyze the occurrence of V-Z infection in all types of malignant lymphoma admitted for staging evaluation from August 1970 to April 1973 and to compare its incidence between the group of patients subjected and that not subjected to splenectomy during the same period of time. In both groups, V-Z is also related to the initial extent of disease and to subsequent treatment.

MATERIAL AND METHODS

Staging laparotomy with splenectomy in Hodgkin's and non-Hodgkin's lymphomas was introduced at the National Cancer Institute of Milan in August, 1970. The charts and the follow up information of 232 patients splenectomized from that period of time to April, 1973 were reviewed. The same retrospective study was performed on 175 patients admitted to our Institute during the same length of time but who were not subjected to laparotomy. In fact, surgical staging was not carried out in 20 patients older than 70 years, in 10 with cardiovascular disturbances, in 21 with marked obesity, and in those (94 patients) who showed diffuse extralymphatic disease with conventional diagnostic methods (X-ray examination, bone marrow aspiration or biopsy, liver biopsy). Furthermore, laparotomy was not carried out in 30 patients who refused surgery.

Splenectomized patients

Of 232 patients subjected to splenectomy, 131 were males and 101 were females. Their mean age was 39 years (range: 6–70 years). Histologic diagnosis was interpreted as follows: Hodgkin's disease, 139 patients, non-Hodgkin's lymphoma, 93 patients (histiocytic 62, lymphocytic 25, mixed histiocytic–lymphocytic type 6). In this group of patients, 187/232 (81%) were untreated previously. In the remaining patients, laparotomy with splenectomy was carried out as reassessment study in presence of lymph node relapse and/or systemic symptoms. This group had been previously treated in other hospitals with limited irradiation and/or single agent chemotherapy.

Non splenectomized patients

Of 175 patients of this group, 111 were males and 64 were females. The mean age was 46 years (range: 4–83 years). Histologic diagnosis was interpreted as Hodgkin's disease in 45 patients, non-Hodgkin's lymphoma in 130 patients (histiocytic 87, lymphocytic 35, mixed histiocytic–lymphocytic type 7, Burkitt's lymphoma 1). There were 140 (80%) untreated patients, while previously treated patients as described above, numbered 35 (20%).

Staging

After all clinical diagnostic tests were carried out, patients were classified according to the criteria proposed at Ann Arbor for Hodgkin's disease and recently adopted also for non-Hodgkin's lymphomas [9]. This classification includes a clinical staging based upon physical, radiographic and laboratory evidence of disease as well as a pathologic staging based upon splenectomy, multiple intra-abdominal specimens, and bone marrow biopsy. After staging, all patients were followed in the Out Patient Clinic. The minimum follow up for those admitted in April is 12 months. Table 1 reports the case series studied from August, 1970 to April, 1973 in relation to splenectomy, prior treatment and stage. Between splenectomy and non splenectomy groups, the percentage of patients with untreated and previously treated Hodgkin's disease and non-Hodgkin's lymphoma appears comparable. In both Hodgkin's and non-Hodgkin's lymphomas, the percentage of patients with Stage IV disease is higher in cases previously treated and in those with no splenectomy as compared to untreated and splenectomized patients. Clinical measurement of adaptive immunity was not systematically determined at diagnosis. In about one fourth of patients, cutaneous reactivity (DNCB, PPD, Dermatophytin and mumps skin tests) was measured. In few patients, *in vitro* lymphocyte blastogenesis was carried out.

The type of treatment (radiotherapy and/or combination chemotherapy) was carried out in

relation to stage and type of lymphoma. In each major group of lymphoma (Hodgkin's disease and non-Hodgkin's lymphoma) treatment was about the same, irrespective to splenectomy or non splenectomy [10, 11].

A patient was recognized as having localized herpes zoster when dermatomal vesicular involvement was observed. Dissemination was noted when progressive widespread vesicular lesions followed the original dermatomal involvement. Varicella was diagnosed when classical generalized eruption occurred without specific dermatomal involvement, and multiple papular and vesicular lesions developed at the same time. Patients with localized herpes zoster were given only supportive therapy while those with disseminated infection were treated with gamma globulins.

Table 1. *Case series from August* 1970 *to April* 1973

		Total	Untreated	Stage I	Stage II	Stage III	Stage IV	Previously treated	Stage I	Stage II	Stage III	Stage IV
Splenectomy	Hodgkin's lymphoma	139	108 (78%)	28	36	37	7 (6·4%)	31 (22%)	2	8	17	4 (13%)
	Non-Hodgkin's lymphoma	93	79 (85%)	19	20	28	12 (15%)	14 (15%)	3	3	4	3 (21%)
		232	187 (81%)				19 (10%)	45 (19%)				7 (16%)
No splenectomy	Hodgkin's lymphoma	45	32 (71%)	10	2	10	10 (31%)	13 (29%)	0	3	2	8 (62%)
	Non-Hodgkin's lymphoma	130	108 (83%)	21	19	6	62 (57%)	22 (17%)	2	0	3	17 (77%)
		175	140 (80%)				72 (51%)	35 (20%)				25 (71%)

RESULTS

Table 2 reports for both groups of Hodgkin's and non-Hodgkin's lymphoma the incidence of V-Z in relation to sex and histologic variant irrespective to splenectomy and non splenectomy. No difference was observed between males and females. In Hodgkin's disease, the lymphocytic depletion type was found to show the highest incidence of V-Z compared to the other histologic subtypes. On the contrary, no significant difference was observed among histiocytic, lymphocytic and mixed type lymphomas.

Table 3 outlines the incidence of V-Z in relation to the initial clinical or pathologic stage. In the present series, Stage IV patients did not show a higher percent incidence of infection compared to other stages, with the exception of the non splenectomized patients with Hodgkin's disease. The highest incidence of V-Z occurred in Stage III Hodgkin's disease after splenectomy (30%).

The infection occurred in Hodgkin's disease more often (17%) than in non-Hodgkin's lymphomas (9%), both in splenectomy and in non splenectomy groups. Furthermore, there was a statistical difference between the incidence of V-Z in the whole group of splenectomized (16%) and that of non splenectomized (9%) patients ($P < 0{\cdot}05$). On the contrary, within each of the two major groups of lymphomas there was no significant difference between splenectomized and non splenectomized patients (Hodgkin's disease 19% vs 11%, non-Hodgkin's lymphomas 12% vs 8%). Also, no statistical difference was observed within each of the four groups in the mean age of patients with and without V-Z.

In 47/52 patients (90%) the infection developed initially as localized zoster while in 5 it occurred as generalized eruption (varicella). In 3/5 patients, splenectomy had been previously performed. In 9/47 (20%), an initially localized herpes became generalized and 8/9 patients underwent splenectomy. Table 4 reports the time distribution in the 52 infected patients. The incidence of infection during the first 12 months from staging was 58% for Hodgkin's disease and 71% for non-Hodgkin's lymphomas. This could be explained in part by a house epidemic of V-Z infection occurring

from May 15 to August 15, 1972. During the time of outbreak a total of 22 patients with Hodgkin's disease (21 adults and 1 child) and 26 patients with non-Hodgkin's lymphomas (22 adults and 4 children) respectively, were exposed to the infection in the medical ward. The incidence of V-Z infection in the whole group of lymphomas was 17% (Hodgkin's disease 18%, non-Hodgkin's lymphomas 15%). but not significant incidence (27%) of V-Z as compared to non-splenectomized patients (11%). This finding was also observed in patients with non-Hodgkin's lymphomas (15% vs 6%). However, considering both Hodgkin's and non-Hodgkin's lymphomas, the incidence of infection was significantly higher in splenectomized patients who received chemotherapy (22%) compared to the group which was

Table 2. *Varicella-zoster in relation to sex and histologic variant*

Hodgkin's disease			Non-Hodgkin's lymphomas		
Total 184 M 100	17/100	(17%)	Total 223 M 141	12/141	(9%)
F 84	14/84	(17%)	F 82	9/82	(11%)
Lymphocyte predominance	3/33	9%	Histiocytic	15/149	10%
Nodular sclerosis	9/50	18%	Lymphocytic	4/60	7%
Mixed cellularity	7/48	15%	Mixed	2/13	15%
Lymphocyte depletion	12/47	26%	Burkitt's lymphoma	0/1	—
Unclassified	0/6	—			

Table 3. *Varicella-zoster infection in relation to stage and type of lymphoma*

Stage	Splenectomy		No splenectomy	
	Hodgkin's disease	Non-Hodgkin's lymphomas	Hodgkin's disease	Non-Hodgkin's lymphomas
I	4/30 (13%)	2/23 (9%)	1/10 (10%)	123 (4%)
II	5/44 (11%)	3/23 (13%)	0/5	4/19 (21%)
III	16/54 (30%)	6/32 (19%)	1/12 (8%)	2/9 (22%)
IV	1/11 (9%)	0/15	3/18 (17%)	3/79 (4%)
Total	26/139 (19%)	11/93 (12%)	5/45 (11%)	10/130 (8%)
	37/232 (16%)		15/175 (9%)	

Two patients 6 and 8 years old with classic varicella were excluded from evaluation.

In this particular series, the occurrence of V-Z was lower in lymphomas of adults who underwent splenectomy (8%) compared to those who had no splenectomy (29%).

The total maximal dermatomal incidence of V-Z occurred in sacral and thoracic areas, the lowest in the cranial area (Table 5). In 37/52 patients (71%), localized herpes zoster developed in an irradiated area. All 5 patients with varicella had received prior radiation therapy, and 3/5 patients underwent splenectomy.

V-Z developed in patients with (32%) and without (67%) active disease. As shown in Table 6, splenectomized patients with Hodgkin's disease treated with intensive cyclic combination chemotherapy showed a higher splenectomized but not treated with chemotherapy (7%). Death following viral infection occurred in 3 patients who had no clinical evidence of lymphoma. In 2/3 patients, the eruption occurred as varicella and both patients died of fulminant bronchopneumonia. In the third patient, an initially localized herpes zoster became generalized within three days from clinical onset, and the patient died of acute encephalomyelitis. All three patients had been previously splenectomized 10, 11 and 13 months, respectively. Six out of 7 patients who developed generalized herpes zoster from an initially localized infection underwent prior splenectomy. From our data, we cannot state that V-Z *per se* influenced unfavourably the course of disease, since in only 11/47 patients

(23%) did the disease relapse with a median of 3 months (range: 0–7) from beginning of infection. In this group of patients, zoster occurred within 6 months from staging in 4 patients, between 6 and 12 in 5, and after 12 months in 2. Six out of 11 patients died because of progressive lymphoma with a median of 6 months from infection.

DISCUSSION

It was already well established [2, 5, 6] that patients with malignant lymphoma, are unusually susceptible to V-Z infection. The development of this viral infection was found to be increased in the past few years. The reason for this high incidence could be attribut-

Table 4. *Time distribution of varicella-zoster in* 52 *patients*

Histology	Patients with V–Z	Months from staging* 0–6	7–12	13–18	19–24	> 24
Hodgkin's disease	31/184 (17%)	C 1/45 P 3/139	2/35 12/114	1/25 5/62	1/20 4/33	0/13 2/20
Non-Hodgkin's lymphomas	21/223 (9%)	C 4/130 P 4/93	2/72 5/68	0/45 0/45	1/27 2/21	3/14 0/8
Total	52/407 (13%)	C 5/175 (3%) P 7/232 (3%)	5/107 (4%) 17/182 (9%)	1/70 (1%) 5/107 (5%)	2/47 (4%) 6/54 (11%)	3/27 (11%) 2/28 (7%)

*Total/at risk
C: Clinical stage
P: Pathological stage

Table 5. *Varicella-zoster infection in relation to dermatomes and to the number of radiation therapy fields*

Primary dermatomal location	Hodgkin's disease Total with V–Z	Hodgkin's disease Total infected /total fields	Non-Hodgkin's lymphomas Total with V–Z	Non-Hodgkin's lymphomas Total infected /total fields	Total incidence of V–Z
Cranial	2	0/3	0	0/80	2
Cervical	1	1/142 (0·7%)	4	2/99 (2%)	5
Thoracic (1–6)	7	7/142 (5%)	3	2/33 (6%)	10
Thoracic (7–12)	8	7/141 (5%)	3	0/32	11
Lumbar	3	3/83 (4%)	3	1/55	6
Sacral	6	5/60 (8%)	7	4/49 (8%)	13
Varicella	4	4	1	1	5
Total	31	27/571 (5%)	21	10/348 (3%)	52 (100%)

Table 6. *Influence of combination chemotherapy and splenectomy*

	No splenectomy No chemotherapy	No splenectomy combination chemotherapy	Splenectomy No chemotherapy	Splenectomy Combination Chemotherapy
Hodgkin's disease	2/18 (11%)	3/27 (11%)	5/62 (8%)	21/77 (27%)
Non-Hodgkin's lymphomas	4/32 (12·5%)	6/98 (6%)	2/32 (6%)	9/61 (15%)
Total	6/50 (12%)	9/125 (7%)	7/94 (7%)	30/138 (22%)

ted in part, to the wide use of intensive treatment with radiotherapy and/or combination chemotherapy [5, 8]. The modern aggressive therapy in fact, has been suggested to be responsible, through depression of immune reactivity, for the increased occurrence of V-Z infection in early stages [8]. The fact that patients with extralymphatic involvement are now controlled for longer periods of time would explain the more frequent observation of clinical infection in advanced disease. Our findings in lymphoma patients treated before and after 1970 are consistent with this hypothesis. The mounting evidence of increased incidence of V-Z infection in lymphoma patients could also be due to the fact that this type of lymphoprolipherative disorders is now preferentially concentrated in centers specialized for diagnosis, treatment and follow up of neoplastic disease. Therefore, it is more likely that in these centers an initial episode of V-Z infection could be followed, as in our own experience as well as in that of Schimpff *et al.* [2], be serial propagation either in the ward or in the out patient clinic.

An analysis of factors predisposing malignant lymphomas to V-Z should first take into consideration the histologic type and the extent of disease. Patients with Hodgkin's disease are notoriously more susceptible to V-Z than patients with non-Hodgkin's lymphomas. Furthermore, in Hodgkin's disease, the infection seems to occur preferentially in lymphocytic depletion type as in our series and in mixed cellularity type as reported by Wilson *et al.*, [8]. On the contrary, we did not find a particular difference among the three major histologic expressions of non-Hodgkin's lymphomas. The incidence of V-Z was reported to increase proportionally with the anatomical extent of lymphoma and particularly with Stage IV disease. Our findings however, do not support this observation. This discrepancy could be explained in part, by the fact that tissue diagnosis of extralymphatic disease was carried out earlier than in other series. Among other conditions predisposing to V-Z infection, the most documented is radiotherapy, especially within an irradiated nerve root level. Finally, intensive cyclic chemotherapy after splenectomy has been reported recently by the Stanford group [5] to produce susceptibility to zoster in Hodgkin's disease.

Our results in both untreated and previously treated Hodgkin's and non-Hodgkin's lymphomas support the evidence that splenectomy *per se* increases the incidence of V-Z. This observation is in line with the results of Goffinet *et al.*, [5]. Furthermore, localized zoster becoming generalized and varicella were more often encountered in splenectomized patients. The incidence of infection was also increased in postsplenectomy patients with both Hodgkin's and non-Hodgkin's lymphomas treated with combination chemotherapy. Furthermore, from our experience, the incidence of V-Z was apparently independent from clinical status of lymphoma (clinical remission or active disease).

Although the incidence of bacteremia was not found to be increased in lymphomas undergoing splenectomy [12], splenectomized patients may be occasionally more susceptible to fulminant infections [13]. In our series, three deaths were attributable to uncontrolled infectious complications following either varicella or disseminated zoster, and they all occurred in splenectomized patients. Preventive measures in this potentially contagious disease should first include the recognition of high-risk patients (splenectomized lymphomas treated with radiotherapy and especially with combination chemotherapy). Furthermore, strict isolation of infected patients as well as separation of the exposed patients should be instituted to avoid large outbreaks among susceptible cases. Finally, the prophylactic administration of gamma globulin and of hyperimmune plasma should by tried in exposed patients in the attempt to prevent or attenuate the eruption. Recent data reported by Geiser *et al.*, [14] showed that in children with leukemia or other neoplasms, prophylaxis with zoster immune plasma (ZIP) significantly decreased the incidence of varicella. Their results clearly indicate that ZIP was more effective than gamma globulin.

In conclusion, we believe that laparotomy with splenectomy is still an important diagnostic procedure especially in patients with Hodgkin's disease, since accurate staging is the most important initial step for a correct and potentially curative treatment. Physicians should be aware that evidence is emerging that splenectomy increases the incidence of V-Z infection. This is especially true in splenectomized patients who require intensive combination chemotherapy. Splenectomized patients appear also to be more susceptible to disseminated zoster. This condition may further be complicated with fatal infections, although from available data this occurrence appears to be rare in adult patients.

REFERENCES

1. E. T. WRIGHT and L. H. WIENER, Herpes zoster and malignancy. *Arch. Derm.* **84,** 242 (1961).
2. S. SCHIMPFF, A. SERPICK, B. STOLER, B. RUMACK, H. MELLIN, J. M. JOSEPH and L. BLOCK, Varicella zoster infection in patients with cancer. *Ann. intern. Med.* **76,** 241 (1972).
3. L. H. MILLER and P. A. BRUNNEL, Zoster, reinfection or activation of latent virus? *Amer. J. Med.* **49,** 480 (1970).
4. D. RIFKIND, The activation of varicella-zoster virus infections by immunosuppressive therapy. *J. Lab. clin. Med.* **68,** 463 (1966).
5. D. R. GOFFINET, E. J. GLATSTEIN and T. C. MERIGAN, Herpers zoster-varicella infections and lymphoma. *Ann. intern. Med.* **76,** 235 (1972).
6. J. E. SOKAL and D. FIRAT, Varicella-zoster infection in Hodgkin's disease. *Amer. J. Med.* **39,** 452 (1965).
7. J. G. MERSELIS, D. KAYE and E. W. HOOK, Disseminated herpes zoster. *Arch. intern. Med.* **113,** 679 (1964).
8. J. F. WILSON, G. W. MARSA and R. E. JOHNSON, Herpes zoster in Hodgkin's disease. *Cancer (Philad.)* **29,** 461 (1972).
9. U. VERONESI, R. MUSUMECI, F. PIZZETTI, L. GENNARI and G. BONADONNA, The value of staging laparotomy in non-Hodgkin's lymphomas (With emphasis on the histiocytic type). *Cancer (Philad.)* **33,** 446 (1974).
10. M. DE LENA, S. MONFARDINI, G. BERETTA, F. FOSSATI BELLANI and G. BONADONNA, Clinical trials with intensive chemotherapy and radiotherapy in Hodgkin's disease. *Nat. Cancer Inst. Monogr.* **36,** 403 (1973).
11. G. BONADONNA, M. DE LENA, A. LATTUADA, F. MILANI, S. MONFARDINI and G. BERETTA, Combination chemotherapy and radiotherapy in non-Hodgkin's lymphomas. *Brit. J. Cancer* 1974 (In press).
12. S. S. DONALDSON, M. R. MOORE, S. A. ROSENBERG and K. L. VOSTI, Characterization of postsplenectomy bacteremia among patients with and without lymphomas. *New Engl. J. Med.* **287,** 69 (1973).
13. R. K. DESSER and J. E. ULTMANN, Risk of severe infection in patients with Hodgkin's disease and lymphoma after diagnostic laparotomy and splenectomy. *Ann. intern, Med.* **77,** 143 (1972).
14. C. F. GEISER, Y. BISHOP, M. MYERS and N. JAFFE, Prophylaxis of varicella with zoster immune plasma in children with neoplastic diseases. *Proc. Amer. Ass. Cancer Res.* **15,** (Abstr. 761) 174 (1974).

Europ. J. Cancer Vol. 11, pp. 59–63. Pergamon Press 1975. Printed in Great Britain

Human Mammary Carcinoma Cell Line: Infection by an Avian Myxovirus as a Prerequisite for Immunopotentiation*

CHRISTIAN SAUTER[1], THOMAS BÄCHI[2] and JEAN LINDENMANN[2]
[1]*Division of Oncology, Department of Medicine and* [2]*Division of Experimental Microbiology, Institute of Medical Microbiology, University of Zürich,* 8006 *Zürich, Switzerland*

Abstract—*In view of the proven potentiation of tumor antigen activity produced by infecting tumor cells with myxoviruses, a strain of avian influenza virus (A/Turkey/England/63, Hav 1, Nav 3) previously adapted to grow in HeLa cells was passed serially 8 times in the human mammary carcinoma cell line BT 20. Comparisons of growth curves of the starting virus with the eighth passage level showed that adaptation had taken place. Immune electron microscopy using ferritin-labelled antiviral antibody revealed viral particles budding at and viral antigens incorporated in cell membranes. The possible use of such a virus for "virus-assisted immunotherapy" is discussed.*

INTRODUCTION

AUGMENTATION of tumor cell immunogenicity by infection with viruses has been shown in several systems [1–5]. Enveloped viruses, mostly influenza viruses, were used for this purpose, since the association of viral and host antigens at membrane sites is apparently important for the augmentation of tumor cell immunogenicity [6].

An essential step for the use of viruses as immunological potentiators in the immunotherapy of human neoplasms is the successful replication of an enveloped virus in the tumor cell in question. Fowl plague virus (FPV), an influenza A virus, has been adapted to several types of human tumor cells [7, 8] and has been shown to replicate regularly in primary cultures of human leukemic cells [9]. In this communication, we report the adaptation of the same virus strain to a human mammary carcinoma cell line as a first step towards obtaining a virus capable of growing in primary cultures of human mammary carcinoma.

Accepted 17 June 1974.

*This investigation was supported by the Julius Müller Foundation for Cancer Research, Zürich, and the Cancer Research Institute, Zürich Branch.

MATERIAL AND METHODS

Virus

The fowl plague virus (A/Turkey/England/63, Hav 1, Nav 3, Langham strain) which we used in these experiments had the following passage history: Obtained from H. G. Pereira, World Influenza Center, Mill Hill, London, England, after 4 allantoic passages; the virus was passed 3 times in ICR mouse kidney cell cultures, 18 times in KB cells, twice in HeLa cells, once in the chick allantois and once in a primary culture of human bronchial carcinoma cells. This virus will be referred to as FPV_0 (having had no passage in mammary carcinoma cells). The subscript numbers following FPV will indicate the number of passages in mammary carcinoma cells.

Human mammary carcinoma cell line

The human mammary carcinoma cell line BT 20 [10] was obtained from L. Ozzello, University of Lausanne, Switzerland.

Culture media

BT 20 cells were grown in RPMI 1640 (Grand Island Biological Company, Grand Island, N.Y., USA), supplemented with 2% fetal calf serum (FCS). Virus adaptation, virus replication, and electron microscopy were done on cells maintained in Eagle's minimum essential medium (MEM) supplemented with 2% FCS.

Virus adaptation

The virus for each passage was taken from the previous passage at the time of first detection of hemagglutinin. Every 12–24 hr the supernatant was checked for hemagglutinin. For each passage, a 25 cm^2 Falcon plastic bottle (Falcon Plastics, Oxnard, Calif. USA) containing a complete monolayer of BT 20 cells was washed with MEM and infected with 0·33 ml of a virus dilution in MEM containing between $10^{1·0}$ to $10^{3·9}$ $TCID_{50}$ (50% tissue culture infectious dose) (see Table 1). The infected culture was incubated for 15 min at 37°C with agitation every 5 min for virus adsorption. Four-point-six-six ml of MEM with 2% FCS were then added and incubation was continued until detection of hemagglutinin. At this time the supernatant was harvested, and aliquots were sealed in sterile glass ampules and stored at −80°C until infectivity titrations or a further adaptation passage could be performed.

Virus replication curves

To compare the replication of FPV_0 and FPV_8 in BT 20 cells the following experiment was done: two 75 cm^2 Falcon plastic bottles containing a complete monolayer of BT 20 cells were washed with MEM. To one bottle was added 1 ml of FPV_0 containing about $10^{2·5}$ $TCID_{50}$, to the other bottle, 1 ml of FPV_8 containing also about $10^{2·5}$ $TCID_{50}$. The infected cultures were incubated for 15 min at 37°C with agitation every 5 min for virus adsorption. Nineteen ml of MEM with 2% FCS were then added and incubation was continued. Five ml of supernatant of each bottle were removed (and replaced by 5 ml of fresh medium) at 4, 22, 46, 70, 94, and 176 hr of incubation. The removed samples were checked for hemagglutinin, put into sterile glass ampules and kept at −80°C until infectivity titrations could be performed.

Virus titrations

Virus infectivity assays were performed in 16 x 100 mm glass tubes containing chicken embryo fibroblast monolayer cultures. The monolayers were infected with serial 10-fold dilutions of virus in MEM without serum. Six tubes per dilution were used. After 72 hr of incubation at 37°C, the tubes were screened for cytopathic effects. Tubes with incomplete destruction of the monolayers were checked for hemagglutinin. The $TCID_{50}$'s were calculated according to the method of Reed and Muench [11]. The HA titrations were done according to WHO standard procedures.

Antisera

Antiserum was prepared to FP virus grown in embryonated hens' eggs. Allantoic fluid with an HA titer of 512 was stored at −80°C until used for immunization. Rabbits immunized by 5 weekly intramuscular injections of 2 ml virus with 2 ml complete Freund's adjuvant were bled 14 days after the last injection. The sera, after inactivation (56°C, 30 min) and exhaustive absorption with washed human erythrocytes (0/Rh+), showed a hemagglutination inhibition titer of 1280 against FP virus as tested with fowl red cells. Guinea pig anti-rabbit IgG serum conjugated with ferritin was prepared as described elsewhere [12].

Electron microscopy

Cells from infected (16 hr after inoculation with FP virus) or uninfected cultures were washed three times with cold Earle's balanced salt solution (EBSS). The monolayers were incubated with rabbit anti-FP serum for 30 min at 4°C) washed three times with cold EBSS, incubated (30 min, 4°C) with guinea pig anti-rabbit IgG conjugated with ferritin and washed again three times with cold EBSS. The cells were then fixed (30 min, 4°C), with a mixture of 3% glutaraldehyde and 3% acrolein in 0·05 M cacodylate buffer, pH 7·2. All cells were post-fixed for 60 min at 4°C with 2% osmium tetroxide in 0·1 M cacodylate buffer, pH 7·2. The fixed material was left overnight in a 2% solution of uranyl acetate before dehydration and embedding in Epon–Araldite. The thin sections were stained with lead citrate.

RESULTS

Adaptation of FPV to BT 20 cells

FPV was passed serially in BT 20 cells in order to get an optimal adaptation to this cell line. In Table 1, the growth characteristics of the different passages are shown. The virus inputs varied between $10^{1·0}$ to $10^{3·9}$ $TCID_{50}$ due to the fact that the viral dilutions had to

be chosen arbitrarily, the infectivity titer of the previous passage being unknown at the time of infection of the following passage. To select for particles capable of replicating in BT 20 cells, the dilutions from one passage to the next were at least 100-fold.

Based on our experience with FPV in HeLa cells [7], we expected that a few passages would suffice for optimal adaptation. From the fifth passage on, hemagglutination was regularly detected at 46 hr of incubation. Regardless of the input, the $TCID_{50}$ was always around 10^6/ml at 46 hr of incubation in later passages. We therefore stopped the adaptation experiments at the eigth passage. Comparing the data of the second and sixth passage (same virus inputs, Table 1) the virus

Table 1. *Serial passage of FPV in BT* 20 *monolayer cultures*

Passage number	Virus input* (Virus from previous passage)	Time to detection of hemagglutinin (hr)	$TCID_{50}$/ml at the time of first detection of hemagglutinin
1	$10^{3.9}$	102	$10^{4.25}$
2	$10^{2.0}$	84	$10^{4.75}$
3	$10^{1.3}$	72	$10^{5.5}$
4	$10^{1.0}$	57	$10^{5.0}$
5	$10^{2.5}$	46	$10^{5.5}$
6	$10^{2.0}$	46	$10^{6.0}$
7	$10^{3.5}$	46	$10^{6.0}$
8	$10^{3.0}$	46	$10^{5.5}$

*$TCID_{50}$ per monolayer.

replication in the later passage was definitely better with respect to replication speed as well as to infectivity titer.

Optimal adaptation may have already been reached by the third or fourth passage, since the delay in hemagglutinin appearance and the somewhat lower infectivity titer could have been due to the very low input in these passages. A good adaptation of FPV_0 to BT 20 cells therefore seemed to require at least two, but probably four, serial passages.

Comparison of the replicating of FPV_0 and FPV_8 in BT 20 *cells*

Figure 1 shows the growth curves of FPV_0 (no passage in BT 20 cells) and FPV_8 (8 passages in BT 20 cells). The drop of the infectivity titer until four hr of incubation was about the same for both viruses. FPV_8 then replicated much faster and to a higher titer than FPV_0. For FPV_8, hemagglutinin reached a maximal titer of 32 at 176 hr of incubation, whereas for FPV_0, no hemagglutinin could be detected until the end of the experiment at 176 hr.

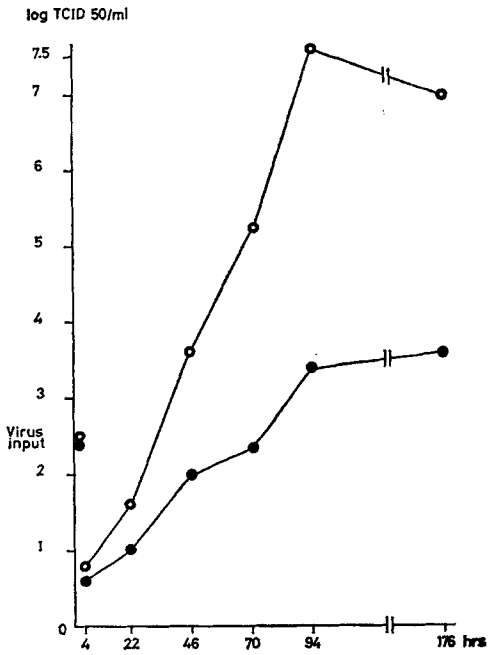


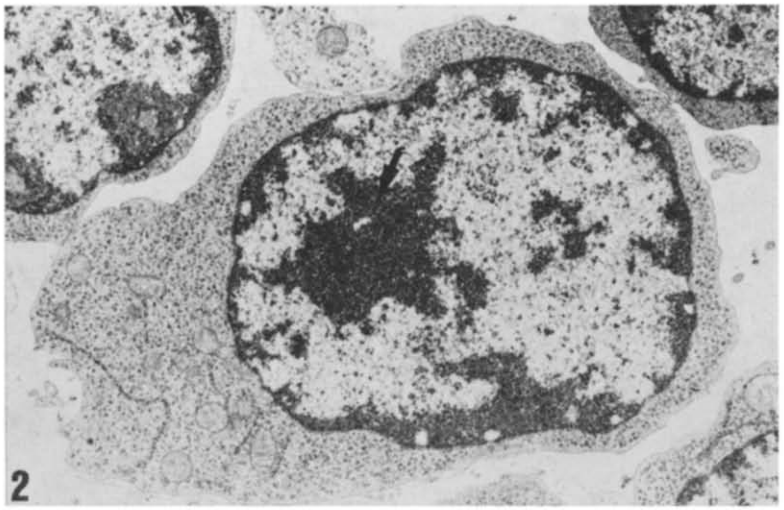
Fig. 1. *Replication of FPV_0 (●——●, no passages in BT* 20 *cells) and FPV_8 (○——○, eight passages in BT* 20 *cells) in BT* 20 *cell monolayers.*

Cytopathogenic effect of FPV on BT 20 *cells*

A slight cytopathogenic effect (CPE) of FPV on BT 20 cells was seen at the time of the first detection of hemagglutinin. At this stage, cytoplasmic vacuolization and a few rounded cells were observed. Twenty-four hours later, an almost complete CPE was seen (Fig. 2B). Most of the cells had detached from the bottom of the plastic bottle, were round, and took up trypan blue stain.

Electron microscopy

In cultures fixed 16 hr after inoculation with FP virus, virions were frequently found to be attached to cell surfaces (Fig. 3). Occasionally, budding of virus was observed at bulging segments of the cytomembrane (Fig. 4). In addition to the morphological features characterizing these structures as viruses, immunological tagging by ferritin labelled antibody revealed viral antigenicity (Figs. 3–6). Attached or budding virus particles invariably were tagged heavily with labelled antibodies visualized by the electron dense core of the ferritin molecule. The specificity of the reaction was established by treatment of uninfected cultures with the same reactants; here, all ferritin molecules were washed out because the antiviral antibody was not bound to the cells (Fig. 7). Reaction of antibodies with membranes of infected cultures not revealing any characteristic viral differentiation demonstrated the presence of viral antigens also in segments of



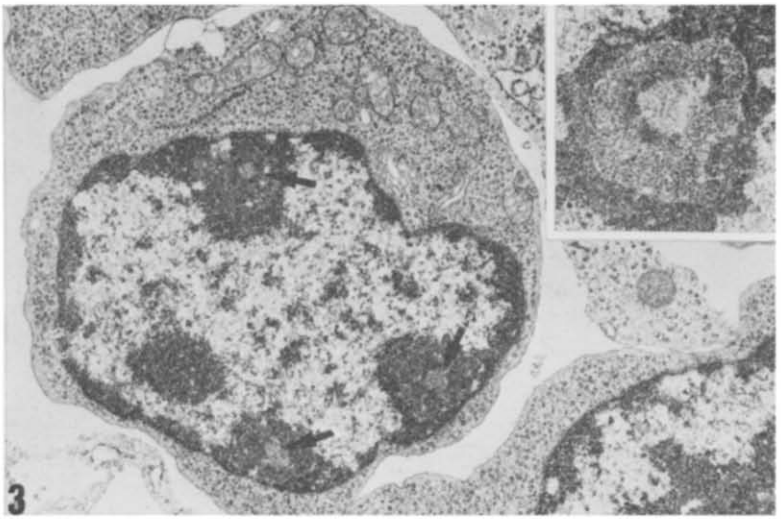




Fig. 2. *Typical lymphoblast: the dense chromatin is disposed in a thin peripheral rim; the nucleolus (arrow) is compact.* (× 8250).

Fig. 3. *Ring-shaped nucleolus cell: the nucleolar (arrow) components are disposed in a characteristical ring-like disposition (inset). The dense chromatin is similar to that observed in typical lymphoblasts.* (× 9500; *inset:* × 20700).

Fig. 4. *X cell: the chromatin is condensed in an irregular peripheral strand and in blocks dispersed in the nucleoplasm and around the large nucleolus (arrow).* (× 10750).

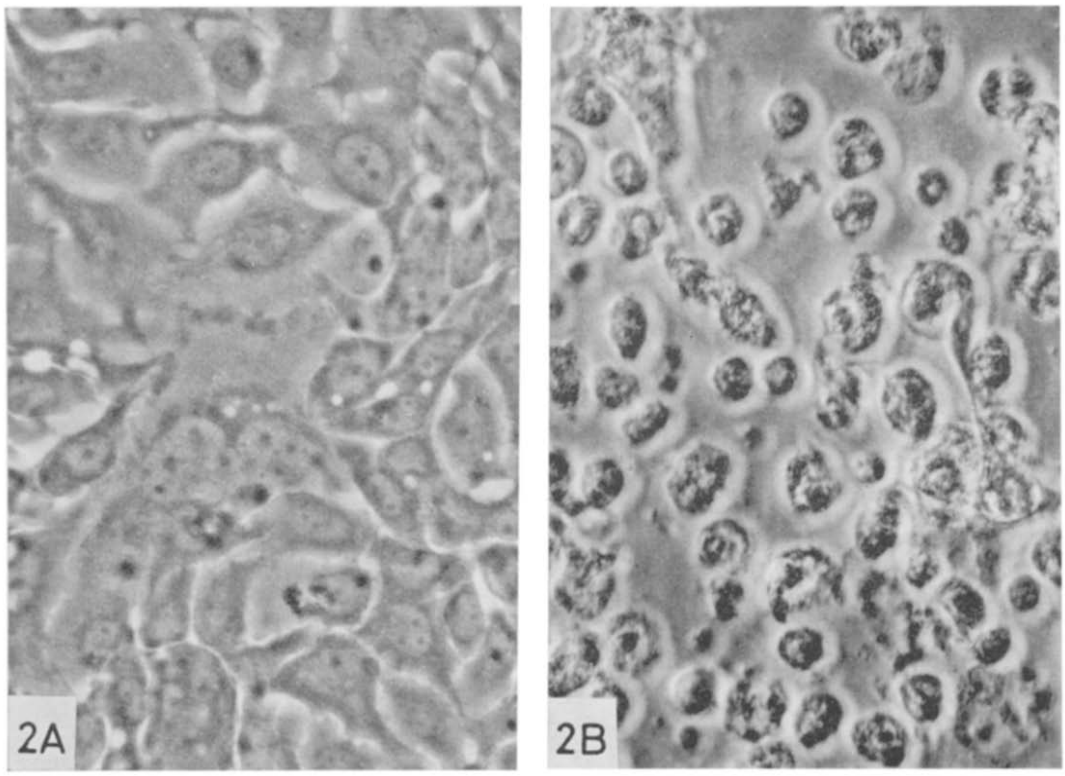


Fig. 2. Phase-contrast photomicrographs of BT 20 cell monolayers. × 320. A. Uninfected control culture. B. 94 hr after infection with FPV_8.

(to face p. 62)

cellular surfaces where neither budding nor adsorbed virus particles were seen (Figs. 3 and 6).

In infected cultures, a few cells were seen which completely failed to react with the antisera and which showed no structural signs of virus replication (Fig. 5). At the stages of infection studied in the electron microscope, the cytopathic effects (16 hr) were inconspicuous. The aspect and structural integrity of nucleus and cytoplasmic organelles in uninfected cells treated under the same conditions of incubation (Fig. 7) were essentially the same as in infected cultures (Fig. 6).

DISCUSSION

In the present report we have shown that an avian influenza A virus (fowl plague virus), after several adaptation passages, replicated well in a human mammary carcinoma cell line (BT 20). Virological as well as immunotherapeutical aspects of this finding will now be discussed.

Virological aspects

In contrast to human influenza A strains and to the Brescia [13, 14] and Dutch strains of avian influenza A [15, 16], the avian influenza A we used in these experiments proved to be easily adaptable to human tumor cells.

For adaptation to BT 20 cells we chose FPV already adapted to HeLa cells, since both cell lines have an epithelial origin. After serial passage of the HeLa adapted FPV in human leukemic cells (which have a mesothelial origin), adaptation to HeLa cells was partially lost [7], indicating that there might be such a property as "epithelial adaptation".

That we were still dealing with Fowl Plague virus and not with some contaminant picked up during the long passage history of our strain was indicated by the electron microscopical findings: the budding particles had the typical morphology of myxoviruses. Their reactivity with an antiserum prepared against the egg grown virus not passaged in BT 20 cells showed their antigenic similarity to the starting virus. The same antiserum, incidentally, reacted to high titer with a subline of the original strain passed only a few times in eggs.

Immunological aspects

In order for immunological potentation of cellular antigens by viruses to occur, it seems essential that a full cycle of viral replication in the relevant cells be obtained [17]. This condition appears to have been met in the present experiments. The budding of virus particles was strikingly similar to that seen in Ehrlich ascites tumor cells infected with the WSA strain of influenza virus [18], a system where immunological potentiation has been studied extensively [1, 2]. The use of ferritin labelled antiviral antibody also revealed incorporation of viral antigens at membrane sites showing no other evidence of viral activity (Fig. 3 and 6). This indicated intimate associations of viral and cellular antigens at various stages of the infectious process, thus creating numerous opportunities for those hapten-carrier relationships to become established which are thought to form the basis of immunological potentiation [17].

With the virus in its present state of adaptation to a stable cell line of human mammary tumor origin, it should be easier to grow it in newly established tissue cultures from fresh mammary tumors. This, of course, would be a prerequisite should "virus-assisted immunotherapy" of cancer patients, using their own virus-infected autologous tumor as a vaccine, be contemplated. If one were to become convinced that certain established tumor cell lines, such as the BT 20 line used in the present experiments, contained antigens shared by spontaneously occurring tumors and amenable to immunological potentiation by viruses, then a much easier technical approach would become feasible: BT 20 cells could be grown in vast quantities and infected with virus by procedures which have become standard practice in the manufacture of viral vaccines. To prove, however, that BT 20 cells, or any other established tumor cell line carries relevant antigens will require some sort of immunological test showing clearly a correlation between a patient's capacity to react against these cells and his clinical course.

Acknowledgements—The authors wish to acknowledge the excellent assistance of Mrs. M. Badoux, Mrs. S. Ekenbark, Miss R. Keller, and Miss R. Leemann. We thank Prof. L. Ozzello for supplying the BT 20 cell line.

REFERENCES

1. J. Lindenmann and P. A. Klein, Viral oncolysis: increased immunogenicity of host cell antigen associated with influenza virus. *J. exp. Med.* **126,** 93 (1967).

2. J. LINDENMANN and P. A. KLEIN, Immunological aspects of viral oncolysis. *Rec. Results Cancer Res.* **9,** 1 (1967).
3. C. BOONE, K. BLACKMAN and P. BRANDCHAFT, Tumor immunity induced in mice with cell-free homogenates of influenza virus-infected tumor cells. *Nature (Lond.)* **231,** 265 (1971).
4. C. W. BOONE, M. PARANJPE, T. ORME and R. GILLETTE, Virus-augmented tumor transplantation antigens: evidence for a helper antigen mechanism. *Int. J. Cancer* **13,** 543 (1974).
5 M. D. EATON, J. A. HELLER and A. R. SCALA, Enhancement of lymphoma cell immunogenicity by infection with non-oncogenic virus. *Cancer Res.* **33,** 3293 (1973).
6. J. LINDENMANN, The use of viruses as immunological potentiators. *Ciba Foundation Symposium* **18,** (new series) 197 (1973).
7. A. GERBER, CHR. SAUTER and J. LINDENMANN, Fowl plague virus adapted to human epithelial tumor cells and human myeloblasts *in vitro*—I. Characteristics and replication in monolayer cultures. *Arch. ges. Virusforsch.* **40,** 137 (1973).
8. A. GERBER, CHR. SAUTER and J. LINDENMANN, Fowl plague virus adapted to human epithelial tumor cells and human myeloblasts *in vitro*—II. Replication in human leukemic myeloblast cultures. *Arch. ges. Virusforsch.* **40,** 255 (1973).
9. CHR. SAUTER, U. BAUMBERGER, S. EKENBARK and J. LINDENMANN, Replication of an avian myxovirus in primary cultures of human leukemic cells. *Cancer Res.* **33,** 3002 (1973).
10. E. Y. LASFARGUES and L. OZZELLO, Cultivation of human breast carcinomas. *J. nat. Cancer Inst.* **21,** 1131 (1958).
11. L. J. REED and H. MUENCH, A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* **27,** 493 (1938).
12. M. AGUET and TH. BÄCHI, Comparison of the direct and indirect immunoferritin tagging of Sendai virus antigen. *Ann. Immunol. (Inst. Pasteur)* **124 C,** 407 (1973).
13. C. HALLAUER, Studien über die Variabilität des Hühnerpestvirus im Gewebeexplantat. *Arch. ges. Virusforsch.* **1,** 70 (1939).
14. C. HALLAUER and G. KRONAUER, Weitere Versuche zur Züchtung und Abeandlung von klassischem Geflügelpestvirus in menschlichen Gewebeexplantaten. *Arch. ges. Virusforsch.* **9,** 232 (1959).
15. H. E. PEREIRA, Multiplication of fowl plague virus in chicken embryo cell suspensions. *J. Path. Bact.* **65,** 259 (1953).
16. D. M. CHAPRONIERE and H. G. PEREIRA, Propagation of fowl plague and of Newcastle disease viruses in cultures of embryonic human lung. *Brit. J. exp. Path.* **36,** 607 (1955).
17. J. LINDENMANN, Viruses as immunological adjuvants in cancer. *Biochim. Biophys. Acta (Review)* **290,** 555 (1974).
18. TH. BÄCHI, W. GERHARD, J. LINDENMANN and K. MÜHLETHALER, Morphogenesis of influenza A virus in Ehrlich ascites tumor cells as revealed by thin-sectioning and freeze-etching. *J. Virol.* **4,** 769 (1969).

Europ. J. Cancer Vol. 11, pp. 65–69. Pergamon Press 1975. Printed in Great Britain

Ultrastructural Demonstration of Peculiar Lymphoid Cells in the Thymus During the Perinatal Period in Mice. Correlation with Radioleukemogenesis

J. L. CARPENTIER, J. BONIVER,* E. H. BETZ and L. J. SIMAR

Laboratoire d'Anatomie Pathologique, Université de Liège, 1, rue des Bonnes Villes B-4000 Liège, Belgium

Abstract—*Ultrastructural investigation of the thymus in C57B1 foetus and new-born mice reveals the morphological heterogeneity of the "immature cell population". This last one contains typical lymphoblasts and peculiar blast cells, i.e. "ring-shaped nucleolus cells" and "X cells" identical to those observed after leukemogenic fractionated irradiation in the adult animal.*

This observation confirms that an X-ray treatment induces a recapitulation of neonatal condition in the adult mouse which induces a facilitation of the expression of the leukemogenic virus.

INTRODUCTION

IN A PREVIOUS work on radioleukemogenesis in adult C57B1 mice, we have shown that the development of thymic lymphomas is preceded by an accumulation of peculiar lymphoid blast cells in the thymus [1]. This ultrastructural study demonstrated the morphological heterogeneity of this blastic population. While the cytoplasm of these blast cells is always characterized by a high amount of ribosomes and scarce endoplasmic reticulum their nuclear structure varies and allows one to distinguish 3 cell types. Some cells are typical lymphoblasts, identical to those observed in adult normal lymphoid tissues. The others, called by us respectively "X cells" and "ring-shaped nucleolus cells" [1, 2] have a peculiar nuclear ultrastructure not frequently observed in normal adult animals over two months of age. These blast cells are likely to correspond to the "immature lymphoid cells" considered by Kaplan [3] as the target cells for the leukemogenic viruses.

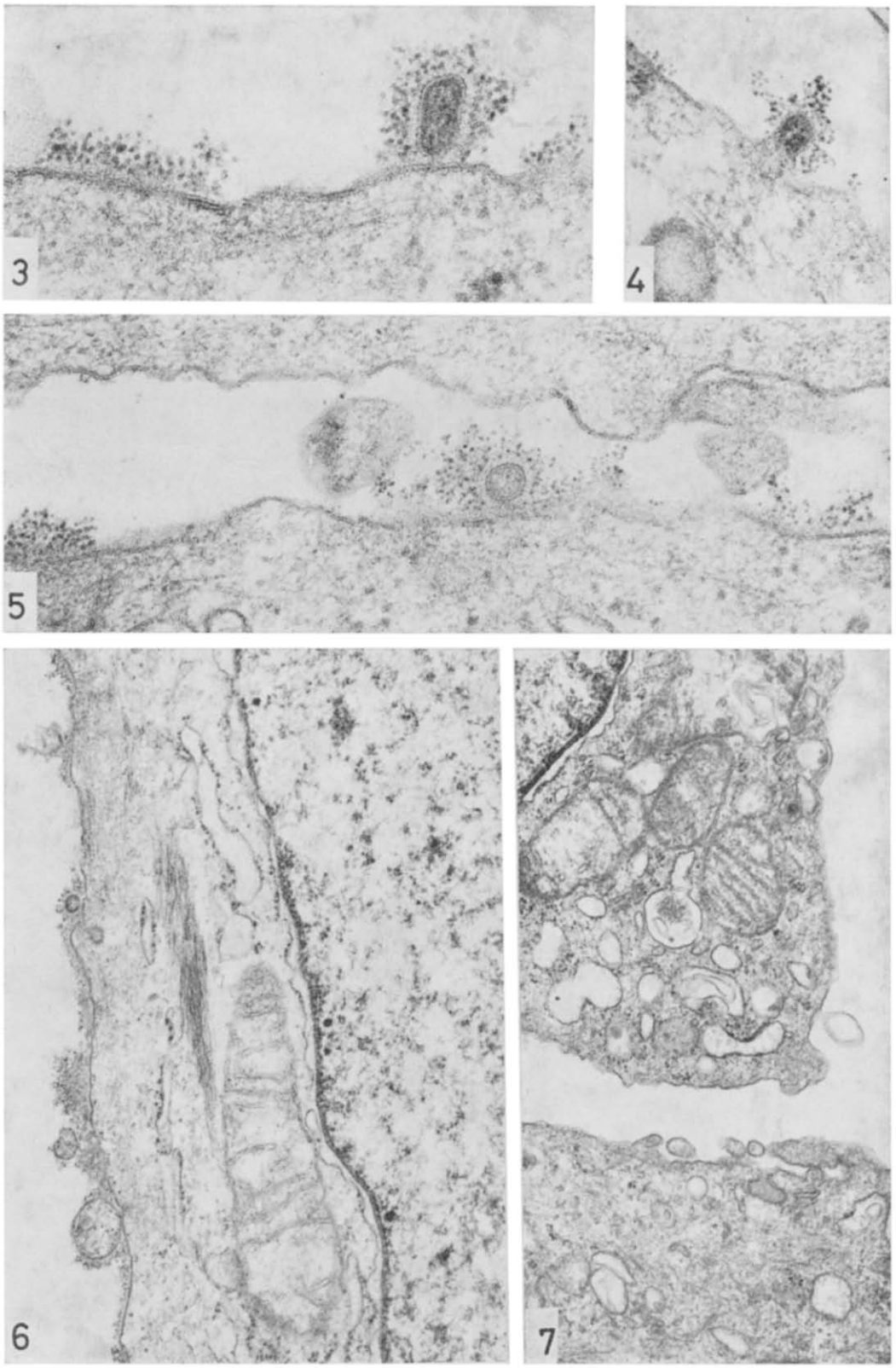
Some results of our group indicate that the peculiar blast cells, i.e. the X cells, appearing in bone marrow after irradiation, are ancestral remnant cells, normally repressed in the adult animal. The destruction of a large proportion of irradiated marrow cells, especially X cells, by a mouse embryo antiserum suggests that the could bear embryonic antigens [4, 5]. Furthermore, X cells, which are never present in the adult marrow, have been found in the bone marrow of normal foetal and newborn mice [6].

Kaplan [3] has postulated that "whole-body irradiation, by simultaneously injuring both the thymus and bone marrow, appears to rekindle thymic susceptibility to the leukemogenic action of the virus, by recapitulating the new-born conditions of the thymus, with respect to its population of immature lymphoid cells." The previous observations on the blast cells developing in the bone marrow of whole-body irradiated mice [5, 7] are in favour of Kaplan's hypothesis.

Accepted 16 May 1974.

*Aspirant du Fonds National de la Recherche Scientifique.

Address reprint requests to: Dr. J. Boniver, Laboratoire d'Anatomie Pathologique, Université de Liége, 1, rue des Bonnes Villes, B-40000 Liége Belgique.



Figs. 3–7. *Material in which viral antigen was labelled by incubation with rabbit anti-FP virus serum and by a subsequent reaction with ferritin labelled guinea pig anti-rabbit IgG.*

Fig. 3. *FP virus at cell surface. The ferritin reveals segments of the cell membrane in which viral antigen is incorporated without structural differentiation.* × 100,000.

Fig. 4. *FP virus budding at BT* 20 *cell surface. Virus is heavily tagged with ferritin.* × 75,000.

Fig. 5. *Surface of two cells of an FP virus infected culture, one being unreactive with the antisera, the other showing tagged membrane segments with a virus particle.* × 75,000.

Fig. 6. *Reaction of cell surface with sera demonstrating a virus specific change after infection.* × 30,000.

Fig. 7. *Uninfected cell of control experiment. Note the absence of ferritin label.* × 25,000.

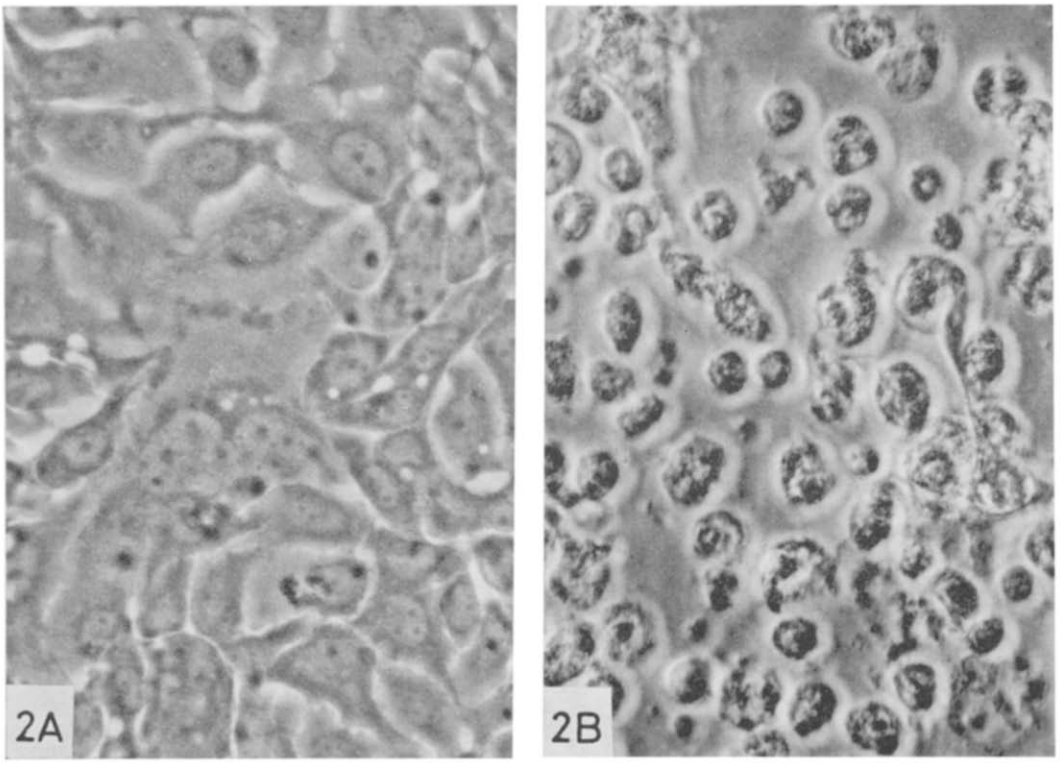


Fig. 2. Phase-contrast photomicrographs of BT 20 cell monolayers. × 320. A. Uninfected control culture. B. 94 hr after infection with FPV_8.

(to face p. 62)

The aim of the present work was to investigate the blast cell population of foetal and new-born thymuses and to see if X cells and ring-shaped nucleous cells are normally present in this organ at that stage of development. It seems necessary indeed to analyze by ultrastructural investigation the composition of the lymphoid blast cell population described by other authors using the light microscope [8, 9].

MATERIAL AND METHODS

Histological procedure

Twelve foetuses of C57Bl mice were obtained at daily intervals between the 17th and 20th days of gestation. Three new-born mice were sacrificed every day between day 1 and 6 as well as on day 10.

The thymuses were removed carefully, cut into small pieces and fixed in 4% distilled glutaraldehyde solution in a phosphate buffer at pH 7·4 for 45 min. and post-fixed for 15 min. in 1% osmium tetroxide diluted in a phosphate buffer at pH 7·4. After dehydration, the tissues are embedded in epon. Semi-thin sections were stained with 1% toluidine blue. Ultra-thin sections of selected zones are contrasted with uranyl acetate and lead citrate [10] and examined with a Siemens Elmiskop 101.

Quantitative analysis

At each delay and for each animal, 400–600 cells were examined with the electron microscope in the whole thymic cortex. This way, at least 1200 cells per day were used for the calculation. They were classified into 5 different groups; the first corresponding to the non-lymphoid elements and the four others to the different aspects of lymphoid cells. The S.E. of the mean was calculated for each group of 3 animals.

RESULTS

Qualitative analysis

From the 17th day of gestation to the 10th day after birth, the thymus contains a high number of blast cells infiltrating the whole thick cortical zone (Fig. 1).

In all these cells, the cytoplasm contains a large amount of ribosomes, usually grouped in small clusters, whereas the endoplasmic reticulum and other cytoplasmic organelles are poorly developed. If the cytoplasmic structure is rather uniform, the morphology of the nucleus varies and permits a distinction between 3 major cell types.

In the *typical lymphoblasts* (Fig. 2), the dense chromatin, which is scarce, is accumulated along the membrane and around the compact nucleolus in which the components are intermingled.

In a small proportion of blast cells (Fig. 3), the nucleolus has a different ultrastructure. Instead of being intermixed, the two ribonucleoprotein components are well separated: the fibrillar material forms a thin shell around a light central zone and is surrounded by the granular substance. In previous work, we called these peculiar cells "ring-shaped nucleolus cells" [1].

In some other blast cells (Fig. 4), the dense chromatin is more abundant than in the lymphoblasts. It forms an irregular layer beneath the nuclear membrane and numerous blocks dispersed in the nucleoplasm and around the large nucleolus. In this latter organelle, the fibrillar components and granular zone are clearly distinguishable but do not constitute concentric rings. This aspect corresponds to the ultrastructure of "X cells" [2].

Furthermore, it seems that some transitional aspects between these three different cell types may be observed.

Quantitative analysis: (Table 1 and Fig. 5)

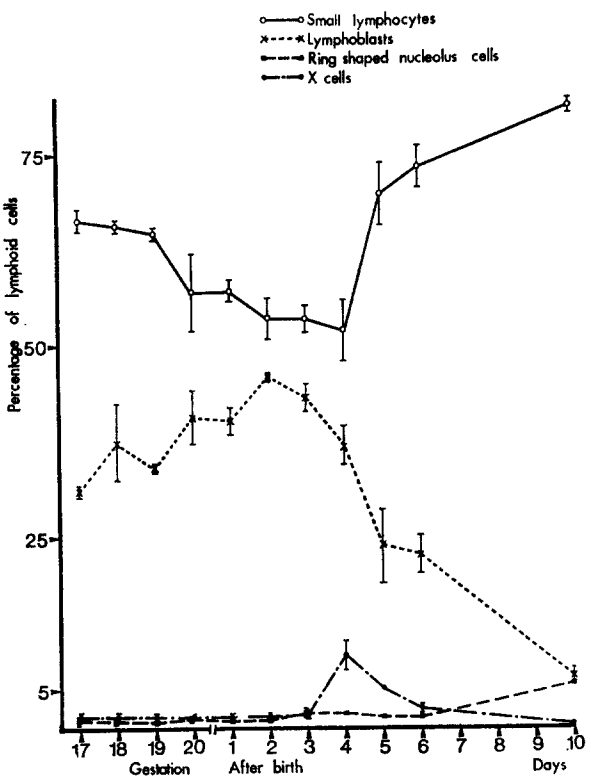


Fig. 5. Variations of the percentage of the different lymphoid cells in relation to the total number of lymphoid cells in the whole thymic cortex during the perinatal period.

Table 1. Percentage of the different lymphoid cells in relation to the total number of lymphoid cells in the whole thymic cortex. (Mean value ± standard error of the mean)

	Gestation day				Day after birth						
Cell types	17th	18th	19th	20th	1st	2nd	3rd	4th	5th	6th	10th
Small lymphocytes	66·4 ± 1·3	65·7 ± 0·7	64·8 ± 0·6	56·8 ± 4·7	57·2 ± 1·31	53·6 ± 1·7	53·7 ± 1·6	51·8 ± 4·0	69·7 ± 4·5	73·2 ± 2·7	81·1 ± 1·1
Lymphoblasts	31·0 ± 0·8	37·4 ± 4·9	34·2 ± 0·4	40·7 ± 3·5	40·3 ± 1·5	45·8 ± 0·6	43·2 ± 1·7	36·8 ± 2·3	24·0 ± 4·7	22·7 ± 2·6	6·7 ± 1·0
X Cells	1·4 ± 0·5	1·6 ± 0·4	1·6 ± 0·4	1·5 ± 0·6	1·4 ± 0·3	1·5 ± 0·4	1·8 ± 0·4	9·0 ± 0·2	4·8 ± 0·2	2·6 ± 0·4	0·5 ± 0·2
Ring-shaped nucleolus cells	1·3 ± 0·4	1·0 ± 0·3	0·7 ± 0·1	1·2 ± 0·5	1·1 ± 0·1	1·0 ± 0·2	1·8 ± 0·4	2·0 ± 0·2	1·4 ± 0·2	1·4 ± 0·2	5·6 ± 0·2

The total amount of blast cells (lymphoblasts, ring-shaped nucleolus cells, X cells) varies during the perinatal period in inverse ratio to that of small lymphocytes. The percentage of small lymphocytes decreases from the 19th day of gestation to the 4th day of life. Afterwards, they increase while the proportion of blast cells decreases sharply. On day 10, the percentage of the different cell types in the thymic cortex is similar to that observed in the young adult.

The ultrastructural study reveals that the composition of the blast cell population itself varies during the first days of life. Lymphoblasts always form the most common cell type. They represent more than 30% of the lymphoid cells up to the 4th day. During the same period, X cells and ring-shaped nucleolus cells are very scarce and represent less than 2% of the lymphoid population. On the contrary, by the fourth day, X cells become transiently more frequent and form 9% of the lymphoid cells, whereas the percentage of lymphoblasts decreases rapidly by day 10, the end of the study period. At this age, the proportion of ring-shaped nucleolus cells increases; they constitute subcapsular islets similar to those observed in the young adult animals [1].

DISCUSSION

Our ultrastructural study demonstrates that during the perinatal period, the blast cell population of the mouse thymus differs from what is observed in the adult animals [1]. It contains a small proportion of "X cells" and "ring-shaped nucleolus cells" similar to those we described previously in the adult thymus during regeneration after leukemogenic fractionated irradiation [1]. These results prove that "X cells" and "ring-shaped nucleolus cells" are normal constituents of the thymus in new-born conditions. The irradiation induces a "recapitulation" [3] of these new-born conditions.

In a previous work, Boniver and co-workers [1] suggested that the various thymic blast cells belong to the same cell line. Lymphoblasts, ring-shaped nucleolus cells and X cells have exactly the same cytoplasmic ultrastructure. Moreover, we have observed intermediate stages between these three cell types. The variations of the nuclear aspect could be due to different functional stages. It is well-known that lymphoblasts are actively proliferating. In the same way, X cells are able to incorporate H^3-thymidine [11]. Whereas these two blast cells correspond to active forms of the same cell, the "ring-shaped nucleolus cells" could be the "resting cells". The peculiar structure of these "ring-shaped nucleolus" is interpretated by Smetana and co-workers [12–16] as the morphological expression of their poor ribonucleoprotein synthesis. This last hypothesis, which is not yet demonstrated definititively, could explain our observations. During the perinatal period, when the thymus proliferation is very important, lymphoblasts and X cells are numerous while "ring-shaped nucleolus cells' are scarce. Cell kinetic studies are in progress in order to check our hypothesis.

After the first week of life, the number of thymic blast cells, and particularly X cells, decreases rapidly. As no picture of local destruction has been observed, this phenomenon could be explained by 3 mechanisms which perhaps occur simultaneously: arrest of production, migration from the thymus, transformation into mature normal lymphocytes. The two last possibilities do not deserve further comment. That the X cells occurring in regenerating bone marrow are repressed by mature marrow lymphocytes is strongly suggested by previous experiments [5, 11, 17]. We have also shown that the injection of normal marrow cells inhibits the development of X cells in the thymus of whole-body irradiated mice [1, 18, 19].

Our results show that during the perinatal period the C57Bl mouse thymus contains lymphoid blast cells identical to those which accumulate in the same organ after a fractionated irradiation. This observation is an important argument in favour of the hypothesis that this irradiation induces the reappearance of an immature condition in the thymus, which facilitates the expression of the leukemogenic virus [1, 3, 8]. Further experiments are needed in order to see if the 3 cell types we have found among the blast cell population are equally sensitive to the virus responsible for the development of radiation-induced lymphomas.

REFERENCES

1. J. Boniver, M. Delrez, L. J. Simar and J. Haot, Modifications of the thymic cell population during the development of the radiation-induced lymphoma in mice. *Beitr. Path.* **150,** 229 (1973).

2. L. J. SIMAR, J. HAOT and E. H. BETZ, Etude ultrastructurale de la moelle hématopoiétique au cours de la régénération après irradiation. *Europ. J. Cancer* **4,** 529 (1968).
3. H. S. KAPLAN, The role of radiation in experimental leukemogenesis. *Nat. Cancer Inst. Monogr.* **14,** 207 (1964).
4. J. HAOT, E. MÖLLER, K. HIESCHE and L. REVESZ, Cytotoxic effect of a xenogeneic antiserum on bone marrow cells from normal or sublethally irradiated mice. *Experientia* **28,** 956 (1972).
5. J. HAOT, L. REVESZ and E. H. BETZ, Characterization of a new lymphoid cell line detected in the irradiated mouse. *Nature (Lond.)* **244,** 211 (1973).
6. L. J. SIMAR, J. L. CARPENTIER, J. HAOT and J. BONIVER. *Exp. Hemat.* in press (1974).
7. M. DELREZ and J. BONIVER (In preparation) (1974).
8. H. S. KAPLAN, The role of cell differentiation as determinant of susceptibility to virus carcinogenesis. *Cancer Res.* **21,** 981 (1961).
9. A. A. AXELRAD and H. G. VANDERGAAG, Susceptibility to lymphoma induction by Gross passage A virus in C3Hf/Bi mice of different ages: relation to thymic cell multiplication and differentiation. *J. nat. Cancer Inst.* **29,** 1065 (1962).
10. J. H. VENABLE and R. A. COGGESHALL, A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25,** 407 (1965).
11. J. HAOT, Etude quantitative et fonctionnelle de la population lymphocytoïde au cours de la régénération médullaire après irradiation corporelle totale. Thèse d'Agrégation de l'Enseignement Supérieur, Université de Liège (1972).
12. H. BUSCH and K. SMETANA, *The Nucleolus*, Academic Press, New York. (1970).
13. M. POTMESIL and K. SMETANA, The effect of chromomycin A_3 on RNA synthesis and formation of ring-shaped nucleoli in cell cultures. *Folia biol. Praha* **14,** 132 (1968).
14. K. SMETANA, F. GYORKEY, P. GYORKEY and H. BUSCH, Comparative studies on the ultrastructure of nucleoli in human lymphosarcoma cells and leukemic lymphocytes. *Cancer Res.* **30,** 1149 (1970).
15. M. POTMESIL and A. GOLDFEDER, Nuclear morphology, nucleic acid synthesis and growth rates of experimental tumors. *Cancer Res.* **31,** 789 (1971).
16. M. POTMESIL and A. GOLDFEDER, Nucleolar morphology and maturation of thymic lymphocytes. *J. Cell Biol.* **53,** 832 (1972).
17. M. DELREZ, J. HAOT and E. H. BETZ, Regenerating bone marrow cell lines in two different X-irradiation conditions. *Int. J. Rad. Biol.* **15,** 405 (1969).
18. E. H. BETZ, J. BONIVER, L. J. SIMAR, M. DELREZ and J. HAOT, Ultrastructural study of thymic regeneration after whole-body irradiation. Effect of bone marrow treatment. *Abstr. of the Tenth International Cancer Congress, Houston*, p. 674 (1970).
19. J. BONIVER, M. DELREZ, J. HAOT, L. J. SIMAR and E. H. BETZ, Ultrastructural characterization of a thymic X cell type in sublethally irradiated mouse. Correlations with the bone marrow X cell population. *Beitr. Path.* **152,** 81 (1974).

Europ. J. Cancer Vol. 11, pp. 71–78. Pergamon Press 1975. Printed in Great Britain

Allograft Reactivity of Mice Against a Radiation-Induced Lymphoma Incompatible for the *H-2K-Ir* Regions of *H-2**

ANNA BONMASSAR†, MARIA CRISTINA FIORETTI†, ENZO BONMASSAR† and ABRAHAM GOLDIN‡

†*Laboratory of Tumor Immunology and Chemotherapy, Institute of Pharmacology, University of Perugia,* 06100 *Perugia, Italy,* ‡*National Cancer Institute, Division of Cancer Treatment, National Institute of Health, Bethesda, Maryland* 20014, *U.S.A.*

Abstract—*B*10.*A*(5*R*) *mice reject* 10^7 *cells of a radiation-induced lymphoma of B*10.*A origin* (*LAF*-17) *incompatible for the H-2K-Ir regions of the H-2 complex. Most of the mice of B*10.*A*(5*R*) *strain succumb after a challenge of* 10^4 *cells of the same lymphoma. The immune response of B*10.*A*(5*R*) *mice against the tumor allograft has been studied* 10–12 *days after the challenge of* 10^7 *or* 10^4 *tumor cells. The inhibition of B*10.*A plaque-forming cells or radioactive LAF*-17 *cells injected intraperitoneally into untreated or pre-challenged B*10.*A*(5*R*) *mice was used to study the allograft reactivity of the mice. The results indicate that the mice challenged with* 10^7 *lymphoma cells showed a strong allograft reaction, whereas the allograft reaction of the animals injected with* 10^4 *cells was weak or absent, in spite of a rapid increase of antigen availability due to allogeneic tumor growth. In addition, it was observed that another radiation-induced lymphoma of B*10.129(5*M*) *origin was immunosuppressant for allogeneic mice and prevented their allograft response to high inocula of LAF*-17 *cells. It was concluded that the challenge of incompatible mice with a limited number of LAF*-17 *tumor cells did not provide initially an adequate antigenic stimulus and immunosuppressed the recipients. When the transplanted tumor reached the critical size necessary to promote an efficient allograft response, the host was incapable of rejecting the tumor and succumbed with generalized lymphoma.*

INTRODUCTION

IT HAS been previously reported that intravenous inocula of 10^7 cells of a number of radiation-induced lymphomas originating in C57Bl/10 mice and related congenic-resistant strains were rejected by *H-2K-Ir* incompatible recipients. However, when a limited number of lymphoma cells were transferred into the same strain of allogeneic mice, the tumor escaped from the immune surveillance and killed the majority of recipient animals [1, 2].

The term dilution escape (DE)§ was used to indicate this phenomenon and studies were conducted to elucidate the escape mechanism. Enhancement or antigenic simplification did not seem to play a significant role [2]. On the other hand it was found that a diffusible and ultrafiltrable factor associated with lymphoma cells could prevent tumor rejection in allogeneic

Accepted 27 September 1974.

*Project P-41 of the U.S.-Italy Cooperative Science Program supported by the Consiglio Nazionale delle Ricerche, Rome, Italy.

§The abbreviations used are: DE, dilution escape of tumor cells from host surveillance (3); *H, histocompatibility gene*; IDF, immunodepressive factor associated with tumor cells; PFC, plaque forming cells.

mice [3]. As the lymphoma-associated factor did not stimulate tumor growth, it was reasonable to assume that the factor depressed the allograft response of the host. Thus the factor, of probable viral origin [4], was called immunodepressive factor, or IDF. It was considered that the reduced inoculum, incapable of adequate antigenic stimulation, releases enough IDF to depress the immunological reactivity of the recipient within 48 hr [2], thus allowing progressive tumor growth. On the contrary, large inocula of incompatible lymphomas, induce a massive allograft response before the IDF is capable of acting on the host's immune system.

The current studies were conducted to verify the proposed model for DE, by analyzing the antitumor allograft response of mice undergoing dilution escape, and evaluating the immunodepressive activity of IDF in two host–tumor systems. For investigation of the proposed model, techniques were developed which do not require animal sacrifice, thus allowing determination of tumor growth and mortality among the animals tested for the immune response. One technique involves the evaluation of target cell disappearance from the peritoneal cavity and is related to that reported by Mann [5]. The second technique involves determination of target cell proliferation in the peritoneal cavity of untreated or prechallenged allogeneic recipients. In these studies mice challenged with large inocula of allogeneic lymphomatous cells promptly reacted against the tumor-associated alloantigens, whereas a weak or no response was found in animals injected with small inocula of cells even after the cell population of the "escaped" tumor had apparently exceeded the "large inoculum" size. In addition, pretreatment with an antigenically unrelated lymphoma carrying IDF was found to inhibit the allograft reaction of B10.A(5R) or B10 mice challenged with large inocula of *H*-2 incompatible tumor cells.

MATERIAL AND METHODS

Mice

Inbred mice of the congenic-resistant strains C57Bl/10 ScSn [abbreviated as B10 (H-2^b)], B10.A (H-2^a) and B10.A(5R) (H-2^i) were obtained from the Mammalian Genetics and Animal Production Section of the National Cancer Institute. The B10.A(5R) strain differs for the *H-2D* region of *H*-2 from the B10 strain, and for the *H-2K-Ir* regions of *H*-2 from the B10.A strain [6, 7]. Mice of both sexes 4–6 months old were used.

Tumors

Two radiation-induced lymphomas, LAF-17 of B10.A (H-2^a) origin and L5MF-22 of B10.129(5M) [H-2^b] origin have been used. The tumors were obtained through the courtesy of G. Cudkowicz in 1969 and kept in a frozen state. As previously reported, both tumors are IDF positive [3]. Tumor cells removed from the tissue bank were used for experiments after at least 5 passages by the i.v. route in syngeneic mice. Both tumors grew as generalized lymphomas and grossly enlarged spleens were used as the tumor source. Spleen cell suspensions were prepared in medium 199 and transplanted by the i.v. route in 0·5 ml at the desired concentration. The survival of transplanted mice was recorded for at least 60 days after tumor challenge, and the possible presence of lymphoma growth was confirmed by gross autopsy.

Irradiation of tumor cells in vitro

Tumor cells were inactivated by exposure to 5500 rads of γ-rays *in vitro*, in a ^{60}Co γ-irradiator (Hot-Spot MK-IV, Harwell, England) at an irradiation rate of 2390 rads/min.

Recovery of injected plaque-forming cells from the peritoneal cavity of living recipients

B10A(5R), B10 or B10.A male mice were immunized with 4×10^8 sheep or burro red blood cells i.p. Four days later, at the time of the maximum PFC response, the spleens of the mice were removed and a cell suspension was made in medium 199, gently teasing the organs with flat-end forceps. The cells were washed three times in chilled medium 199 and resuspended in the same medium. The cell suspension was then adjusted to the final concentration of $1{\cdot}6 \times 10^8$ viable cells/ml. Generally the viability, tested by means of Trypan Blue uptake, ranged from 75 to 85%. B10.A(5R)-recipient mice controls or prechallenged with LAF-17 tumor cells were then injected intraperitoneally with 0·5 ml of B10 or B.10A spleen PFC suspension. Aliquots of the same suspension were kept at 4°C until the plaque assay was performed on all samples at the end of the experiment. Preliminary experiments, not reported here, showed that the loss of PFC content of washed spleen cell suspension stored 24 hr at 4°C was not more than 10%. More consistent loss (30–40%) of PFC was observed during the washing procedure.

At different time intervals after PFC administration, the recipient mice were injected intraperitoneally with 5 ml of medium 199, using a 27 gauge needle. The enlarged abdomen

was gently massaged and 5 min later the peritoneal fluid was collected inserting a 16 gauge needle in the peritoneal cavity of the animal. The liquid was recovered rapidly and approximately 4 ml were collected in a test tube from each mouse (80% recovery of the injected medium). All animals survived in excellent condition following the above procedure, allowing further observations on tumor growth and mortality in the same mice.

In other experiments, a double-label method was applied. B.10A(5R) mice, untreated or prechallenged with B10.A lymphoma LAF-17, were injected intraperitoneally with a mixture of antisheep PFC of B10.A strain and antiburro PFC of B10.A(5R) strain. After peritoneal washing, double plaque assay for sheep and burro red blood cells was performed in each sample, in order to test the specific decrease of B10.A target cells, labeled as antisheep PFC, in comparison with syngeneic B10.A(5R) cells, labeled as antiburro PFC.

Plaque assay

The classic method of Jerne *et al.* [6] was followed with minor modifications, as already described [7].

Recovery of injected radioactive tumor cells from the peritoneal cavity of living recipients

To test the host's immune response against the transplanted tumor, lymphoma cells labeled *in vivo* with 5-iodo-2'-deoxyuridine-I^{125} (^{125}IUdR), were used [8]. B10.A male mice were injected intravenously with 10^7 LAF-17 lymphoma cells. Four days later, the animals were injected intraperitoneally with 25μg of 5-fluoro-2'-deoxyuridine (FUdR) and 1 hour later with 2μCi of ^{125}IUdR (I.R.E. c/o SCK-CEN, Belgium, Boeretang, specific activity = 10·4mCi/mg). Twenty-four hours later, the spleens were removed and a cell suspension of the labeled tumor was prepared in medium 199. The suspension was washed twice and brought to a concentration of $1{\cdot}6 \times 10^8$ cells per ml in the same medium. B10.A(5R) male recipient controls or prechallenged with LAF-17 lymphoma were injected with 0·5 ml of the labeled suspension. Twenty-four hours later, the animals were subjected to the same peritoneal washing procedure described for PFC recovery. Two ml of the peritoneal liquid was centrifuged, the supernatant discarded and the pellet read in a crystal scintillation counter (Packard, mod. 5142). As the radioactivity of the supernatant was usually low, it was not necessary to wash the labeled cells recovered from the peritoneum. The results were expressed in counts per minute per pellet of 2 ml suspension, or in counts per minute calculated for the entire peritoneal fluid injected (i.e. the value obtained with 2 ml multiplied times 2·5).

Evaluation of tumor cell division in the peritoneal cavity: ^{125}IUdR uptake of unlabeled cells injected i.p.

The detailed technique is reported elsewhere [9]. In brief, recipient mice were injected intraperitoneally with unlabeled tumor cells suspended in 0·25 ml of medium 199. Five hours later, the mice received an intraperitoneal injection of 25μg of FUdR and one hour later 1μCi of ^{125}IUdR. Twenty-four hours after ^{125}IUdR administration, peritoneal radioactive cells were recovered and counted as already described. Since all dividing cells incorporate the radiolabeled precursor into their DNA [10], ^{125}IUdR uptake was considered a function of the rate of tumor cell multiplication in the peritoneal cavity [8]. As cell division is inhibited by cytotoxic immune presponses [11], this technique was found highly sensitive for detecting the host's allograft reaction against rapidly dividing cells in mice [9].

RESULTS

Peritoneal recovery of PFC in animals presensitized with allogeneic lymphoma cells

Figure 1 reports the number of PFC of B10 donors, recovered from the peritoneum of B10.A(5R) recipient mice 4, 8 and 16 hr after the injection of 26,000 B10 PFC per mouse i.p. No difference was found between B10.A(5R) recipients untreated or challenged intravenously 12 days earlier with 10^7 cells of LAF-17 lymphoma of B10.A origin. The data, repeatedly confirmed, showed that the immune response of recipient B10.A(5R) mice directed against the B10.A alloantigens of LAF-17 tumor cells, did not influence the rate of disappearance from peritoneum of spleen cells of B10 donors. Figure 2 reports the number of PFC of B10.A donors recovered at various intervals from the peritoneum of B10.A(5R) recipient mice. The recipients were untreated or prechallenged with 10^7 LAF-17 cells of B10.A origin. The number of recovered B10A PFC was significantly lower in prechallenged recipient animals, as compared with the number of PFC recovered from untreated mice. Data not reported indicated that the pattern of recovery of syngeneic B10.A(5R) PFC in untreated recipients or in mice prechallenged with 10^7 cells

of LAF-17 12 days earlier was similar to that observed for allogeneic PFC injected into untreated controls.

The results of the above experiment (Fig. 2) along with previous studies of Mann [5] indicate that the higher rate of disappearance

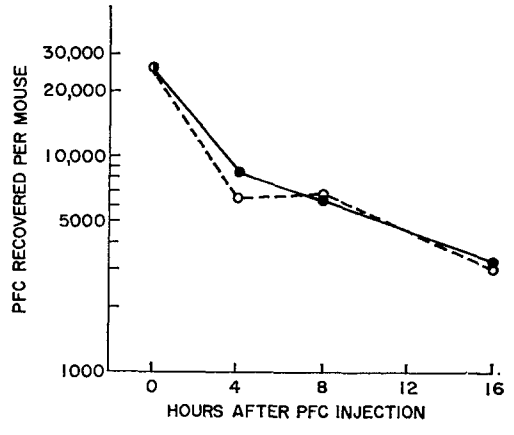


Fig. 1. Total number of anti-sheep plaque-forming cells (PFC) recovered from the peritoneum of B10.A(5R) male mice at various intervals after i.p. injection of 26,000 plaque-forming cells of B10 male mice.

Solid line = control mice

Dotted line = mice injected with B10.A lymphoma LAF-17, 10^7 cells i.v. 12 days before PFC injection.

Each point represents the mean of five mice. The value reported at time 0 represents the number of B10 plaque-forming cells injected, evaluated after 18 hr storage at +4°C.

The statistical analysis (Student's t test) did not reveal any significant difference between the two groups at any time.

from the peritoneum of target PFC in specifically immunized recipients is the result of the allograft response of the host against transferred allogeneic cells. This technique was, therefore, considered applicable to test the immune reactivity of B10.A(5R) mice challenged with large (10^7 cells) or small (10^4 cells) inocula of LAF-17 of B10.A origin. Table 1 reports the data of a double-label experiment in which B10.A(5R) male mice, untreated or challenged with 10^7 or 10^4 LAF-17 lymphoma cells, were injected intraperitoneally 10 days later with antisheep PFC of B10.A donors and antiburro PFC of syngeneic B10.A(5R) male donors. The recovery of PFC 16 hr later,

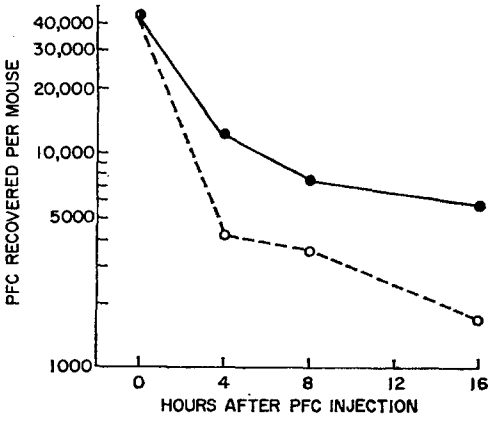


Fig. 2. Total number of anti-sheep plaque-forming cells (PFC) recovered from the peritoneum of B10.A(5R) male mice at various intervals after i.p. injection of 43,000 plaque-forming cells of B10.A male mice.

Solid line = control mice

Dotted line = mice injected with B10.A lymphoma LAF-17, 10^7 cells i.v. 12 days before PFC injection.

Each point represents the mean of five mice. The value reported at time 0 represents the number of B10.A plaque-forming cells injected, evaluated after 18 hr storage at +4°C.

The statistical analysis (Student's t test) revealed significant difference ($P < 0.05$) between the two groups at 4, 8 and 16 hr after PFC injection

indicated that only recipients pretreated with 10^7 tumor cells were capable of reducing the number of B10.A PFC as compared with untreated controls. The animals injected with 10^4 LAF-17 cells did not show an allograft reaction and 4 over 7 animals died on day 12 after challenge, with generalized lymphoma in

Table 1. Recovery of B10.A(5R) and B10.A plaque-forming cells from the peritoneum of B10.A(5R) male mice previously injected with LAF-17 lymphoma, i.v.

Group no.	No. of B10.A (5R) recipient mice	i.v. treatment on day 0	Number of PFC injected on day + 10 i.p.* PFC of B10.A (5R) strain†	Number of PFC injected on day + 10 i.p.* PFC of B10.A strain‡	PFC per mouse§ Recovered 16 hr later PFC of B10.A (5R) strain†	PFC per mouse§ Recovered 16 hr later PFC of B10.A strain‡	MST‖	Dead Total
1	7	Untreated	1856	19200	1375 ± 170	12050 ± 1375	—	0/7
2	7	LAF-17 10^4 i.v.	1856	19200	1580 ± 122	15380 ± 1192	12	4/7
3	6	LAF-17 10^7 i.v.	1856	19200	1210 ± 157	4810 ± 811(o)	—	0/6

*Tested after 16 hr at 4°C.

†Antiburro plaque-forming cells.

‡Antisheep plaque-forming cells.

§Total amount of PFC collected from the peritoneum of each mouse. Mean ± standard error.

‖Median survival time of tested mice.

(o) $P < 0.01$ (Student's t test analysis).

accordance with the previously described phenomenon of dilution escape [1, 2]. No deaths occurred among the animals transplanted with 10^7 lymphoma cells. Another experiment conducted with female mice, not reported here, gave similar results.

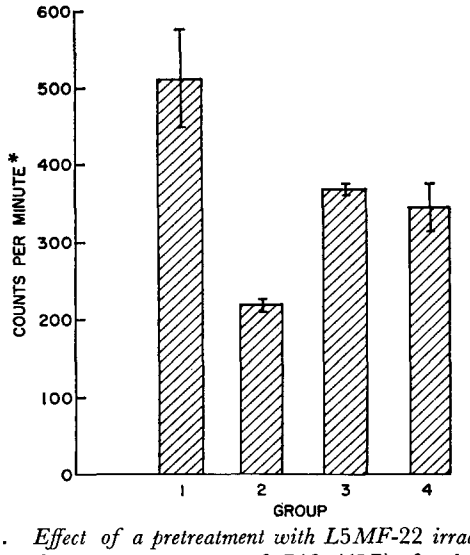


Fig. 3. Effect of a pretreatment with L5MF-22 irradiated cells on the immune response of B10.A(5R) female mice inoculated intravenously with 4×10^7 *LAF-17 irradiated cells. Recovery of LAF-17 cells labeled with* ^{125}*IUdR, inoculated i.p. 11 days after i.v. injection of the same tumor (experimental conditions similar to those reported in Table 2).*

Group 1 = Non-immunized mice (5 mice).

Group 2 = Mice immunized with 4×10^7 *irradiated LAF-17 cells i.v. on day-11 (6 mice).*

Group 3 = Immunized mice, as in Group 2, and also treated on day-14 with L5MF-22 irradiated cells, 10^6 *i.v. (6 mice).*

Group 4 = Non-immunized mice treated on day-14 with L5MF-22 irradiated cells, 10^6 *i.v. (6 mice).*

**Counts per minute of the pellet obtained after centrifugation of 2 ml of the peritoneal fluid collected 5 min after i.p. injection of 5 ml of medium 199.*

Statistically significant difference was found between groups 1 and 2 ($P < 0{\cdot}01$). No significant difference was found between groups 1, 3 and 4. (Student's t test)

Recovery of radioactive lymphoma cells

To confirm results obtained with PFC recovery, the peritoneal host-versus-graft reaction was studied using ^{125}IUdR labeled LAF-17 tumor cells as the target, instead of B10.A plaque forming cells. Table 2 reports the results obtained with B10.A(5R) recipient female mice, untreated or challenged with LAF-17, 10^7 or 10^4 cells i.v., and injected i.p. with radioactive LAF-17 cells 10 days later. Twenty hours later, the recovery of radioactive tumor cells from the peritoneal cavity of mice pretreated with 10^4 tumor cells indicated a limited but not statistically significant reduction as compared with untreated controls. On the other hand, the animals pretreated with 10^7 tumor cells showed a significant decrease of radioactivity in cells recovered from the peritoneal cavity.

Influence of IDF on the allograft reaction

It was hypothesized that an immunodepressive factor (IDF) is released by various radiation-induced lymphomas [2–4]. To test this hypothesis, the techniques of peritoneal recovery of radiolabeled cells or peritoneal radiolabeling were used.

Figure 3 reports the recovery of cell-bound radioactivity from the peritoneum of four groups of B10.A(5R) recipient mice injected intraperitoneally with ^{125}IUdR-labeled LAF-17 cells. One group was a previously untreated control, and two other groups were injected on day minus 11 with an immunizing dose (4×10^7 cells i.v.) of irradiated LAF-17 lymphoma. One of the immunized groups was pretreated with irradiated L5MF-22 lymphoma (10^6 cells i.v.) three days before immunization (day minus 14). The L5MF-22 tumor is not antigenically related to LAF-17 for B10.A(5R)

Table 2. Recovery of radioactive LAF-17 lymphoma cells from the peritoneum of B10.A(5R) female mice previously injected with LAF-17 lymphoma i.v.*

Group no.	No. of B10.A(5R) recipient mice	Treatment on day 0	Counts/min of radiolabeled cells injected on day 10 i.p.	Counts/min‡ of recovered cells from peritoneum 20 hr later
1	7	Untreated	7598	2041 ± 50
2	5	LAF-17 10^4 i.v.	7598	1510 ± 275
3	5	LAF-17 10^7 i.v.	7598	$743 \pm 206^{(o)}$

*8×10^7 radiolabeled cells per mouse.

‡Counts per minute of cell pellet after centrifugation of 2 ml of peritoneal liquid collected 5 minutes after intraperitoneal injection of 5 ml of medium 199. The data have been multiplied by 2·5 to give the total radioactivity present in 5 ml of peritoneal liquid. Mean ± standard error.

(o) $P < 0{\cdot}01$ (Student's t test analysis).

mice, but does carry the immunodepressive factor [3]. The fourth group was treated on day minus 14 with L5MF-22 alone. The results show that animals immunized with irradiated LAF-17 tumor cells had a reduced number of labeled tumor cells detectable in their peritoneum. On the other hand, the animals immunized with LAF-17 and pretreated with L5MF-22 tumor (group 3), or pretreated with L5MF-22 tumor only (group 4), showed peritoneal levels of radioactive cells relatively close to those found in non-immunized controls. The data of group 3 indicate that a pronounced inhibition of the allograft reaction was induced by irradiated L5MF-22 lymphoma cells.

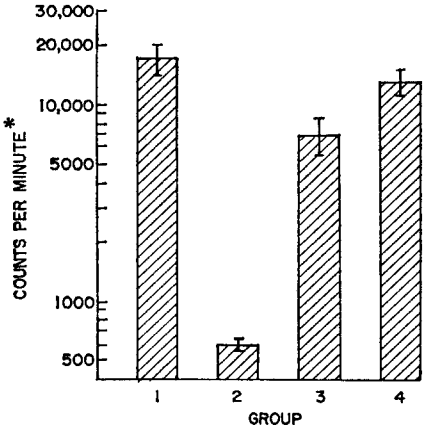


Fig. 4. Effect of a pretreatment with an ultrafiltrate preparation obtained from frozen and thawed L5MF-22 cells on the immune response of B10 male mice inoculated intravenously with 10^7 viable LAF-17 cells. Peritoneal uptake of ^{125}IUdR by 20×10^6 unlabeled LAF-17 cells injected i.p. 4 days after the i.v. injection of the same tumor.

Group 1 = Control mice (10 mice).

Group 2 = Mice injected intravenously with 10^7 LAF-17 lymphoma cells 4 days before the intraperitoneal injection of the same tumor (10 mice).

Group 3 = Mice subjected to the same treatment as Group 2, but pretreated with L5MF-22 ultrafiltrate four days before the intravenous injection of LAF-17 tumor (10 mice).

Group 4 = Mice pretreated with L5MF-22 ultrafiltrate, but not pretreated intravenously with LAF-17 tumor (10 mice).

**Counts per minute of the pellet obtained after centrifugation of 2 ml of the peritoneal fluid collected 5 min after the i.p. injection of 5 ml of medium 199.*

Statistically significant difference was found between groups 2 and 3 ($P < 0.001$) and both were significantly lower than group 1 ($P < 0.001$). (Student's t test)

Figure 4 reports the peritoneal uptake of ^{125}IUdR of four groups of B10 male mice injected intraperitoneally (day 0) with 2×10^7 unlabeled LAF-17 tumor cells and treated with FUdR and ^{125}IUdR. In addition to a non-pretreated control (group 1), two groups (groups 2 and 3) were preinjected with 10^7 viable LAF-17 lymphoma cells i.v. on day minus 4 to elicit a strong allograft reaction in B10 recipients incompatible with LAF-17 for the entire *H*-2 complex. One of the preinjected groups received 0·2 ml i.p. of an ultrafiltrate preparation of frozen-thawed L5MF-22 cells [3] on day minus 8 (group 3). The fourth group of mice was pretreated with the ultrafiltrate only on day minus 8. Two additional groups of mice not reported in Fig. 4 were treated with FUdR and ^{125}IUdR but were not injected intraperitoneally with unlabeled tumor cells, and were used as "background" controls for the intraperitoneal isotope uptake. The latter two groups were untreated control mice and animals injected intravenously with 10^7 viable LAF-17 cells on day minus 4. The results of this experiment indicate that allogeneic tumor cell multiplication was drastically inhibited in the *H*-2 incompatible host previously sensitized with the same tumor (group 2). In contrast, sensitized mice pretreated with IDF showed only a moderate reduction of DNA synthesis of target cells (group 3) confirming the immunodepressive effect of IDF. Pretreatment with IDF alone (group 4) did not exert any significant influence on tumor cell division. ^{125}IUdR uptake of "background" controls (not reported in the figure) was minimal and, therefore, did not interfere with the interpretation of the results. All animals pretreated with IDF and challenged intravenously with 10^7 viable LAF- 17cells (group 3) died with generalized lymphoma, reflecting the immunodepressive activity of IDF. No tumor deaths occurred in the other groups of mice, which rejected both i.v. or i.p. tumor cells, presumably after an initial period of growth. As previously reported [2, 3], the inhibition of allograft reactivity induced by IDF was sufficient to allow lethal tumor growth only in those mice injected with the allogeneic lymphoma by the intravenous route (group 3). It did not prevent tumor rejection in mice injected with *H*-2 incompatible tumor by the intraperitoneal route (group 4).

DISCUSSION

The increased incidence of progressive tumor growth and death of the animals on reduction of the number of transplanted cells of antigenic neoplasms has been reported by a number of investigators [1–3, 12, 13]. In our laboratory, it was found that several radiation-induced lymphomas transplanted into congenic strains of mice incompatible for the *H-2K* region of *H*-2 exhibited this phenomenon defined as dilution escape (DE). Three primary hypotheses could be applicable in attempting to

account for the progressive growth of allogeneic tumor cells that have escaped from host surveillance: (a) tumor growth is supported by enhancing antibodies, (b) tumor growth occurs in spite of a potentially efficient allograft reaction of the host because of failure of early recognition of the tumor cells ("sneak through" phenomenon) [14], (c) tumor growth occurs since the animals undergo tumor-related immunodepression.

The present studies were conducted to measure *in vivo* the allograft reaction of the host against the escaped tumor cells. Techniques were specifically designed for this purpose and the results favor the hypothesis that lymphoma growth resulted from immunodepression of the host following tumor challenge.

To evaluate the host's allograft response *in vivo* two methods were developed, namely (a) the disappearance of allogeneic target cells from the peritoneum ("disappearance test") and (b) the inhibition of DNA synthesis in the peritoneal cavity following transfer of target tumor cells ("inhibition test"). These techniques have the advantage of detecting allograft reactivity of the recipients starting 3 days after alloantigenic stimulus (5 and unpublished observations). In addition, the animals under test are not sacrificed and can be observed for tumor growth and mortality.

In the disappearance test, the mechanism accounting for a greater reduction of allogeneic cells in the peritoneum of specifically immunized recipients may be complex. It could be based on target cell destruction and/or accelerated diffusion from the peritoneum induced by mediators released during the allograft reaction in the peritoneal cavity. Although further studies are required to identify the cellular and humoral components involved in the peritoneal host-versus-graft reaction, the techniques employed here have nevertheless provided information on the allograft response of the presensitized host. The data reported in Figures 2 and 3 and Tables 1 and 2 show that in B10.A(5R) mice subjected to large inocula of LAF-17 lymphoma, there was an acceleration of the disappearance of target B10.A lymphoma or B10.A spleen cells (plaque-forming cells) from the peritoneal cavity. Thus, B10.A(5R) mice sensitized with 10^7 cells of the *H-2K-Ir* incompatible LAF-17 tumor were capable of mounting an efficient allograft reaction against both antigens of the same tumor cells (as also confirmed by Figure 4) and tumor-associated B10.A alloantigens.

In contrast, the data reported in Tables 1 and 2 show that the disappearance rate of target cells in the peritoneal cavity of B10.A(5R) mice previously subjected to small inocula of LAF-17 lymphoma was similar to that of non-prechallenged controls. This finding with low inocula could be explained on the basis of two hypotheses: (a) the mice have a high level of cytotoxic cellular and/or humoral antibodies in the peritoneal cavity, but their activity against target cells is blocked by enhancing antibodies, or (b) the peritoneal level of cytotoxic antibodies is low. Since the former hypothesis would appear to be weakened by other experiments [2], the latter seems to be favored. The animals in which the tumor escaped host surveillance were exposed a few days after challenge to large amounts of antigens associated with the rapidly proliferating lymphoma cells. Therefore, at the time when the peritoneal test was performed, they might have shown a strong peritoneal allograft response against tumor cells as well as against associated B10.A alloantigens. The lack of such a reaction could have been the result of tolerance induction, immune paralysis, "exhaustion" of cytotoxic cellular and humoral antibodies by antigen excess due to tumor cell overloading, or IDF release from lymphoma cells [3, 4]. At the present time, these questions remain open, although the last possibility received support from the results of the experiments reported in Fig. 3 and 4. These experiments provide further evidence that a radiation-induced lymphoma may exert non-specific inhibition of the allograft reaction [3]. The immunological response of B10.A(5R) mice to LAF- 17 tumor cells was abolished or strongly reduced by pretreatment with non-viable cells of an antigenically unrelated radiation-induced lymphoma of B10.129 (5M) origin (Fig. 3) or with an ultrafiltrate of the same tumor (Fig. 4). It is noteworthy that IDF alone did not increase the rate of tumor cell division in non-sensitized mice (Figure 4, group 4) in confirmation of the previous report showing that IDF did not stimulate tumor growth [2]. The latter observation rules out the hypothesis that the mechanism of action of IDF is related to increased tumor cell multiplication capable of overwhelming the normal allograft reaction of an *H-2* incompatible host.

In conclusion, the data reported in the present communication indicate that an immune reaction is detectable in animals injected with large inocula of a lymphoma incompatible for the *H-2K-Ir* regions of *H-2* but not in animals challenged with small inocula and carrying the escaped tumor. The results provide support for the hypothesis that the immunodepressive

activity of a tumor-associated factor plays a role in the escape of a limited number of antigenic tumor cells from host surveillance. Studies are in progress to analyze the humoral and cell-mediated anti-lymphoma immune response of *H-2K-Ir* incompatible mice *in vitro*.

REFERENCES

1. E. Bonmassar, A. Goldin and G. Cudkowicz, Differential reactivity of mice to alloantigens associated with the D and K end of *H*-2. Preliminary studies with bone marrow and lymphoma grafts. *Transplantation* **12,** 314 (1971).
2. E. Bonmassar, E. Menconi, A. Goldin and G. Cudkowicz, Escape of small numbers of allogeneic lymphoma cells from immune surveillance. *J. nat. Cancer Inst.* **53**, 475 (1974).
3. E. Bonmassar, A. Bonmassar, A. Goldin and G. Cudkowicz, Depression of antilymphoma allograft reactivity by tumor-associated factors. *Cancer Res.* **33,** 1054 (1973).
4. A. Iorio, A. Barzi, A. Bonmassar and A. Goldin, Evidence for the viral nature of an immunodepressive factor (IDF) associated with various transplantable tumors. *Fed. Proc.* **33,** 781 (1974).
5. L. T. Mann Jr., Suppression of transferred allogeneic plaque-forming cells in mice. Effect of presensitization of the recipients. *Transplantation* **13,** 580 (1972).
6. N. K. Jerne, A. A. Nordin and C. Henry, The agar plaque technique for recognizing antibody producing cells. In *Cell Bound Antibodies.* Edited by B. Amos and H. Koprowski, Wistar Institute Press, Philadelphia, p. 109 (1963).
7. E. Bonmassar, A. Bonmassar, S. Vadlamudi and A. Goldin, Immunologic response of mice bearing leukemia L1210. *Cancer (Philad)* **30,** 1356 (1971).
8. K. G. Hofer and L. Huges, Incorporation of Iododeoxyuridine-^{125}I into the DNA of L1210 leukemia cells during tumor development. *Cancer Res.* **30,** 236 (1970).
9. M. C. Fioretti, M. Liberati, E. Bonmassar and G. Cudkowicz, Immune inhibition of allogeneic lymphoma cells in the peritoneal cavity of mice. *Cancer Res.* In press (1975).
10. M. Bennett, G. Cudkowicz, R. S. Foster, Jr. and D. Metcalf, Hemopoietic progenitor cells of W anemic mice studied *in vivo* and *in vitro*. *J. Cell Physiol.* **71,** 211 (1968).
11. R. Evans and P. Alexander, Role of macrophages in tumor immunity. Involvement of a macrophage cytophilic factor during syngeneic tumour growth inhibition. *Immunology* **23,** 627 (1972).
12. S. R. Humphreys, G. P. Glynn, M. A. Chirigos and A. Goldin, Further studies on the homograft response in BALB/c mice with L1210 leukemia and a resistant subline. *J. nat. Cancer Inst.* **28,** 1053 (1962).
13. E. Klein, Tumor immunology: escape mechanisms. *Ann. Inst. Pasteur* **122,** 593 (1972).
14. G. Klein, Recent trends in tumor immunology. *Israel J. Med. Sci.* **2,** 135 (1966).

Europ. J. Cancer Vol. 11, pp. 79-86. Pergamon Press 1975. Printed in Great Britain

The Specificity of Cellular Immune Reactions to Three DNA Virus Induced Tumours, as Measured by the Macrophage Migration Inhibition Test*

R. C. REES,† C. W. POTTER and JENNIFER SHELTON

The University of Sheffield Medical School, Department of Medical Microbiology, Beech Hill Road, Sheffield, and Department of Pathology, Weston Park Hospital, Sheffield, England

Abstract—*Groups of hamsters were immunized with three weekly doses of X-irradiated tumour cells, from transplanted tumours induced by either adenovirus* 12, *SV*40 *or CELO virus. Peritoneal exudate cells from the animals were then tested for cellular reactivity using the macrophage migration inhibition test with tumour extracts from the homologous and heterologous tumours. The migration of macrophages from hamsters immunized with SV*40 *tumour cells or CELO virus tumour cells were inhibited by* 10% *(v/v) homologous tumour extract. Macrophages from hamsters immunized with adenovirus* 12 *tumour cells were inhibited by* 10% *and* 2% *adenovirus* 12 *tumour extract and* 10% *SV*40 *tumour extract. In contrast, hamsters immunized with DNA virus or irradiated virus-induced tumour cells showed only specific transplantation immunity; thus the results indicate that the latter test was insensitive to cross-reacting antigens present in tumour extracts. The cross-reacting antigen(s) present in SV*40 *tumour extract may not therefore be tumour specific transplantation antigen(s), since transplantation immunity tests did not detect their presence. These cross-reacting antigens may be related to embryonic or "S" antigens.*

INTRODUCTION

TRANSPLANTATION immunity to DNA virus-induced tumour cells may be produced by immunization with the homologous virus or killed, intact homologous tumour cells [1–3]; for an adenovirus 12-induced tumour of CBA mice, immunity was induced with cell-free tumour extracts [4, 5]. The immune reaction was specific, since immunization of hamsters with chick embryo lethal orphan (CELO) virus or various adenovirus serotypes did not produce immunity to transplanted SV40 virus-induced tumour cells [6]. Using adoptive transfer tests, transplantation immunity to adenovirus 12-induced tumours of CBA mice was shown to be a cellular immune reaction [7]; this type of immunity can also be measured by the macrophage migration inhibition test [8, 9]. In particular, the macrophage migration inhibition test has been used to demonstrate cellular immunity in chemically-induced and virus-induced tumour systems [10–14].

In the present study, cell-mediated immune responses were demonstrated in hamsters immunized against transplanted tumours induced by three different DNA viruses, using the macrophage migration inhibition test. The hamster tumours were induced by SV40 virus, adenovirus 12 and CELO virus. The specificity of the reaction was investigated using both

Accepted 27 September 1974.

*This work was partly financed by the Cancer Research Campaign and the Ernest and Minnie Dawson Cancer Research Fund, Preston, Lancashire.

†Present address: Cancer Research Campaign Laboratories, The University of Nottingham, University Park, Nottingham NG7 2RD.

homologous and heterologous tumour extracts, and the results were compared with those obtained by *in vivo* transplantation rejection tests.

MATERIAL AND METHODS

Animals

All experiments were carried out using Syrian hamsters from a closed, randomly-bred colony at the University of Sheffield. The hamsters were weaned at age 3–4 weeks, and immunization schedules were begun immediately.

Viruses

The preparation of viruses has been previously described [6]. The infectivity titre of SV40 virus in African green monkey kidney cells (21 days reading) was $10^{7\cdot5}TCID_{50}$/ml; that of adenovirus type 12 in primary human embryo kidney cells (14 days reading) was $10^{6\cdot7}TCID_{50}$/ml; and for CELO virus, the preparation had a titre of $10^{8\cdot5}TCID_{50}$/ml as measured in primary chicken kidney tissue cultures. All virus pools were stored at −80°C.

Tumour cells

Cells from three transplanted DNA virus-induced hamster tumours were used for immunization schedules and for tumour cell challenge experiments. The transplanted adenovirus 12-induced tumour was obtained from Dr. F. C. Chesterman, Imperial Cancer Research Fund, London; the CELO virus-induced tumour of hamsters was induced in our laboratory [15]; and the SV40 tumour was obtained by subcutaneous inoculation of hamsters with SV40-induced hamster tumour cells obtained from Flow Laboratories Limited. All three tumour cell lines were maintained *in vivo* by subcutaneous transplantation of tumour fragments at 2–3 week intervals into weanling hamsters.

Single cell suspensions of tumour cells were prepared in an identical manner for each of the three tumours. Transplanted tumours of 20–30 mm dia were excised, finely chopped and treated with 0·25% trypsin.

The cells obtained were washed with Eagle's minimal essential medium containing 2% inactivated calf serum, and resuspended to the desired concentration.

Tumour cell extracts

Hamsters bearing tumours 20–30 mm dia were killed by ether anaesthetic and the tumours excised. Necrotic tissue was removed, and the tumour tissue chopped finely and washed with phosphate buffered saline (PBS.). The fragments were deposited by centrifuging at 600 ***g*** for 15 min, and resuspended in PBS to a 20% concentration (v/v). The fragments were then homogenized briefly in a mechanical blender, freeze-thawed at −80°/20°C and centrifuged at 3000 rev/min for 15 min. The supernatant fluids were removed and stored at −80°C in 1–2 ml aliquots. The protein concentration of the extracts prepared in this manner was 3·0–4·0 mg/ml [16].

Preparation of peritoneal exudate (PE) cells

Groups of hamsters were inoculated with either 5×10^4 SV40 tumour cells, 1×10^5 adenovirus 12 tumour cells, or 1×10^5 CELO tumour cells; the tumour cells were X-irradiated with 2000 rads, and three subcutaneous inoculations of tumour cells were given at weekly intervals in an 0·1 ml volume. One week later the hamsters, together with normal animals, were inoculated intraperitoneally with 5·0 ml of sterile paraffin oil to induce peritoneal exudate.

Seven–ten days after inoculation with paraffin oil, the hamsters were killed by ether anaesthetic, exsanguinated, and the peritoneal cavity opened and washed out with 100 ml of Hank's saline. The peritoneal washings were placed in a separating funnel to allow the paraffin oil to separate, and the lower layer of macrophages was collected and centrifuged at 500 ***g*** for 5 min. The deposited cells were washed three times in medium "199" and resuspended in medium "199" to a 10% (v/v) concentration. Peritoneal washings from both normal and immunized hamsters were shown to consist of approximately 70% macrophages, 10% lymphocytes and 20% polymorphonuclear cells.

Macrophage migration inhibition (MMI) tests

The direct MMI test was used to study cellular immunity in hamsters bearing virus-induced tumours [8, 9]. Drummond microcapillary tubes (Drummond Scientific Company, U.S.A.) of 10 ul. volume were filled with peritoneal exudate (PE) cells from hamsters immunized with tumour cells or from control animals, sealed at the one end with Cristoseal (Hawksley and Sons Limited, Lancing, Sussex) and centrifuged at 500 ***g*** for 5 min. The tubes were then cut at the sedimented cell-liquid interface, and the portion containing the packed cells placed in the bottom of a plastic migration chamber (Sterilin Limited) and held

in position by silicone grease. Up to 5 microcapillary tubes were placed in each well, which was then filled with medium "199" containing 10% (v/v) heat-inactivated foetal calf serum and 2% or 10% (v/v) test antigen; control wells were filled with medium "199" and serum only. The wells were sealed with a glass coverslip fixed with silicone grease, and incubated at 37°C. The area of migration of the macrophages was measured at 24 and 48 hr by planimetry. Two to 6 capillary tubes were used for each test, and the percentage of inhibition calculated according to the formula:

$$\% \text{ inhibition} = 100 - \left\{ \frac{\text{average migration of cells with antigen}}{\text{average migration of cells with no antigen}} \times 100 \right\}$$

The standard deviation from the mean was calculated from the results obtained, and the statistical significance of the results was calculated using students *t* test.

produce tumours in 50% or more of the animals. The animals were examined weekly for palpable tumours, and the final incidence of tumours was calculated five weeks after tumour cell inoculation.

RESULTS

SV40 virus-induced tumours

(*a*) *Macrophage migration inhibition test.* The migration of peritoneal exudate (PE) cells from immunized and normal hamsters was measured in the presence of 2% or 10% (v/v) tumour extracts, prepared from each of the three hamster tumour lines, by the direct macrophage migration inhibition test. The area of migration was measured after 24 and 48 hr incubation, and compared with the area of cells migrated in the absence of antigen. The migration areas at 24 and 48 hr of PE cells from normal hamsters and hamsters immunized with SV40-irradiated tumour cells incubated in the presence of tumour extracts are shown in Table 1. Inhibition of macrophage migration was 78% at 24 hr, and 84% at 48 hr for cells from immunized hamsters incubated with 10% SV40 tumour extract; this difference was shown to be statistically significant ($P = < 0{\cdot}05$). The SV40 tumour extract caused some inhibition of migration of macrophages from normal hamsters, but this degree of inhibition was not significant. Tumour extracts from adenovirus 12-induced hamster tumours did not cause significant inhibition of migration of

Table 1. *The production of MIF from PE Cells from hamsters immunized with SV40 virus-induced tumour cells*

PE cells from	Tumour extract (final conc)	Macrophage migration area ± S.D. (24 hrs)*	% Inhibition	Macrophage migration area ± S.D. (48 hrs)	% Inhibition
immunized hamsters	—	2·10 ± 0·23	—	3·15 ± 1·05	—
	SV40 tumour extract (10%)	0·47 ± 0·32	78†	0·50 ± 0·14	84†
	Adenovirus 12 tumour extract (10%)	1·57 ± 0·60	25	2·22 ± 0·60	29
normal hamsters	—	2·17 ± 0·25	—	3·0 ± 0·14	—
	SV40 tumour extract (10%)	1·67 ± 0·46	23	2·02 ± 0·95	33
	Adenovirus 12 tumour extract (10%)	2·02 ± 0·67	7	2·47 ± 1·24	18

*Average area of macrophage migration (2–6 tests) ± standard deviation.
†Significant inhibition of macrophage migration ($P = < 0{\cdot}05$).

Transplantation immunity tests

Groups of hamsters were inoculated subcutaneously with three, 0·2 ml doses of undiluted virus; further groups of animals were immunized with three doses of X-irradiated tumour cells, as described above for the preparation of macrophages. Two weeks following the third immunization, the hamsters were divided into three groups and inoculated subcutaneously with live tumour cells. The animals received either homologous or heterologous tumour cells, and the number of tumour cells given was calculated by prior titration to

macrophages from hamsters immunized with SV40 tumour extract or from normal hamsters.

(*b*) *Transplantation immunity tests.* The incidence of tumours in hamsters immunized with SV40 virus or with X-irradiated SV40 tumour cells, and subsequently challenged with live virus-induced tumour cells is shown in Table 2, exp. 1. Of 35 hamsters immunized with SV40 virus, none developed tumours following inoculation with live SV40 tumour cells; in contrast, 19 of 35 non-immunized hamsters developed tumours, and a similar proportion of tumours was found in hamsters previously immunized with adenovirus 12 or CELO virus. The incidence of tumours in hamsters immunized with X-irradiated tumour cells also demonstrated specific transplantation immunity (Table 2 exp. 2). Thus, all six non-immunized hamsters developed tumours following inoculation with SV40 tumour cells, but only one of five hamsters previously inoculated with SV40 tumour cells developed a tumour. Immunization with adenovirus 12 or CELO virus induced tumour cells did not induce immunity to transplanted SV40 virus tumour cells.

Table 2. Cross-transplantation immunity tests with three DNA virus-induced tumour lines

Exp. no.	Hamsters immunized with	Hamsters challenged with tumour cells induced by: SV40 (1×10^4 viable cells)	Adenovirus 12 (5×10^5 viable cells)	CELO (1×10^5 viable cells)
1,	SV40	0/35*	17/24	24/31
	Adenovirus 12	21/35	2/26	NT
	CELO virus	17/35	16/25	1/34
	Nil	19/35	14/27	19/29
2,	SV40-induced tumour cells (500 rads.)	1/5	NT	3/4
	Adenovirus 12-induced tumour cells (500 rads.)	4/5	NT	6/6
	CELO virus-induced tumour cells (500 rads.)	7/7	NT	1/7
	Nil	6/6	NT	6/7

*No. of animals with tumours/ No. of animals inoculated
NT Not tested.

CELO virus-induced tumours

(*a*) *Macrophage migration inhibition tests.* The results of macrophage migration inhibition tests using PE cells from hamsters previously immunized with CELO virus-induced tumour cells is shown in Table 3. Significant inhibition was observed in the presence of tumour extracts from CELO virus-induced tumours. Thus, at 24 hr, the inhibition of migration was 30% and at 48 hr the inhibition was 33%; both these observations were statistically significant ($P = < 0{\cdot}05$). Some inhibition was observed for macrophages from hamsters immunized with CELO virus tumour cells and incubated with adenovirus 12 tumour extract, but this was not significant (Table 3); this tumour extract also caused a measurable but insignificant inhibition of migration of macrophages from normal hamsters.

(*b*) *Transplantation immunity tests.* Of 34 hamsters immunized with CELO virus, only one developed a tumour when subsequently challenged with live CELO virus-induced tumour cells. In contrast, 19 of 29 non-immunized hamsters developed tumours following inoculation with the same dose of CELO virus-induced tumour cells (Table 2, exp. 1). Immunization of hamsters with SV40 virus did not reduce the transplantability of CELO virus-induced tumour cells although this procedure produced significant transplantation immunity to SV40 tumour cells. For animals immunized with X-irradiated CELO virus-induced tumour cells, similar results were recorded. Thus, this procedure induced significant immunity to transplanted CELO virus-induced tumour cells, but immunization with SV40 virus or adenovirus 12 tumour cells gave no demonstrable immunity (Table 2).

Table 3. The production of MIF from PE cells from hamsters immunized with CELO virus-induced tumour cells

PE cells from	Tumour extract (final conc)	Macrophage migration area ± S.D. 24 hr*	% Inhibition	Macrophage migration area ± S.D. 48 hr	% Inhibition
immunized hamsters	—	1·42 ± 0·19	—	4·3 ± 0·65	—
	CELO tumour extract (10%)	0·99 ± 0·34	30†	2·9 ± 0·94	33†
	Adenovirus 12 tumour extract (10%)	1·22 ± 0·28	14	3·43 ± 0·24	20
normal hamsters	—	1·04 ± 0·32	—	2·62 ± 1·08	—
	CELO tumour extract (10%)	1·05 ± 0·25	1	3·44 ± 1·19	—
	Adenovirus 12 tumour extract (10%)	0·87 ± 0·23	16	2·49 ± 1·36	5

*Average area of macrophage migration (2–6 tests) ± S.D.

†Significant inhibition of macrophage migration ($P = < 0·05$).

Table 4. The production of MIF by PE cells from hamsters immunized with irradiated adenovirus12-induced tumour cells

Exp. no.	PE cells from	Tumour cell extract (final conc.)	Macrophage migration are ± S.D. (24 hr)*	% Inhibition	Macrophage migration area ± S.D. (48 hr)	% Inhibition
1.	immunized hamsters	—	1·4 ± 0·61	—	5·13 ± 1·02	—
		Adenovirus 12 tumour extract (10%)	1·02 ± 0·34	27	4·56 ± 0·60	11
		Adenovirus 12 tumour extract (2%)	0·49 ± 0·36	65†	3·02 ± 0·56	41†
		SV40 tumour extract (10%)	0·59 ± 0·16	58†	3·11 ± 0·70	39†
		SV40 tumour extract (2%)	0·85 ± 0·39	39	5·21 ± 0·62	2
	normal hamsters	—	0·71 ± 0·20	—	3·09 ± 0·90	—
		Adenovirus 12 tumour extract (10%)	0·74 ± 0·29	—	3·27 ± 1·11	—
		Adenovirus 12 tumour extract (2%)	0·57 ± 0·21	20	3·49 ± 1·25	—
		SV40 tumour extract (10%)	0·66 ± 0·11	7	2·99 ± 1·02	3
		SV40 tumour extract (2%)	0·70 ± 0·15	1	3·31 ± 1·32	—
2.	immunized hamsters	—	0·87 ± 0·28	—	2·87 ± 0·44	—
		Adenovirus 12 tumour extract (10%)	0·26 ± 0·09	70†	1·28 ± 0·50	55‡
		CELO tumour extract (10%)	0·95 ± 0·14	—	5·03 ± 0·65	—
	normal hamsters	—	1·02 ± 0·35	—	3·53 ± 0·91	—
		Adenovirus 12 tumour extract (10%)	1·30 ± 0·10	—	3·59 ± 0·28	—
		CELO tumour extract (10%)	1·31 ± 0·20	—	6·03 ± 0·26	—

*Average area of macrophage migration (2–6 tests) ± S.D.
†Significant inhibition of macrophage migration ($P = < 0·05$).
‡Significant inhibition of macrophage migration ($P = < 0·001$).

Adenovirus 12-induced tumours

(*a*) *Macrophage migration inhibition tests.* Table 4 shows the areas of migration of PE cells from normal hamsters and hamsters immunized with adenovirus 12 tumour cells incubated in the presence of virus-induced tumour extracts. Inhibition of migration was observed at 24 and 48 hr in the presence of homologous adenovirus 12 tumour extract at a concentration of 2% and 10% (Table 4, exp. 1); however, although the inhibition was significant for tumour extract at 2% ($P = < 0{\cdot}05$ at 24 hr, and $P = < 0{\cdot}01$ at 48 hr), the inhibition in the presence of 10% extract in this experiment was not significant. Normal PE cells were not significantly inhibited by either concentration of adenovirus 12 tumour extract. Significant inhibition was also found when PE cells from hamsters immunized with adenovirus 12-induced tumour cells and migrated in the presence of SV40 tumour extract, but in this case inhibition was observed for 10% tumour extract but not for 2% extract. Thus, for 10% SV40 tumour extract the inhibition of migration of PE cells from hamsters immunized with adenovirus 12 tumour cells was 58% at 24 hr and 39% at 48 hr, and both these results were statistically significant ($P = < 0{\cdot}05$); SV40 tumour extract did not inhibit the migration of PE cells from normal hamsters (Table 4, exp. 1). In a second experiment (Table 4, exp. 2), PE macrophages from hamsters immunized with adenovirus 12-induced tumour cells were migrated in the presence of 10% adenovirus 12 tumour extract. Significant inhibition of macrophage migration was recorded at both 24 and 48 hr for the homologous extract. Inhibition of migration was not, however, demonstrated for sensitized cells in the presence of heterologous CELO virus tumour extract at a 10% concentration. The migration of PE cells from normal hamsters was not inhibited by a 10% extract prepared from either adenovirus 12 or CELO virus-induced tumours.

(*b*) *Transplantation immunity tests.* The incidence of tumours in hamsters immunized with adenovirus 12 and subsequently challenged with different virus-induced tumour cells is shown in Table 2, exp. 1. Of 26 hamsters immunized with adenovirus 12, 2 developed tumours following subsequent inoculation with adenovirus 12-induced tumour cells; in contrast 14 of 27 non-immunized hamsters developed tumours. The incidence of tumours in hamsters inoculated with adenovirus 12-induced tumour cells following immunization with SV40 virus and CELO virus was similar to that found in non-immunized animals. Both SV40 virus and CELO virus induced significant transplantation immunity to challenge with homologous tumour cells (Table 2, exp. 1).

DISCUSSION

The present results of transplantation immunity tests using SV40, adenovirus 12 and CELO virus induced tumours of hamsters confirm previous findings of specific virus-induced antigens on the surface of tumour cells induced by DNA viruses [17]. Thus, hamsters immunized with SV40 virus developed specific transplant immunity and rejected SV40-induced tumour cells, but did not reject adenovirus 12-induced or CELO virus-induced tumour cells. Specific transplantation immunity was also demonstrated for both the adenovirus 12 and CELO virus tumour systems. In addition, immunization with X-irradiated tumour cells gave similar results. Transplantation immunity to tumour cell challenge has been shown to be a cell-mediated immune response [7, 11, 12, 14] and can be measured *in vitro* using the macrophage migration inhibition test [8, 9]; however, it is not known if this latter test has the same specificity as the *in vivo* transplantation immunity test.

In the present study, PE cells from hamsters immunized with X-irradiated SV40 tumour cells showed significant inhibition of migration in the presence of SV40 tumour extract, but not in the presence of adenovirus 12-tumour extract. PE cells from hamsters immunized with X-irradiated CELO virus tumour cells showed significant inhibition of migration in the presence of CELO tumour extract, but not in the presence of adenovirus 12 tumour extract. Thus, the macrophage migration inhibition test would appear to show a specificity similar to that seen for transplantation immunity tests. Adenovirus 12 tumour extract at 10% and 2% concentration inhibited the migration of PE cells from hamsters immunized with the homologous tumour cells, but not macrophages obtained from normal hamsters; or hamsters immunized with SV40 or CELO virus-induced tumour cells. However, significant inhibition of migration was obtained for PE cells, from hamsters immunized with adenovirus 12-induced tumour cells when migrated out in the presence of 10% SV40 tumour extract, but this tumour extract did not inhibit the migration of PE cells from normal hamsters. These results suggest that lymphoid cells from hamsters immunized with

adenovirus 12-induced tumour cells are sensitized to antigen(s) present in SV40 tumour extract; and that this cross reactivity can be diluted out, since inhibition was not detected using SV40 tumour extract at a 2% concentration. Using an indirect fluorescent antibody test, antigen(s) common to both adenovirus 12 and SV40 tumours have been demonstrated; these antigen(s) were shown to react with anti SV40 "S" (surface) antiserum, and further investigation showed that they were possibly of embryonic origin since the antigens were found present in early embryo cells [18]. The cross-reactivity detected in the present study may be due to common embryonic antigen(s) on different tumours. Alternatively, the cross-reacting antigen may be a weak antigen demonstrable in the *in vitro* test but not measured in transplantation immunity tests. Further work is required to establish the nature of the cross-reacting antigens.

Previous studies have shown that the macrophage migration inhibition test can be used to study cell mediated immunity to tumours. In addition, the properties of an adenovirus 12-induced tumour antigen which produced transplantation immunity in CBA mice could be assayed by either the transplantation immunity test or the macrophage migration test with comparable results [19, 20]. However, the present study suggests that the transplantation immunity test is either not influenced by, or not sensitive for, cross-reacting antigens present on the surface of DNA virus-induced tumour cells.

Attempts to show cross-reacting tumour-associated antigens by using a smaller tumour cell challenge dose were also unsuccessful (Rees and Potter—unpublished data). In contrast, the macrophage migration inhibition test is sensitive to common antigens. In order to use the latter test specifically, a method of tumour extract preparation to exclude cross-reacting antigens may be necessary, and the optimal concentration of antigen used in the test may have to be calculated. Further studies are required to establish the exact nature of the cross-reactions, and whether they are due to common embryonic or surface antigens.

Acknowledgements—We would like to thank Professor Sir Charles Stuart-Harris and Professor M. G. McEntegart for their advice and criticism, and Mr. Allen Clegg for excellent technical assistance.

REFERENCES

1. J. J. Trentin and B. Estelle, Immunization of hamsters and histoisogenic mice against transplantation of tumours induced by human adenovirus type 12. *Proc. Amer. Assoc. Cancer Res.* **5,** 64 (1964).
2. L. D. Berman, On the nature of transplantation immunity in the adenovirus tumour system. *J. exp. Med.* **125,** 983 (1967).
3. L. W. Law, Studies of the significance of tumour antigens in induction and repression of neoplastic diseases: Presidential address. *Cancer Res.* **29,** 1 (1969).
4. C. W. Potter and J. S. Oxford, Transplantation immunity following immunization with extracts of adenovirus 12 tumour cells. *Int. J. Cancer* **6,** 410 (1970).
5. R. C. Rees and C. W. Potter, Specific enhancement of transplantation immunity with heat-killed mycobacterium butyricum and immunizing extracts from adenovirus 12-induced tumour cells. *Brit. J. Cancer* **26,** 139 (1972).
6. C. W. Potter, J. M. Hoskins and J. S. Oxford, Immunological relationships of some oncogenic DNA viruses. *Arch. ges. Virusforsch.* **27,** 73 (1969).
7. R. C. Rees and C. W. Potter, *In vivo* studies of cell-mediated and humoral immune responses to adenovirus 12-induced tumour cells. *Arch. ges. Virusforsch.* **41**, 116 (1973a).
8. M. George and J. H. Vaughan, *In vitro* cell migration as a model for delayed hypersensitivity. *Proc. Soc. exp. Biol. (N.Y.)* **111,** 514 (1962).
9. J. R. David, S. Al-Askari, H. S. Lawrence and L. Thomas, Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens. *J. Immunol.* **93,** 264 (1964).
10. W. J. Halliday, Blocking effect of serum from tumour-bearing animals on macrophage migration inhibition with tumour antigens. *J. Immunol.* **106,** 855 (1971).
11. W. J. Halliday, Macrophage migration inhibition with mouse tumour antigens: properties of serum and peritoneal cells during tumour growth and after tumour loss. *Cell Immunol.* **3,** 113 (1972).
12. J. Vaage, R. D. Jones and B. W. Brown, Tumour-specific resistance in mice detected by inhibition of macrophage migration. *Cancer Res.* **32,** 680 (1972).

13. J. C. Churchill, A. A. Nordin and K. Y. Brunner, Detection of cellular immunity to tumour antigens of a guinea pig hepatoma by inhibition of macrophage migration. *J. nat. Cancer Inst.* **48,** 54 (1972).
14. R. C. Rees and C. W. Potter, Immune response to adenovirus 12-induced tumour antigens, as measured *in vitro* by the macrophage migration inhibition test. *Europ. J. Cancer* **9,** 497 (1973b).
15. G. C. Schild, J. S. Oxford and C. W. Potter, Demonstration of a tumour and transplantation antigen in hamster tumours induced by an avian adenovirus (CELO). *Arch. ges. Virusforsch.* **29,** 25 (1970).
16. H. O. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
17. G. Klein, Tumour antigens. *Ann. Rev. Microbiol.* **20,** 223 (1966).
18. L. D. Berman, The SV40 S antigen; A carcino-embryonic type antigen of the hamster. *Int. J. Cancer* **10,** 326 (1972).
19. E. G. Brown, C. W. Potter and J. S. Oxford, Properties of transplantation antigen present in cell-free extracts of adenovirus 12 tumours. *Europ. J. Cancer* **8,** 9 (1971).
20. C. W. Potter and R. C. Rees, Some physico-chemical properties of adenovirus 12-induced TSTA(s) as measured using the macrophage migration inhibition test. *Europ. J. Cancer.* In press.

Europ. J. Cancer Vol. 11, pp. 87–90. Pergamon Press 1975. Printed in Great Britain

An Immuno-Ultrastructural Study of Surface Immunoglobulins in Metastasizing and Non-Metastasizing Hamster Lymphomas*

R. MACHINAMI†, R. L. CARTER and M. S. C. BIRBECK

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London SW3 6JB, England

Abstract—*Cells from two transplantable hamster lymphomas, one of which (ML) metastasizes while the other (NML) grows only at the site of implantation, were examined by an immuno-histochemical (immuno-peroxidase) technique to demonstrate surface immunoglobulins. About 90% of ML cells showed positive surface staining with a consistent pattern comprising a localized "cap". NML cells showed little or no immunoglobulin staining and the reaction product (when present) exhibited no consistent pattern; capping was never seen. The association between surface immunoglobulin on ML cells and the capacity of this tumour to metastasize is discussed and two interpretations of the surface staining are considered. The immunoglobulin may be* host *in origin and bound to ML cells in antigen-antibody complexes; or it may be synthesised by the* tumour cells *as an intrinsic, self-generating mechanism to potentiate metastatic growth.*

INTRODUCTION

SEVERAL studies have been made of two homotransplantable lymphomas in hamsters which, despite closely similar morphology, differ widely in their capacity to metastasize: one of them (ML) metastasizes widely while the other (NML) does not normally metastasize at all [1–7]. As a broad generalization it appears that metastasis is inhibited by "cellular" immune mechanisms in NML and facilitated by "humoral" mechanisms in ML. Comparing the ultrastructure of ML and NML, Birbeck and Carter [7] observed that ML cells often contained small stacks of endoplasmic reticulum; no such structures were seen in NML cells. The question therefore arose as to whether ML cells synthesize and release proteins, particularly immunoglobulins. The possibility that cells from a metastasizing tumour might elaborate their own potentiating or "blocking" antibodies is an intriguing one and, as a preliminary to further investigations, it was decided to examine ML and NML cells by an ultrastructural immunological technique to determine whether surface immunoglobulins were indeed present on ML cells.

A previous immunofluorescence study using rabbit-anti-hamster γ globulin conjugated with fluorescein [8] had shown that immunoglobulins were present in ML but not NML cells; but acetone-fixed sections were used in this investigation and such a procedure demonstrates intracellular rather than surface immunoglobulin. The more refined method described here was accordingly used (a) to confirm the

Accepted 26 September 1974.

†Present Address: Department of Pathology, Faculty of Medicine, University of Tokyo, Hongo, Tokyo 113, Japan.

*This work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the Cancer Research Campaign.

presence of immunoglobulin on ML (but not NML cells), and (b) to attempt to localize it in a more precise fashion.

MATERIAL AND METHODS

Tumours and control tissues

The metastasizing and non-metastasizing lymphomas (ML and NML) have been maintained by serial transplantation in this laboratory. Solid fragments were implanted into the subcutaneous tissues of the flank of female hamsters aged about 10 weeks and weighing approximately 100 g. Tumours were obtained at various times from 8 to 21 days after grafting from the flank and (in the case of ML) from liver metastases. Ascites forms of ML and NML were also examined.

Spleens from normal hamsters were used as controls.

Reagents

The rabbit anti-hamster immunoglobulin (Ig) serum was obtained from Behringwerke Ag. Two goat-anti-rabbit Ig-peroxidase conjugates were used; one was obtained from Cappel Laboratories while the other was prepared from anti-rabbit serum and horseradish peroxidase by the method of Kawaoi and Nakane [9].

Cell labelling and electron microscopy

Cell suspensions of ML and NML (from subcutaneous grafts), from ML (hepatic metastases), and from normal spleens were prepared by homogenizing solid tissues (1–2 gm in the case of the tumours) in 1 ml tissue culture medium (TC 199, Bio-cult Laboratories, Glasgow) containing 1% bovine serum albumin. Suspensions of the ascites tumours were made by spinning 2ml of ascites fluid at 1000 ***g*** for 3min and resuspending the cells in the same tissue culture medium.

The cells were labelled by resuspending them in 1 ml of culture medium containing 0·1 ml rabbit-anti-hamster serum for 30 min at room temperature. They were then washed twice with culture medium and suspended in 1ml medium containing 0·1 ml of the peroxidase conjugate for 30 min at room temperature. The cells were subsequently washed twice with medium and treated with diamino-benzidine (DAB) reagent (3mg DAB in 10 ml phosphate-buffered saline (PBS)—0·1 ml 1% H_2O_2).

After a further brief wash with medium, the cells were fixed in 1% glutaraldehyde in 0·07M cacodylate buffer for 1 hour at room temperature, post-fixed in 1% osmium tetroxide for 30 min and embedded in Araldite. Ultrathin sections were stained with lead solution [10] and examined in a Philips EM300 microscope.

RESULTS

It was not easy to obtain uniformly satisfactory cell suspensions from all specimens. NML in the ascites form was sometimes contaminated by erythrocytes and the ML solid tumour, with its rather fibrous stroma, was on occasions difficult to homogenize without damaging many of the tumour cells. But technically satisfactory suspensions for counting were eventually obtained from the following specimens: normal hamster spleen, ML ascites cells (20 days), ML solid tumour (21 days), NML solid tumours (13 and 15 days) and ML liver metastases (16 days). The other preparations, though unsuitable for quantitative examination, contained adequate numbers of well-preserved cells for qualitative appraisal.

The proportion of intact stained cells in the suspensions was determined by viewing consecutive fields under the electron microscope until about 50 separate tumour or spleen cells had been counted. Damaged or necrotic cells were excluded; so, too, were erythrocytes though they sometimes showed membrane staining. In some instances the experiments were performed twice using two different conjugates.

Control spleen cells from normal hamsters

The results of the cell counts are shown in Table 1. The cell suspensions contained large numbers of erythrocytes and lymphoid cells together with a few eosinophils, neutrophils and macrophages. Roughly half the lymphoid cells showed a consistent pattern of staining in the form of a "cap" over one sector of the cell, about 600 Å thick (Fig. 1). Eosinophils, neutrophils and macrophages were not stained.

ML tumour cells

Suspensions were examined quantitatively from a solid tumour of 21 days, an ascites tumour of 20 days and a hepatic metastasis (16 days). The results are summarized in Table 1. The proportion of stained cells was high, and the consistent capping pattern of staining observed (Fig. 2) was similar to that seen in lymphoid cells from normal spleens (see below). Most eosinophils and macrophages in these preparations were unstained. Lymphoid cells, which were relatively common in suspensions from the hepatic metastases, were stained in

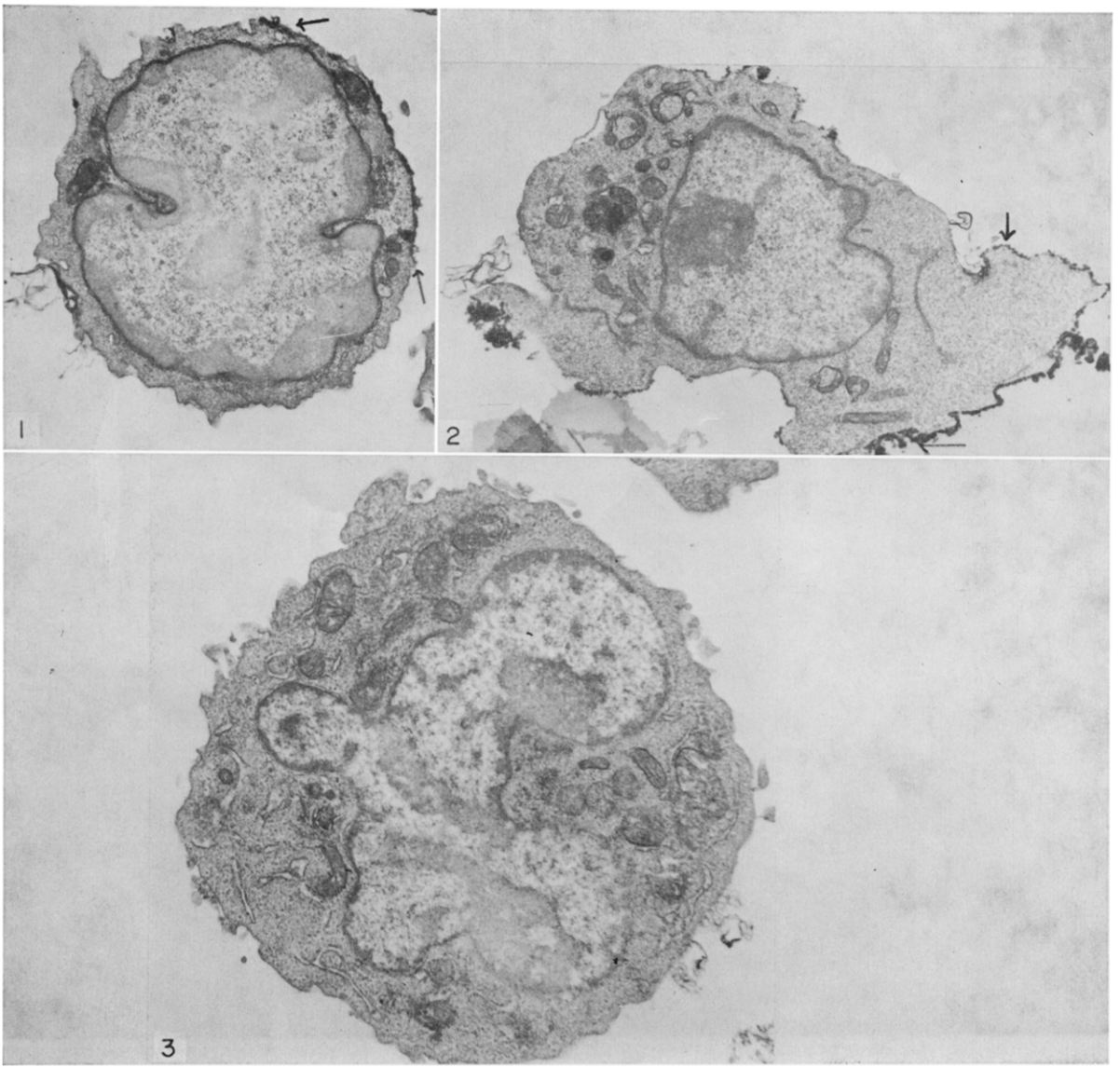


Fig. 1. *A lymphoid cell from normal hamster spleen showing positive immunoglobulin staining. The surface staining (between two arrows) forms a "cap" over one sector of the cell, about* 600 Å *thick*(× 17,000).

Fig. 2. *ML tumour cell showing positive staining. The staining (between two arrows) is similar to that seen in Fig.* 1(× 14,000).

Fig. 3. *NML tumour cell, prepared by the same methods as Figs.* 1 *and* 2. *No reaction product is seen*(× 14,000).

(to face p. 88)

about the same proportion as lymphoid cells from a normal spleen. The thickness of the stain seen on ML tumour cells and on normal lymphoid cells was similar. Although preparations of ML solid tumours obtained at other times were unsatisfactory for quantitation, these further suspensions (made at 8, 12, 14, 16 and 18 days) all contained tumour cells capped with stainable immunoglobulin.

NML tumour cells

Suspensions were prepared from solid tumour at 13 and 15 days; the counts are given in Table 1. The proportion of stained cells was very low and their pattern of staining was strikingly different from that seen in ML cells,

Table 1. *Counts and percentages of normal and neoplastic cells with immunoglobulin demonstrated by immuno-ultrastructural method*

Cell suspensions	Positive	Negative	Total	% (Positive cells)
Normal hamster spleen (lymphoid) cells	49* (26, 23)	55* (25, 30)	104	47
ML ascites tumour (20 days)	98* (39, 59)	22* (17, 5)	120	82
ML solid tumour (21 days)	52	4	56	93
ML liver metastasis (16 days)	59	2	61	97
NML solid tumour (13 days)	10* (2, 8)	112* (66, 46)	122	8
NML solid tumour (15 days)	3	49	52	6

*Two different conjugates were used and the two different numbers in the parentheses correspond to each of the two cell suspensions.

consisting of a few diffusely stained regions arranged inconstantly and irregularly on the cell surface (Fig. 3). No NML cells with a discrete "cap" of stainable immunoglobulin were ever seen.

DISCUSSION

This immuno-ultrastructural investigation has demonstrated a consistent difference in stainable immunoglobulin on the surface of two transplantable metastasizing and non-metastasizing lymphomas (ML and NML) in hamsters. ML cells have stainable surface immunoglobulin in the form of a localized cap; NML cells have virtually none. The proportion of positively-staining ML tumour cells (~90%) was higher than the proportion of positive lymphoid cells in normal spleen (~50%). The latter figure is in agreement with previously published figures for normal spleen cells [11] and has been interpreted as showing that not all spleen cells have immunoglobulin on their surface. It can, however, be argued that the stained cap will not always be included in an ultrathin section of a cell so that the actual number of capped cells may sometimes be underestimated. The extent of this underestimation will depend, *inter alia*, on the size of the cell and the area of its cap; and it will clearly be different for normal lymphoid cells and for larger tumour cells. We therefore suggest that the high proportion of ML tumour cells observed with a stained cap indicates that most (perhaps even all) ML cells carry immunoglobulin on their surface. By contrast, very few NML cells contained any demonstrable reaction product on their cell surfaces. This material was present in only trace amounts, showed no consistent pattern of localization and is probably attributable to non-specific binding of the conjugates.

Although a difference in intracellular immunoglobulins of ML and NML cells had already been described in immunofluorescent studies on fixed cells [8], the presence of more precisely localized γ-globulin on the ML cell surfaces has now been demonstrated; the formation of discrete caps had not been noted previously. Similar surface capping has been reported in an ultrastructural study on normal spleen lymphocytes from the mouse with the immunoferritin technique by de Petris and Raff [12]; these authors also reported a difference in distribution of surface immunoglobulin at cold and room temperatures—0–4°C compared with 20–24°C. Cell surface staining on normal rat lymphoid cells has been described by Gonatas and co-workers [13] who used the peroxidase immunohistochemical method which was employed here. These workers estimated the stained zone to be from 100 Å to 0·2 μm to 0·3 μm in thickness, values not dissimilar from the 600–700 Å observed by us. The present study appears to be the first in which capped immunoglobulin is demonstrable on the surface of a metastasizing lymphoma but virtually absent in an (otherwise closely similar) non-metastasizing tumour.

The functional implications of these observations are intriguing. The consistent pattern of staining observed on the surface of ML cells, and its virtual absence from NML cells, makes it unlikely that the reaction product is non-

specifically adsorbed immunoglobulin. One possibility, noted in the introduction, is that immunoglobulin may be produced by ML cells and exported via the endoplasmic reticulum which is a feature of ML (but not NML) cells. A previous electron microscope study [7] showed that macrophages in the ML tumour are not activated though activation is marked in macrophages in NML; since ML evokes a predominantly humoral response it might be postulated that ML cells themselves elaborate humoral factors that block macrophage-tumour cell interaction. Alternatively, the stained immuglobulin on ML cells may represent specific antibody formed by the host and reacting with antigenic determinants on the tumour cell surface. Further experiments are needed to resolve these various interpretations —in particular, the use of suspensions of fixed ML cells to determine whether immunoglobulin is associated with intracellular structures, notably the endoplasmic reticulum.

Acknowledgements—This work was undertaken while one of the authors (R. M.) was a British Council scholar. We thank Mrs. Norma Barron for technical help and Mrs. Mary Manley for photography.

REFERENCES

1. R. L. Carter and R. K. Gershon, Studies on homotransplantable lymphomas in hamsters. I. Histologic responses in lymphoid tissues and the relationship to metastasis. *Amer. J. Path.* **49,** 637 (1966).
2. R. L. Carter and R. K. Gershon, Studies on homotransplantable lymphomas in hamsters. III. Further observations on the specificity of the histological responses in lymphoid tissues and their relationship to metastasis. *Amer. J. Path.* **50,** 203 (1967).
3. R. K. Gershon, R. L. Carter and K. Kondo, On concomitant immunity in tumour-bearing hamsters. *Nature (Lond.)* **213,** 674 (1967).
4. R. K. Gershon, R. L. Carter and K. Kondo, Immunologic defense against metastasis: impairment by excision of an allotransplanted lymphoma. *Science, N.Y.* **159,** 646 (1968).
5. K. Nomoto, R. K. Gershon and B. H. Waksman, Role of non-immunized macrophages in the rejection of an allotransplanted lymphoma. *J. nat. Cancer Inst.* **44,** 739 (1970).
6. R. K. Gershon and R. L. Carter, Facilitation of metastatic growth by antilymphocyte serum. *Nature (Lond.)* **226,** 368 (1970).
7. M. S. C. Birbeck and R. L. Carter, Observations on the ultrastructure of two hamster lymphomas with particular reference to infiltrating macrophages *Int. J. Cancer* **9,** 249 (1972).
8. R. L. Carter, R. K. Gershon and K. Kondo, Studies on homotransplantable lymphomas in hamsters. V. The significance of immunofluorescent staining in tissues from tumor-bearing animals. *Transplantation* **6,** 313 (1968).
9. A. Kawaoi and P. K. Nakane, An improved method of conjugation of peroxidase with proteins. *Fed. Proc.* **32,** 840 (1973).
10. M. J. Karnovsky, Simple methods for staining with lead at high pH in electron microscopy. *J. biophys. biochem. Cytol.* **11,** 729 (1961).
11. M. C. Raff, Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology* **19,** 637 (1970).
12. S. De Petris and M. C. Raff, Distribution of immunoglobulin on the surface of mouse lymphoid cells as determined by immunoferritin electron microscopy. Antibody-induced, temperature-dependent redistribution and its implications for membrane structure. *Europ. J. Immunol.* **2,** 523 (1972).
13. N. K. Gonatas, J.-C. Antoine, A. Stieber and S. Avrameas, Surface immunoglobulins of thymus and lymph node cells demonstrated by the peroxidase coupling technique. *Lab. Invest.* **26,** 253 (1972).

Europ. J. Cancer Vol. 11, pp. 91–92. Pergamon Press 1975. Printed in Great Britain

Distinctive Cytoplasmic Inclusions in Chronic Lymphocytic Leukaemia

J. C. CAWLEY, J. EMMINES, A. H. GOLDSTONE, T. HAMBLIN, D. HOUGH and J. L. SMITH

Department of Medicine, University of Cambridge and The Tenovus Research Laboratory, University of Southampton, Great Britain

Abstract—*Distinctive cytoplasmic inclusions of a type hitherto given little attention are described in two of a series of thirty cases of chronic lymphocytic leukaemia. The possible nature and significance of these inclusions are discussed and attention is drawn to their similarity to inclusions prominent in leukaemic reticuloendotheliosis.*

INTRODUCTION

A VARIETY of different cytoplasmic inclusions has been found in the lymphocytes of chronic lymphocytic leukaemia (CLL) [1–3]. The purpose of this paper is to report the presence in CLL of distinctive cytoplasmic inclusions of a type which has hitherto received little attention. Similar inclusions have been previously described in only one case of CLL [4] and one case of lymphosarcoma [5], although similar inclusions seem to be a prominent feature of leukaemic reticuloendotheliosis [6–8]. These distinctive cytoplasmic inclusions were present in two of a series of 30 cases of CLL and therefore, although not as common as in hairy cell leukaemia, they are probably not particularly rare in CLL.

MATERIAL AND METHODS

The cytoplasmic inclusions described in this report were observed while screening a series of 30 typical cases of CLL at the ultrastructural level for the presence of immunoglobulin crystals [9]. All cases were typical examples of CLL with regard to clinical picture, cytology and response to therapy, and none resembled leukaemic reticuloendotheliosis.

Leucocytes were separated by dextran sedimentation, fixed in 3% glutaraldehyde in cacodylate–HCl buffer, and then processed for electron microscopy in a routine fashion [2].

Routine Leishman preparations of whole blood and buffy coat were examined in all cases.

Accepted 24 September 1974.

RESULTS

The cytoplasmic inclusions were observed in two of the 30 cases studied. The inclusions could not be identified with any certainty in the Romanowsky preparations, and were only detectable during ultrastructural examination. The morphology of the inclusions is illustrated in Figs. 1 and 2. On longitudinal section (Fig. 1), they are seen to be composed of a central space flanked by two rows of approximately parallel fibrils or lamellae interspersed with ribosome-like particles. In transverse section (Fig. 2), the inclusions had a central space surrounded by several concentric lamellae. Both in longitudinal and transverse section (Figs. 1 and 2), the inclusions were frequently partially or completely surrounded by strands of rough endoplasmic reticulum.

The morphology of those lymphocytes containing inclusions in no way differed from the other lymphocytes present. In both the cases of CLL, the inclusions were present in less than 10% of the lymphocytes.

DISCUSSION

The inclusions described here closely resemble those described by Zucker Franklin [4]

in a single case of CLL and those documented recently by Anday *et al.* [5] in a case of what they regarded as lymphosarcoma. The paucity of previous reports would suggest that these inclusions are rare in CLL. However, our demonstration of the inclusions in two cases of a series of 30 suggests that they may be commoner in CLL than has been recognised hitherto.

Although they probably contain RNA [5], the nature and significance of the inclusions is uncertain, but it is interesting to note that similar inclusions appear to be a prominent feature in at least 50% of cases of leukaemic reticuloendotheliosis [7]. Indeed it has been suggested that they are specific for that condition [6, 8]. However, the present study confirms the conclusion reached by Daniel and Flandrin [7] that the inclusions are present in both CLL and leukaemic reticuloendotheliosis. Since both CLL [10, 11] and leukaemic reticuloendotheliosis [12] are probably B cell diseases, it is tempting to speculate that these inclusions may represent a marker of B cells. However, why, in both CLL and leukaemic reticuloendotheliosis, only some cases, and a minority of cells in these cases, contain inclusions, remains unexplained.

REFERENCES

1. Y. Tanaka and J. R. Goodman, *Electron Microscopy of Human Blood Cells.* Harper & Row, New York (1972).
2. J. C. Cawley and F. G. J. Hayhoe, *Ultrastructure of Haemic Cells.* W. B. Saunders, London (1973).
3. M. Bessis, *Living Blood Cells and their Ultrastructure.* Springer-Verlag, Berlin (1973).
4. D. Zucker-Franklin. Virus-like particles in the lymphocytes of a patient with chronic lymphocytic leukaemia. *Blood* **21,** 509 (1963).
5. G. J. Anday, J. R. Goodman and G. H. Tishkoff, An unusual cytoplasmic ribosomal structure in pathologic lymphocytes. *Blood* **41,** 439 (1973).
6. I. Katayama, C. Y. Li and L. T. Yam, Ulstrastructural characteristics of the hairy "cells" of leukaemic reticuloendotheliosis. *Amer. J. Path.* **67,** 361 (1972).
7. M. T. Daniel and G. Flandrin, Fine structure of hairy cell. *Lab. Invest.* **30,** 1 (1974).
8. D. Catovsky, J. E. Pettit, A. G. Galton, A. S. D. Spiers and C. V. Harrison, Leukaemic reticuloendotheliosis ("hairy" cell leukaemia): a distinct clinico-pathological entity. *Brit. J. Haemat.* **26,** 9 (1974).
9. J. C. Cawley, C. R. Barker, R. Britchford and J. L. Smith, Crystalline inclusions in a case of chronic lymphocytic leukaemia. *J. clin. exp. Immunol.* **13,** 407 (1972).
10. J. L. Preud'homme and M. Seligmann, Surface bound immunoglobulins as a cell marker in human lympho-proliferative diseases. *Blood,* **40,** 777 (1972).
11. A. C. Aisenberg, K. J. Block and J. C. Long, Cell-surface immunoglobulins in chronic lymphocytic leukaemia and allied disorders. *Am. J. Med.* **55,** 184 (1973).
12. D. Catovsky, J. E. Pettit, J. Galetto, A. Okos and D. A. G. Galton, The B-lymphocyte nature of the hairy cell of leukaemic reticuloendotheliosis. *Brit. J. Haemat.* **26,** 29 (1974).

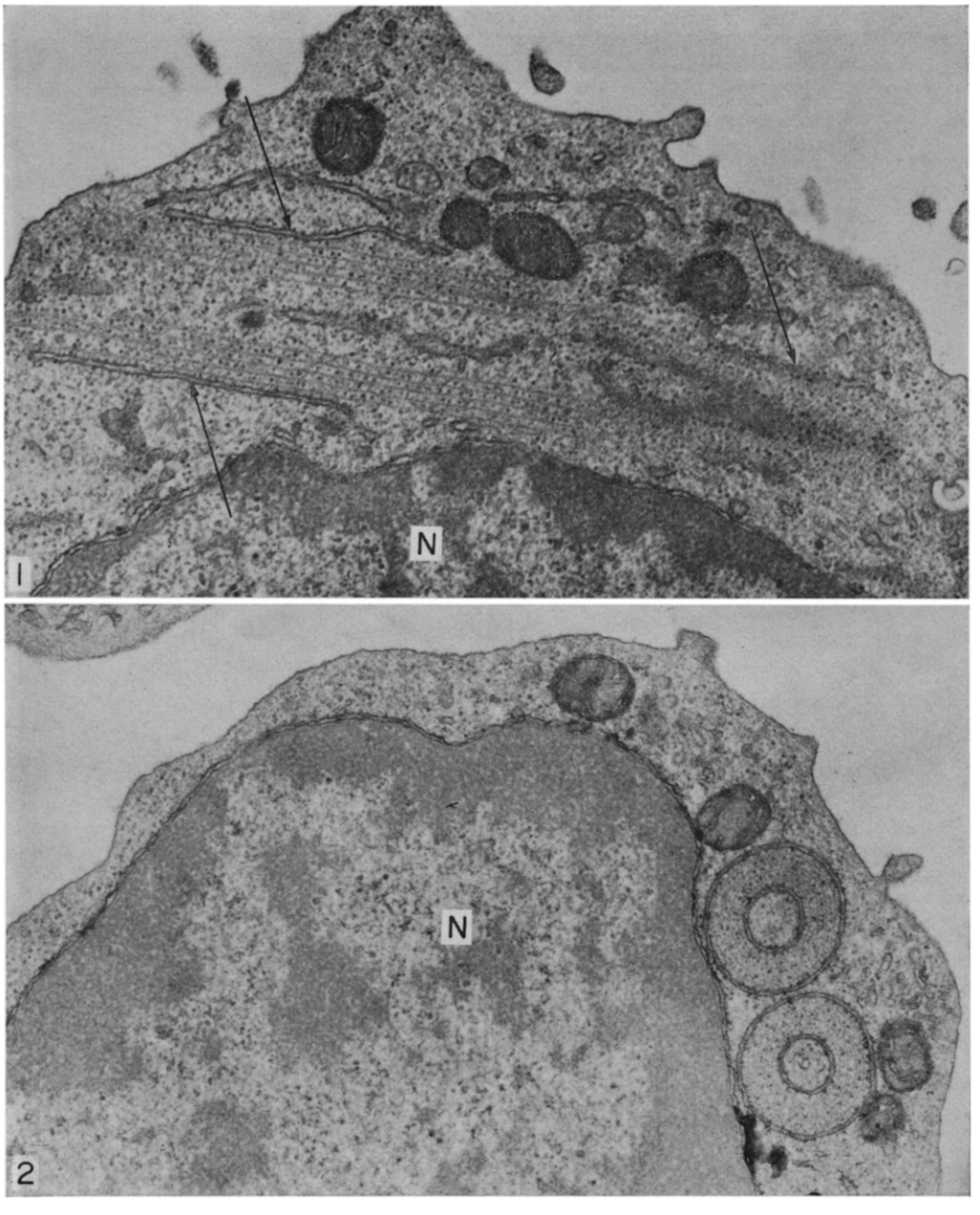


Fig. 1. (×27,500) *A typical cytoplasmic inclusion is shown in approximately longitudinal section. A central area of cytoplasm containing strands of rough endoplasmic reticulum (RER) is flanked by parallel fibrils. Numerous ribosome-like particles are scattered among these fibrils. The inclusion is partially surrounded by strands of RER (↑). The general cytology of the cell is typical of the lymphocytes of CLL; part of the nucleus (N) with heavy peripheral chromatin condensation is shown, and the cytoplasm contains scattered mitochondria, pinocytotic vesicles, RER and free ribosomes.*

Fig. 2. (×27,500) *Two inclusions sectioned transversely are shown. The central area of cytoplasm is enclosed by concentric fibrils interpersed with ribosome-like particles. The inclusion is enclosed, both within and without, by strands of RER. The nucleus with heavily condensed peripheral chromatin is typical of the small lymphocyte of CLL.*

(to face p. 92)

Europ. J. Cancer Vol. 11, pp. 93–96. Pergamon Press 1975. Printed in Great Britain

Repeated Cloning from Untreated Diploid XC-cells of Hypotetraploid XC-cells with Altered Morphology, *in vivo* Growth, Electric Mobility and Cyclic AMP Content*

P. EBBESEN†, J. HESSE†, T. VAN DEN BERGH†, K. CAPITO‡ and J. VISFELDT§

†*Department of Tumor Virus Research, Institute of Medical Microbiology, University of Copenhagen* 22 *Juliane Maries Vej*, ‡*Department of Biochemistry A, University of Copenhagen*, 30 *Juliane Maries Vej and* §*The University Institute of Pathlogical Anatomy*, 16 *Juliane Maries Vej, DK*-2100 *Copenhagen Ø, Denmark*

Abstract—*Repeated double-cloning of an ordinary diploid XC-cell culture established so far three cultures of hypotetraploid XC-cells that give fewer syncytial plaques than ordinary XC-cells when exposed to murine leukemia virus infected fibroblasts. Extracts of the ordinary diploid XC-cells and the hypotetraploid cells did not yield virus detectable by the XC-test. Infection of the two types of XC-cells with Rauscher virus did not alter the karyotype.*

The hypotetraploid cells were found also to differ from ordinary XC-cells in: (1) *containing fewer small multinucleated cells in uninfected culture,* (2) *having a slightly higher anodic mobility in cell electrophoresis,* (3) *a progressive growth in Wistar rats, and* (4) *a lower content of cyclic AMP.*

INTRODUCTION

By DOUBLE cloning an XC-cell line (E2B4) was established which differed from ordinary diploid XC-cells (A1) in: (1) being hypotetraploid, and (2) forming plaques with murine leukemia virus in the XC-test with a decreased efficiency [1]. A detailing of other characteristics of these E2B4 XC-cells that might be relevant to their altered reaction to leukemia virus infections is given in the paper.

Accepted 24 September 1974.

*This investigation was supported by the Danish Cancer Society, P. Carl Petersens Fond, Daell Fonden Anders Hasselbalchs fond til leukaemiens bekaempelse, the Danish Medical Research Council, the Danish Fund to the Advancement of Medical Science. Ingemann O. Bucks Fond, Novo's Fond and WHO.

MATERIAL AND METHODS

XC-cells

An ordinary hypodiploid line (A_1) [2], and a double-cloned hypotetraploid line (E2B4), derived from A_1 [1] were used. The chromosome constitution of the cell lines was redetermined after every fifth *in vitro* passage, and found unchanged. Two more hypotetraploid lines have been double-cloned [1] from A_1.

Growth of XC-cells and test for Rauscher murine leukemia virus was done by the XC-test [2], as previously described [1]. Minimum essential medium (Eagle) with Hanks' buffered salt (MEM) was used. Direct infection of XC-cells was done by adding 10^6 XC-units of Rauscher virus to 5×10^5 subconfluent DEAE-pretreated cells.

Transplantation

Log dilutions of from 10^6 to 10^1 cells were given. Each of these six doses was administered i.p. to two litters of newborn C3H mice, to 5 newborn Wistar rats and to 2 adult rats.

Cell electrophoresis

After trypsinization and washing, 10^6 cells per ml were incubated for 60 min at 20° C (rocking) in MEM and MEM containing 25 ug/ml of polyion. Following three times washing in MEM, electrophoresis was done, as described [3]. The polyions used were: (1) Polycations, Diethylaminoethyl dextran (DEAE-dextran) m.w. 2×10^6, Pharmacia, Uppsala, Sweden, and Polybrene (hexadimethrine bromide) m.w. 3500, Abbot Lab., Aldrich Chemical Comp. Inc., Wis. 53210, USA; (2) Polyanions, Dextran sulphate m.w. 5×10^5, Pharmacia, Uppsala, Sweden, and Heparin, Novo Industri A/S, 2880 Copenhagen, Denmark.

Cyclic AMP (c-AMP) content in freshly harvested cells was determined by a protein-binding radio assay. The c-AMP-containing extract was prepared by boiling the cells in assay phosphate buffer containing a phosphodiesterase inhibitor (aminophyllin) [4]. We have compared this method with the $BaSO_4$-precipitation method [5], with the following results: (a) the values for total c-AMP were found to be the same, (b) the recovery of c-AMP added before boiling or acidification was $100 \pm 15\%$, (c) the c-AMP values decreased to 0 after incubation with phosphodiesterase.

RESULTS

Both the original hypotetraploid XC-cell line E2B4 and the two later isolated hypotetraploid XC-cell lines showed decreased *syncytia* formation after contact with Rauscher-infected BALB/c embryo fibroblasts (XC-test) [1] as compared with ordinary XC-cells. Far the majority of cells in the ordinary XC culture are diploid [1]. *Infection* of two diploid and two tetraploid cell lines with Rauscher virus did not cause alteration in karyotypes. No virus inducing syncytia in the XC-test were recovered from the medium above XC-cells or the XC-cells themselves.

Uninfected recently confluent cultures of ordinary XC-cells contained about 20 small three-nucleated *"giant" cells* per 60 mm petri dish, whereas only a few were present in dishes containing hypotetraploid cells.

The electrophoretic anodic mobility of ordinary hypodiploid XC-cells was found slightly lower than that of the hypotetraploid E2B4 cells (Table 1). Polycations reduced the mobility of hypotetraploid cells. Polyanions increased the mobility of both hypodiploid and hypotetraploid cells.

Growth of *transplanted* tumor cells was successful only with the hypotetraploid cells and only when 10^6 cells were given to newborn rats. After 30 days all these five rats were dying with peritoneal sarcomas. No evidence of metastasis was found by microscopy of lung, liver, spleen, kidney, lymph nodes, thymus, and thyroid.

The *cyclic AMP* content was found to be 1·5 p.M. per 10^6 hypodiploid XC-cells and 0·75 p.M. per 10^6 hypotetraploid cells (3 tests).

DISCUSSION

Chan *et al.* [6] demonstrated that infection with murine leukemia virus can abolish the syncytia-forming capacity of XC-cells. The functional difference between our di- and tetraploid XC-cells, however, cannot be due to infection of the tetraploid cells after their cloning. Firstly, the co-occurrence of tetraploidy and decreased syncytia formation was a repeated finding when cloning was done from our XC-stock. Secondly, no change in karyotype was observed after infecting di- and tetraploid cell lines. Thirdly, using the XC-test no virus was detectable in extracts of our XC-cells.

Table 1. Electrophoretic mobility um/sec/V/cm (±standard deviation) of ordinary hypodiploid XC-cells and of hypotetraploid XC-cells treated with polycation/polyanion

		Polycations		Polyanions	
Treatment	Control	DEAE-d	Polybrene	Dextran-s.	Heparin
Hypodiploid XC-cell line	1·49 ± 0·12	1·45 ± 0·12	1·38 ± 0·15	1·64 ± 0·1	1·82 ± 0·09
Hypotetraploid XC-cell line E2B4	1·72 ± 0·06	1·33 ± 0·06	1·35 ± 0·08	1·97 ± 0·05	1·97 ± 0·06

Although virus may have played a role in "spontaneous" conversion of diploid to tetraploid XC-cells the differences between di- and tetraploid XC-cells now seem linked to the difference in karyotype.

The occurrence of fewer giant cells in uninfected tetraploid than diploid XC-cultures suggests that spontaneous giant cell formation may depend on the same membrane factors determining syncytia formation after infection with murine leukemia virus. This is supported by induction of giant cells in diploid XC-cells by infection with murine leukemia virus [7].

Progressive *in vivo* growth of hypotetraploid cells may be related to the higher negative outer charge. A variant of the murine leukemia cell line 1210 has lower outer negative charge and decreased capability of *in vivo* surviving [8], and treatment with polycations decreases *in vivo* growth of tumors [9, 10]. Also the lacking cell fusion when exposed to C-type virus may be related to the elevated negative outer charge of the tetraploid cells since treatment of cells with polyanions decreases their adhesiveness [11] and polyanion treatment of ordinary XC-cells decreases syncytia formation following exposure to C-type virus [12].

Our finding of a lower concentration of endogeneous cyclic AMP in the hypotetraploid cells may fit with reports on decreased adhesiveness of cells with low AMP level [13, 14] and indirect evidence for involvement of cyclic AMP in the cellular uptake of C-type virus [1]. Mitchell and Korinek, however, reported an inhibitory effect of *exogeneous* c-AMP on syncytia formation in the XC-test [15].

A connection between low c-AMP content and the more malignant behaviour *in vivo* is supported by *in vitro* evidence of a connection between malignancy and c-AMP level [16, 17, 18].

The occurrence of XC-cells with alteration of several characteristics in connection with a chromosome alteration support other evidence of a causal interrelationship between two or more of these cell characteristics.

REFERENCES

1. P. Ebbesen, O. Jørgensen and J. Visfeldt, Decreased formation of syncytial plaques in the XC-test with a double-cloned hypotetraploid XC-cell line and evidence of budding C-type paricles in this and ordinary hypodiploid XC-cell lines. *Europ. J. Cancer* **9,** 31 (1973).
2. W. P. Rowe, W. E. Pugh and J. W. Hartley, Plaque assay techniques for murine leukemia viruses. *Virology* **42,** 1136 (1970).
3. J. A. Forrester and M. H. Salaman, Cell electrophoretic mobilities in Friend virus disease. *Nature (Lond.)* **215,** 279 (1967).
4. R. H. Cooper, M. McPherson and J. G. Schofield, The effect of prostaglandins on pituitary content of adenosine 3′: 5′ cyclic monophosphate and release of growth hormone. *Biochem. J.* **127,** 143 (1972).
5. R. H. Cooper, S. J. H. Ashcroft and P. J. Randle, Concentration of adenosine 3′: 5′-cyclic monophosphate in mouse pancreatis islets measured by a protein-binding radioassay. *Biochem. J.* **134,** 599 (1973).
6. J. L. Chan, N. Vera, J. L. East, S. Hiraki and L. Dmochowski, Lack of syncyticum-formation by a type C-virus-producing XC-cell line in the mixed culture cytopathogenicity test. *Cancer Res.* **34,** 468 (1974).
7. G. S. Johnson, R. M. Friedman and I. Pastan, Analysis of the fusion of XC-cells induced by homogenates of murine leukemia virus-infected cells and by purified murine leukemia virus. *J. Virol* **7,** 753 (1971).
8. D. Kessel and H. B. Bosmann, Studies on a cell line derived for the L1210 murine leukemia with altered surface properties and decreased capacity for tumor production. *Cancer Res.* **34,** 606 (1974).
9. B. Larsen and K. Olsen, Inhibitory effect of polycations on transplantability of mouse leukemia reversed by heparin. *Europ. J. Cancer* **4,** 157 (1968).
10. P. Ebbesen, Influence of DEAE-dextran, polybrene, dextran and dextran sulfate on spontaneous leukemia development in AKR mice and virus induced leukemia in BALB/c mice. *Brit. J. Cancer* **30,** 68 (1974).
11. S. Nordling, Adhesiveness, growth behaviour and charge density of cultured cells. *Acta path. microbiol. scand.* **192,** 1 (1967).
12. J. Hesse and P. Ebbesen, Murine leukemia virus induced giant cell formation in XC sarcoma cell cultures modified by polyions. In preparation.
13. G. S. Johnson and I. Pastan, Cyclic AMP increases the adhesion of fibroblasts to substratum. *Nature New Biol.* **236,** 237 (1972).

14. B. M. Chassy, L. L. Love and M. I. Krichevsky, The acrasin activity of 3′,5′-cyclic nucleotides. *Proc. nat. Acad. Sci. USA* **64,** 296 (1969).
15. W. M. Mitchell and J. Korinek, The effect of adenosine 3′: 5′-cyclic monophosphate (c AMP) on XC rat sarcoma cell growth and function. *Amer. Chem. Soc. Abstract No. 302*, Washington, p. 162d, (1971).
16. G. S. Johnson, R. M. Friedman and I. Pastan, Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine 3′-5′-cyclic monophosphate and its derivative. *Proc. nat. Acad. Sci. USA.* **68,** 425 (1971).
17. J. Otien, J. Bader and G. S. Johnson, A mutation in a Rous sarcoma virus gene that controls adenosine 3′,5′-monophosphate levels and transformation. *J. Biol. Chem.* **247,** 1632 (1972).
18. L. A. Smets, Contact inhibition of transformed cells incompletely restored by dibutyryl cyclic AMP. *Nature New Biol.* **239,** 323 (1972).

Europ. J. Cancer Vol. 11, pp. 97–99. Pergamon Press 1975. Printed in Great Britain

Carcinomas of the Esophagus in Rats Ingesting Diethylnitrosamine

MELVIN D. REUBER

Laboratory of Biology, National Cancer Institute, Bethesda, Maryland 20014, *U.S.A.*

Abstract—*Buffalo strain male and female rats* 12 *weeks of age ingested* 0·0114% *diethylnitrosamine (DEN) in the diet for* 26 *weeks and were returned to the basal diet without DEN for the remainder of the experiment. Rats survived an average of* 28 *weeks. Carcinomas of the esophagus were observed in* 9 *of* 14 *male and* 5 *of* 14 *female rats. The incidence of carcinomas of the esophagus was higher in male rats; however, the incidence of liver carcinomas was higher in female rats. The organ site of induction of carcinomas with the symmetrical and asymmetrical nitrosamines is discussed.*

INTRODUCTION

DRUCKREY *et al.* felt that the N-N-diethylnitrosamines had an affinity for the liver because they were metabolized by and induced carcinomas of the liver [1] and that the asymmetrical nitrosamines were specific for the esophagus and carcinomas in that organ [2, 3].

This report describes carcinomas of the esophagus, as well as liver, in Buffalo strain male and female rats ingesting diethylnitrosamine (DEN) in the diet.

MATERIAL AND METHODS

Inbred Buffalo male and female rats (National Institutes of Health Animal Production), 12 weeks of age, were used. They were given 0·0114% DEN in a semisynthetic diet. Morris diet #272 contained 300 g commercial casein, 2275 g skim milk powder, 6152 g ground hard spring wheat, 200 g brewer's yeast, 200 g desiccated liver, N.P. (Wilson Laboratories, Chicago, Ill.) 140 g sodium chloride, 10 g ascorbic acid, 13 g ferric citrate, 100 g cod-liver oil, and 610 g corn oil.

Animals ingested the carcinogen-containing diet for 26 weeks *ad libitum*. After that time rats were given the basal diet. Animals were weighed weekly. Surviving animals were killed by exsanguination 36 weeks after the start of the experiment.

All organs, except the nasal cavity, were examined grossly and histologically. The esophagus was removed with the thyro-cricoid cartilage and the stomach. The esophagus was opened and lesions described, and the size and location recorded. Tissues were fixed in 4% formaldehyde and stained routinely with hematoxylin and eosin. Special stains, when indicated, included periodic acid-Schiff (PAS).

RESULTS

Animals gained weight until about 2 weeks before death or termination of the experiment, at which time they lost 16–20 g. Both the males and females survived for an average of 28 weeks [range 25–34].

Nine of 14 male rats and 5 of 14 female rats developed carcinomas of the esophagus. The lesions measured 1·5–1·7 cm dia and were located 1·5–2·0 cm (average 1·7) from the gastro-esophageal junction in male rats and half of the female rats; in the remaining half of the females the carcinomas were located at the

Accepted 27 September 1974.

*Present address: 11014 Swansfield Road, Columbia, Maryland 21044, U.S.A.

pharyngeal tracheal level of the esophagus. Histologically the carcinomas were a mixture of undifferentiated, poorly differentiated cells (Figs. 1 and 2) and well-differentiated squamous cells with keratin pearls. There was focal invasion of the submucosa.

Bronchopneumonia, observed in 5 female and 3 male rats with carcinomas of the esophagus, probably resulted from obstruction of the esophagus and regurgitation into the lungs.

Carcinomas of the esophagus occurred most often in rats with either small carcinomas of the liver or in rats without malignant tumors of the liver.

Lesions in organs other than esophagus

Ten female rats had carcinomas and 3 sarcomas of the liver; as compared with 5 carcinomas and 2 sarcomas of the liver in male rats ingesting DEN. This higher incidence of malignant tumors of the liver in females was significant at the $P = < 0{\cdot}05$ level.

Female rats also had random malignant tumors in other organs. These were located in the uterus, brain, and ovary.

Carcinomas were not observed in the oral cavity, tongue or squamous portion of the stomach.

DISCUSSION

Carcinomas of the esophagus were observed regularly by unsymmetrical nitrosamines in young BD strain rats and the authors concluded that the chemicals were specific inducers of carcinomas of that organ [2]. Ethyl-vinyl-nitrosamine, methyl-benzyl-nitrosamine, dimetyl-dinitroso-ethylenediamine, *n*-nitroso-sarkosin, nitroso-sarkosin-ethyl, *n*-nitrosopiperidine and *n*-methyl-*n*-nitrosoaniline all induced carcinomas of the esophagus; whereas rats given ethyl-isopropyl-nitrosamine and ethyl-n-butyl-nitrosamine had carcinomas of the liver in addition to esophageal carcinomas [1–4]. Carcinomas were observed only in the liver in rats given ethyl-ethanol-nitrosamine [2]. Ethyl-alkyl-nitrosamide and diazo-aceto-ester rarely produced carcinoma of the esophagus, but almost always induced squamous cell carcinoma of the stomach [5].

Buffalo strain rats ingesting DEN in the diet developed carcinomas of both the esophagus and liver. The carcinomas of the esophagus occurred in rats without hepatic carcinomas or in rats with small hepatic carcinomas.

Carcinomas of the esophagus were either localized or diffuse in BD strain rats given unsymmetrical nitrosamines. In Buffalo strain rats given DEN carcinomas of the esophagus were focal and located either near the gastro-esophageal junction or at the level of the pharyngeal tracheal bifurcation.

The incidence of carcinomas of the esophagus was higher in male Buffalo strain rats and the incidence of hepatic carcinomas and sarcomas was higher in Buffalo strain female rats ingesting DEN. This finding would indicate that the chemical was first transformed into an active carcinogenic agent in the esophageal mucosa and later reached the liver for additional metabolism, and that the esophagus was more susceptible in males than in female rats. With diffuse localization in the esophagus in BD strain rats given unsymmetrical nitrosamines, it could be predicted that little of the chemicals or their metabolites would reach the liver.

RF mice given DEN in the drinking water developed tumors of the lungs, liver, and stomach and a small number of microscopic tumors of the esophagus [6]. The authors stressed the importance of not relying on gross examination and of doing histological studies of the esophagus. Hamsters developed some liver tumors, but differed from rats and mice by developing tumors of the nasal cavity, trachea, bronchus and lungs regardless of the route by which DEN was given [7–10].

There are differences in the location of tumors in rats given nitrosamines. These differences are also observed in mice, rats and hamsters and may also be related to strain, dose, and route of administration, although to our knowledge there are no studies concerning route of administration in mice and rats. Until further studies are done the difference in location of the tumors in various organs in rats given symmetrical or asymmetrical nitrosamines can be considered to be quantitative rather than qualitative.

REFERENCES

1. H. Druckrey, R. Preussman, D. Schmähl and M. Müller, Chemische Konstitution und carcinogene Wirkung bei Nitrosaminen. *Naturwissenschaften* **48,** 134 (1961).
2. H. Druckrey, R. Preussman, G. Blum, S. Ivankovic and J. Afkham, Erzengung von Korzinomen der Speiseröhre durch unsymmetrische Nitrosamine. *Naturwissenschaften* **50,** 100 (1963).

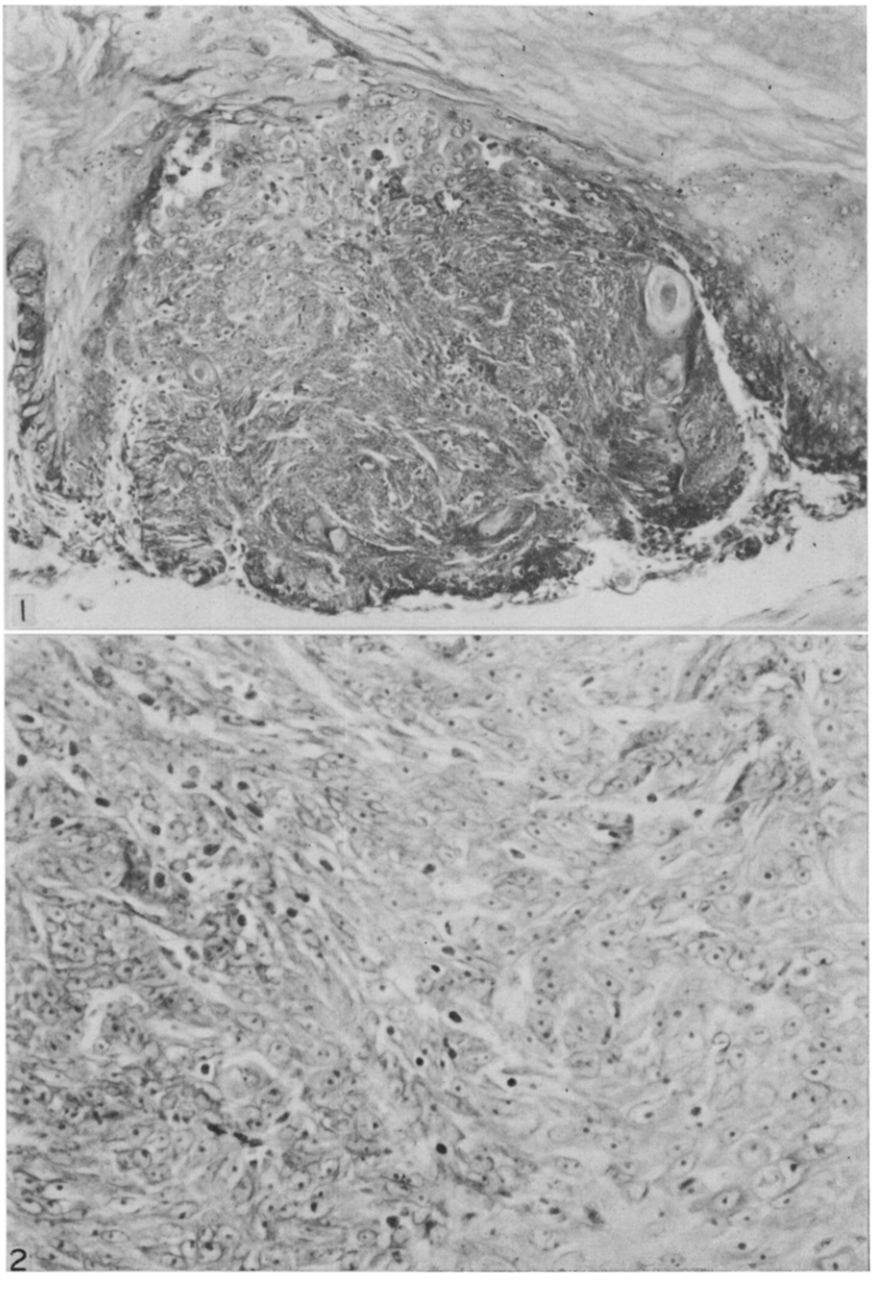


Fig. 1. *A small carcinoma of the esophagus that would have been difficult to observe on gross examination of the esophagus. The carcinoma is well demarcated and is located beneath the surface that is covered by keratin. Hematoxylin and eosin,* × 160.

Fig. 2. *Histologically the carcinoma is undifferentiated. Cells have large nuclei with prominent nucleoli, a small amount of basophilic, cytoplasm, and grow in sheets. Hematoxylin and eosin,* × 440.

(to face p. 98)

3. N. P. NAPOLKOV and K. M. POZHARISSKI, Morphogenesis of experimental tumors of the esophagus. *J. nat. Cancer Inst.* **39,** 903 (1967).
4. H. DRUCKREY, R. PREUSSMAN, D. SCHMÄHL and G. BLUM, Carcinogene Wirkung von N-Methyl-N-Nitroso-Anilin. *Naturwissenschaften* **98,** 722 (1961).
5. H. DRUCKREY, R. PREUSSMAN, D. SCHMÄHL and M. MÜLLER, Erzengung von Magenkrebs durch Nitrosamide an Ratten. *Naturwissenschaften* **48,** 165 (1961).
6. N. K. CLAPP and A. W. CRAIG, Carcinogenic effects of diethylnitrosamine in RF mice. *J. nat. Cancer Inst.* **39,** 903 (1967).
7. K. McD. HERROLD, Effect of route of administration on the carcinogenic action of diethylnitrosamine (N-nitrosodiethylamine). *Brit. J. Cancer* **18,** 763 (1964).
8. K. McD. HERROLD, Epithelial papillomas of the nasal cavity. *Arch. Path.* **78,** 189 (1964).
9. K. McD. HERROLD, Induction of olfactory neuroepithelial tumors in Syrian hamsters by diethylnitrosamine. *Cancer (Philad.)* **17,** 114 (1964).
10. K. McD. HERROLD and L. J. DUNHAM, Induction of tumors in the Syrian hamster with diethylnitrosamine (N-nitrosodiethylamine). *Cancer Res.* **23,** 773 (1963).
11. K. McD. HERROLD, Epidermoid carcinomas of the esophagus and forestomach induced in Syrian hamsters by N-Nitroso-N-methylurethan. *J. nat. Cancer Inst.* **37,** 389 (1966).

Europ. J. Cancer Vol. 11, pp. 101–103. Pergamon Press 1975. Printed in Great Britain

The Determination of High-Affinity Oestrogen Receptors in Human Breast Tumours

Critique of a Method Approved by the EORTC Breast Cancer Cooperative Group

HANNELORE BRAUNSBERG

Department of Chemical Pathology, St. Mary's Hospital Medical School, London W2 1PG, England

Abstract—*Theoretical considerations have shown that inaccurate estimates of receptor concentrations are obtained when charcoal adsorption methods, such as that adopted by the EORTC Breast Cancer Cooperative Group, are applied to tissue extracts containing appreciable concentrations of low-affinity binding components. A method of overcoming this difficulty is proposed.*

In a recent study [1] of a published method [2] for the determination of oestradiol receptors in tumour tissue extracts it was noted that errors in estimates of receptor concentrations and dissociation constants for the oestrogen-receptor complex can arise in the presence of excess low-affinity binding components. These analyses consist of equilibration of tumour cytosol preparations with tritium-labelled oestradiol and subsequent removal of free and loosely-bound steroid by incubation with dextran-coated charcoal. Three sources of inaccuracy were encountered. Occasionally, curved Scatchard [3] diagrams were obtained from which receptor concentrations could not be estimated. In some instances, dissociation of low-affinity complexes appeared to be incomplete giving rise to Scatchard plots [3] which yielded extremely high values for both binding parameters. But even when the low-affinity complex is completely dissociated, the amount of "free" steroid obtained from the difference between total added and the amount determined as bound, can be grossly overestimated. As a result erroneous estimates of receptor concentrations are obtained and experiments with albumin have confirmed this [1].

Since the EORTC Breast Cancer Cooperative Group adopted a modification of this method as a standard assay [4], consideration of the errors involved is of some importance.

Accepted 24 September 1974.

THEORETICAL ASPECTS

By analogy with enzyme kinetics [5], the equilibrium equation for low- and high-affinity binding components competing for oestradiol can be written:

$$\frac{P+Q}{F} = \frac{P_O}{F+K_P} + \frac{Q_O}{F+K_Q} \qquad (1)$$

where P,Q are concentrations of oestradiol bound to high- and low-affinity sites, respectively, F is the concentration of free steroid at equilibrium, and K_P, K_Q are dissociation constants of complexes with, and P_O, Q_O concentra-

tions of total available sites of, high- and low-affinity components, respectively. In the absence of low-affinity binding components, the values of Q and the second term on the right-hand side of equation (1) are zero and

$$\frac{P}{F} = \frac{P_O}{F+K_P} \tag{2}$$

This equation can be rearranged to that of Scatchard [3]:

$$\frac{P}{F} = \frac{P_O}{K_P} - \frac{P}{K_P} \tag{2a}$$

In the absence of low-affinity components or when their concentration (Q_O) is relatively low, correct results will be obtained with or without charcoal incubation.

In the presence of appreciable concentrations of low-affinity binding components, incubation with dextran-coated charcoal removes oestradiol from the low-affinity binding sites and this, together with steroid left free after the first equilibration, is adsorbed by charcoal. The steroid left in solution (i.e. bound to high-affinity receptor) is determined and the difference between this and the total added is now $F+Q$.

At low oestradiol concentrations ($< 5\ nM$), $K_Q \gg F$ and equation (1) approximates to

$$\frac{P+Q}{F} = \frac{P_O}{F+K_P} + \frac{Q_O}{K_Q} \tag{3}$$

This cannot be rearranged to an equation of type (2a). But even if such an equation approximately fitted the data, the plot of $P/(F+Q)$ must give erroneous results for both P_O and K_P.

DETERMINATION OF BINDING CONSTANTS IN THE PRESENCE OF LOW-AFFINITY COMPONENTS

From equations (1) and (3) it follows that an estimate of Q_O/K_Q must be obtained before accurate determination of P_O and K_P is possible. At high oestradiol concentrations ($> 10\ nM$) $P \approx P_O$ and $F \gg K_P$. Then equation (1) approximates to

$$\frac{Q}{F} = \frac{Q_O}{F+K_Q} \tag{4}$$

which can be used in the form

$$\frac{Q}{F} = \frac{Q_O}{K_Q} - \frac{Q}{K_Q} \tag{4a}$$

to estimate Q_O/K_Q without charcoal incubation. Rearranging (3)

$$\frac{P+Q}{F} - \frac{Q_O}{K_Q} = \frac{P_O}{F+K_P}$$

$(P+Q)$ and F can be estimated at low oestradiol concentrations without charcoal incubation and writing

$$A = \frac{P+Q}{F} + \frac{Q_O}{K_Q}$$

we have

$$AF = P_O - AK_P \tag{5}$$

Hence a plot of AF against A has slope $-K_P$ and intercept (for $AF = 0$) P_O. In the absence of receptor, A is zero at low oestrogen concentrations.

DISCUSSION

Studies from several centres have shown that a considerable proportion of patients with tumours containing oestradiol receptors have not responded to endocrine therapy, and that a small proportion with receptor-negative tumours have shown responses [6]. At present there is a possibility that discrimination between responders and non-responders may be improved by using quantitative, rather than qualitative, assessment of receptor content. If this were the case, more accurate data are needed.

A technique involving removal of low-affinity binding sites prior to analysis would be expected to yield the most reliable results. At present no such technique has been developed.

Estimation of total (low- and high-affinity) binding at high and low oestrogen concentration as described in this paper would seem to offer one way of overcoming inaccuracy. It should be noted that this will require larger volumes of cytosol and, therefore, more tissue than the original techniques adopted [2, 4]. Tumours containing exceptionally high concentrations of low-affinity binding sites still present a problem, and their high-affinity receptor content cannot readily be quantitated by any method available at present.

REFERENCES

1. H. Braunsberg, Factors influencing the estimation of oestrogen "receptors" in human malignant breast tumours. Paper submitted to the *Europ. J. Cancer.*

2. P. FEHERTY, G. FARRER-BROWN and A. E. KELLIE, Oestradiol receptors in carcinoma and benign disease of the breast: An *in vitro* assay. *Brit. J. Cancer* **25,** 697 (1971).
3. G. SCATCHARD, The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51,** 660 (1949).
4. EORTC BREAST CANCER COOPERATIVE GROUP, Standards for the assessment of oestrogen receptors in human breast cancer. *Europ. J. Cancer* **9,** 397 (1973).
5. M. DIXON and E. C. WEBB, *Enzymes* p. 87. Longmans, London (1964).
6. W. L. MCGUIRE, P. P. CARBONE and E. P. VOLLMER (Eds.), *Estrogen Receptor in Human Breast Cancer.* Raven Press, New York (1975).

Europ. J. Cancer Vol. 11 pp. 105–109. Pergamon Press 1975. Printed in Great Britain

Enzyme Histochemical Patterns in Human Tumours—I. Hydrolytic Enzymes in Carcinoma of the Colon and the Breast.

JOHAN KOUDSTAAL, BOB MAKKINK and SIEBE H. OVERDIEP
Department of Pathology, "SSD Z", Stichting Samenwerking Delftse Ziekenhuizen, Reynier de Graefweg 7–Delft, Hollande

Abstract—*Enzyme histochemical methods were used to determine the activity of a number of hydrolytic enzymes in mammary and colonic–rectal carcinoma and to compare the activity with that in adjacent normal epithelium. The activity of the hydrolytic enzymes, a possible measure for the degree of differentiation of the cell, shows a general tendency to decrease, exceptions however, being numerous. For instance, a high proportion of the mammary carcinomas have an elevated acid phosphatase activity; some of the colonic–rectal carcinomas have an elevated 5'nucleotidase activity. Variations of the enzyme pattern between different kinds of tumours and between tumours of the same group are large.*

INTRODUCTION

UNDER normal conditions there is a delicately stabilized equilibrium between cells that differentiate and die and cells that provide for the production of cells. Once this equilibrium is disturbed, the basic condition for cancer is established, with production surpassing persistent decay.

In a highly differentiated tumour, a reasonably large proportion of the cells continue to differentiate; in non-differentiated cells this process has ceased.

The cell has enzymes, i.e., biologic catalizers promoting chemical reactions. A dividing cell has a group of enzymes that differ partially from the group of enzymes found in a differentiating cell. Enzyme histochemical techniques make it possible to localize a number of enzymes in a tissue section.

Details on enzyme histochemical methods can be found, in Pearse (1971) [1] and Pfleiderer (1968) [2]. These methods can be used to investigate how much the enzyme patterns of tumours differ from those of the tissues from which they develop.

The problem was studied in two groups of tumours, i.e., mammary carcinoma and colonic–rectal carcinoma. This report concerns the hydrolytic enzymes.

MATERIAL AND METHODS

Surgically excised tumours from patients treated in hospitals at Delft, Schiedam and Vlaardingen were received in the unfixed state. Samples of tumour tissue, whenever possible with adjacent normal tissue, were frozen at −80°C. Sections were cut in a cryostat for enzyme histochemical investigation, and corresponding samples were processed by the paraffin method. A sample of 181 mammary carcinomas was prepared, 120–136 of which allowed comparison of the tumour tissue with normal adjacent epithelium.

The variable number is due to the fact that in some cases one section of a series did not contain sufficient normal tissue so that the same

Accepted 20 May 1974.

number of tumours could not be compared for all of the enzymes. The age groups of the patients are shown in Table 1, where the number of tumours per age group is also indicated.

Table 1. Breast carcinoma (N = 181)

Age group	No. of cases	Age group	No. of cases
20–24	0	55–59	16
25–29	2	60–64	21
30–34	2	65–69	15
35–39	12	70–74	20
40–44	19	75–79	11
45–49	27	80–84	10
50–54	19	85–90	7

The tumours were classified histologically according to the WHO system [3]. The great majority of the neoplasms fell in group II, grades 2 and 3. Of the 136 tumours which could be compared with normal healthy tissue, 60 belonged to group C II, grade 2, and about 50 to group C II, grade 3, most of the remaining material belonging to group C II, grade 1.

Table 2. Colonic and rectal carcinoma (N = 112)

Age group	Male	Female	Age group	Male	Female
35–39	—	1	65–69	14	11
40–44	2	2	70–74	8	11
45–49	—	1	75–79	1	13
50–54	3	3	80–84	4	—
55–59	8	8	85–90	3	3
60–64	5	11			

Of the 112 colonic–rectal carcinomas, about 110 could be compared with normal epithelium. According to the TNM system (International Union Against Cancer), the tumours were graded as G1, G2 or G3. A high proportion were G2 and G3, i.e. 61 and 28 tumours, respectively. The age groups of these patients are given in Table 2.

The following enzyme histochemical reactions were performed: alkaline phosphatase [4], acid phosphatase [5], 5′nucleotidase and adenosine triphosphatase [6], leucine aminopeptidase [7] and α-naphtylacetate esterase [1].

RESULTS

The enzyme pattern of normal secretory epithelium of the breast and normal colonic epithelium is shown in Table 3. In general, the enzyme activity in tumour tissue is extremely variable and the localization in the cell is also more irregular than in a comparable normal cell.

Table 3. Enzyme histochemical pattern in breast and colonic–rectal epithelium

	Secretory epithelium of the breast		Colonic epithelium
	Ducts	Lobules	
Alkaline phosphatase	−	−	−
Acid phosphatase	+/−	++/−	++/±
5′nucleotidase	−	±/−	+/±
Adenosine triphosphatase	±/−	+/±	+++
Leucine aminopeptidase	+/±	+/±	+/±
Unspecific esterase	+/±	+/±	+++/++

− = no activity; ± = trace activity; + = low activity; ++ = moderate activity; +++ = high activity.

Our data are represented in Fig. 1 and 2, showing the hydrolytic enzymes of the group of mammary carcinomas and colonic–rectal carcinomas. The columns represent the percentage of tumours the activity of which was lower, equal to, or higher than the activity of the adjacent epithelium. It is evident from Fig. 2 that the activity of most of the hydrolytic

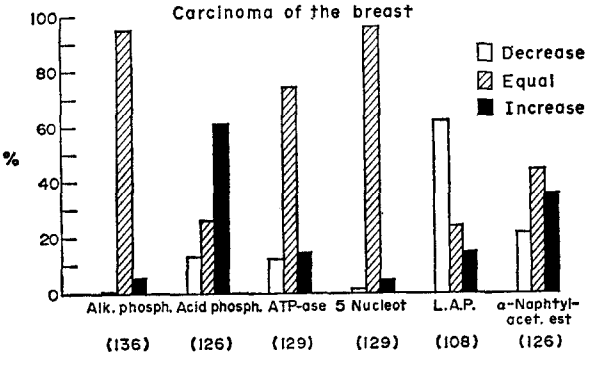


Fig. 1. Diagram showing the enzyme pattern in breast carcinoma. The left-hand column of each group indicates the percentage of the tumours in which enzyme activity was lower than in adjacent epithelium, the middle column, the percentage with the same activity, and the right-hand column, the percentage with increased activity. The value under the name of the enzyme indicates the number of comparable tumours investigated.

enzymes is decreased in colonic and recta carcinomas; particularly as regards acid phosphatase (Fig. 3), adenosine triphosphatase, leucine aminopeptidase, and α-naphthylacetate esterase. Adenosine triphosphatase proved to be an especially sharp indicator (Fig. 4). This general tendency comes up to expectation,

in principle tumour tissue being less differentiated than comparable normal epithelium. On the borderline between neoplastic tissue and normal epithelium, pronounced transitions are sometimes seen in the same acinus (Fig. 5). However, there was a number of tumours in which the activity of one of the hydrolytic enzymes had been increased, particularly 5'nucleotidase. In general, the elevation of the activity of one enzyme did not go together with an increased activity of other hydrolytic enzymes. This divergent reaction seemed to occur rather arbitrarily. Tumour cells with an elevated activity of a hydrolytic enzyme

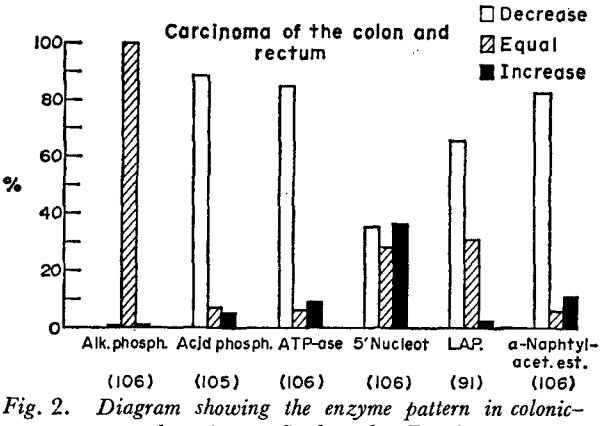


Fig. 2. Diagram showing the enzyme pattern in colonic–rectal carcinoma. See legend to Fig. 1.

showed the same dehydrogenase patterns as most of the other tumours. In breast carcinoma these effects were much less pronounced. Since the activity of the hydrolytic enzymes is also rather low in the breast under normal conditions, differences are less clear. A high activity of alkaline phosphatase occurring in normal acini of the breast may be suspected. This is deceptive, however, the activity being localized in myo-epithelial cells (Fig. 6). Only a few mammary carcinomas are characterized by a high activity of the enzyme alkaline phosphatase. Most breast tumours have a low activity of the hydrolytic enzymes.

A noteworthy exception is acid phosphatase. Tumour cells have a distinct tendency to high activity of this enzyme (Fig. 7). It should be mentioned here that colonic–rectal carcinomas have only a limited acid phosphatase activity, although they are surrounded by numerous macrophages with a high activity of this enzyme. In mammary carcinoma, the reverse phenomenon occasionally may be observed.

DISCUSSION

Normal colonic mucosa and colonic carcinoma generally show no activity of alkaline phosphatase. It confirms the data of literature [8–10]. Furthermore, activity of this enzyme could not be demonstrated in polyps and adenomas of the colonic mucosa [9].

The activity of acid phosphatase is low in colonic carcinoma [8, 10], and according to the results of Czernobilsky and Tsou [9], is lower than in normal colonic mucosa, which is confirmed by our findings, only in a few cases was an activity higher than that in normal colonic epithelium found. Elevated activity was observed mainly in tumour cells in the process of decay. One of the most striking observations was the large number of macrophages containing acid phosphatase and situated in the stroma. Such cells are found in low numbers in the stroma of normal colonic mucosa. Another remarkable finding in the present study is the relatively large number of colonic and rectal carcinomas with elevated 5'nucleotidase activity.

Schmidt-Mattiesen (1959) [11], demonstrated adenosine triphosphatase activity in 6 out of 7 colonic carcinomas. Our results, like those of Czernobilsky and Tsou (1968) [9], show a sharply decreased activity of this enzyme; these authors found the same phenomenon in colonic polyps and adenomas. However, a few of our tumours showed an increased activity of adenosine triphosphatase. Like Filipe (1971) [10], we found little or no activity of aminopeptidase. The diminished esterase activity observed by Wachstein (1962) [12], and Czernobilsky and Tsou (1968) [9], was also confirmed by our results. Activity of hydrolytic enzymes in normal secretory epithelium of the breast is low. Decrease of enzyme activity in carcinomas of the breast is less spectacular than in carcinomas of the colon.

Although alkaline phosphatase does not occur in normal secretory epithelium of the breast, this enzyme was found in some of our mammary carcinomas, which is in accordance with other reports [8, 13–15]. Under normal conditions, alkaline phosphatase is observed in the myo-epithelial cells, reaching a high level during pregnancy. Since myo-epithelial cells are derived from epithelium cells [16, 17], it is conceivable that the epithelial cells of the breast and the myo-epithelial cells both originate from the same stem cell. In view of the reaction of other hydrolytic enzymes in certain tumours, this may account for the activity of alkaline phosphatase in some breast carcinomas. Like Fanger and Barker (1959) [14] and Willighagen (1960) [8], we found breast carcinomas in which the acid phosphatase activity was higher than in the adjacent normal epithelium. In our study, 60% of the

tumours showed this elevation.

Wachstein (1962) [12] found no 5'nucleotidase activity in 13 breast tumours. In general, our results confirm the absence of this activity. Novikoff (1960) [18] found adenosine triphosphatase activity in 12 out of 13 breast carcinomas. In 14% of our breast carcinomas, this enzyme showed an activity higher than the very low activity observed in normal adjacent epithelium. Aminopeptidase is known to occur in breast carcinoma [19, 20]. In a number of our cases, we found a sharp increase in the activity of this enzyme as compared with adjacent epithelium.

Melnick and Bullock (1959) [21] described esterase activity in adenocarcinomas but found no enzyme activity in medullary carcinomas. Organs showing changes under hormonal influences might be expected to show differences in enzyme activity reflecting those changes. Kluck (1974) [22] reported that breast epithelium shows different enzyme patterns before and after ovulation. Leucine aminopeptidase and acid phosphatase, which occur mainly in the secretions of the acini, may here point to differentiation.

In all probability, the hydrolytic enzymes in general are indicators of differentiation, as they become active in developing organs, as we have been able to demonstrate in a 10 week old embryo and a 5 month old foetus. There are some exceptions, however, such as the high activity of alkaline phosphatase in ovocytes which disappears with maturity.

Decrease in activity of the hydrolytic enzymes can be regarded as partially due to a reduction in the differentiation of carcinoma cells. Some cases show an increased activity of a hydrolytic enzyme virtually unaccompanied by increased activity of another enzyme with which it is closely associated in a normally differentiated cell. This divergence points to a severe disturbance in the differentiation of the tumour cell.

It is not clear whether tumour cells with a high activity of a hydrolytic enzyme have a special metabolism or whether their capacity to divide is still intact.

In breast cancer most of the tumours with a high level of acid phosphatase activity showed an active intermediary metabolism, as deduced from the high activity of most of the dehydrogenases, (see, Part II, Koudstaal *et al.*, 1974) [23]. Data in literature [8], indicate that most of the human tumours show a lower acid phosphatase activity than the normal tissue from which they arise. Exceptions are, as already mentioned, mammary carcinoma and also prostatic carcinoma, both of which are tumours developed in tissue with a strong hormonal dependence.

For the time being, we would interpret the general decrease of the enzyme activity and the more or less unrelated increase in the activity of the hydrolytic enzymes as an expression of disturbances in the differentiation process. For the diagnostic importance of hydrolytic enzymes in tumours, we refer to Willighagen (1970, 1973). [24, 25].

Because of the large differences in enzyme activity in one and the same group of tumours, it is necessary to study the enzyme pattern of a large number of the same tumour. The activity of one enzyme may decrease or even disappear, whereas the activity of another enzyme may be increased. These facts are demonstrated by the pattern of two different human tumours.

REFERENCES

1. A. G. E. Pearse, *Histochemistry. Theoretical and Applied Second Edition* London (1971).
2. A. Pfleiderer, Enzymhistochemische Untersuchungen am Carcinom des Corpus uteri. *Fortschr. Geburtschilfe und Gynäkologie*, Vol. 36. Bibliotheca Gynaecologica No. 49. Karger, Basel (1968).
3. R. W. Scarff and H. Torloni, *Histological typing of Breast Tumours*. World Health Organization, Geneva (1968).
4. M. S. Burstone, Histochemical demonstration of phosphatase in frozen sections with naphtol A.S.-phosphatase. *J. Histochem. Cytochem.* **3,** 146 (1961).
5. T. Barka and P. J. Anderson, Histochemical methods for acid phosphatase using hexazonium pararosaline as coupler. *J. Histochem. Cytochem.* **10,** 741 (1962).
6. M. Wachstein and E. Meisel, Histochemistry of hepatic phosphatases at a physiologic pH. *Amer. J. clin. Path.* **27,** 13 (1957).

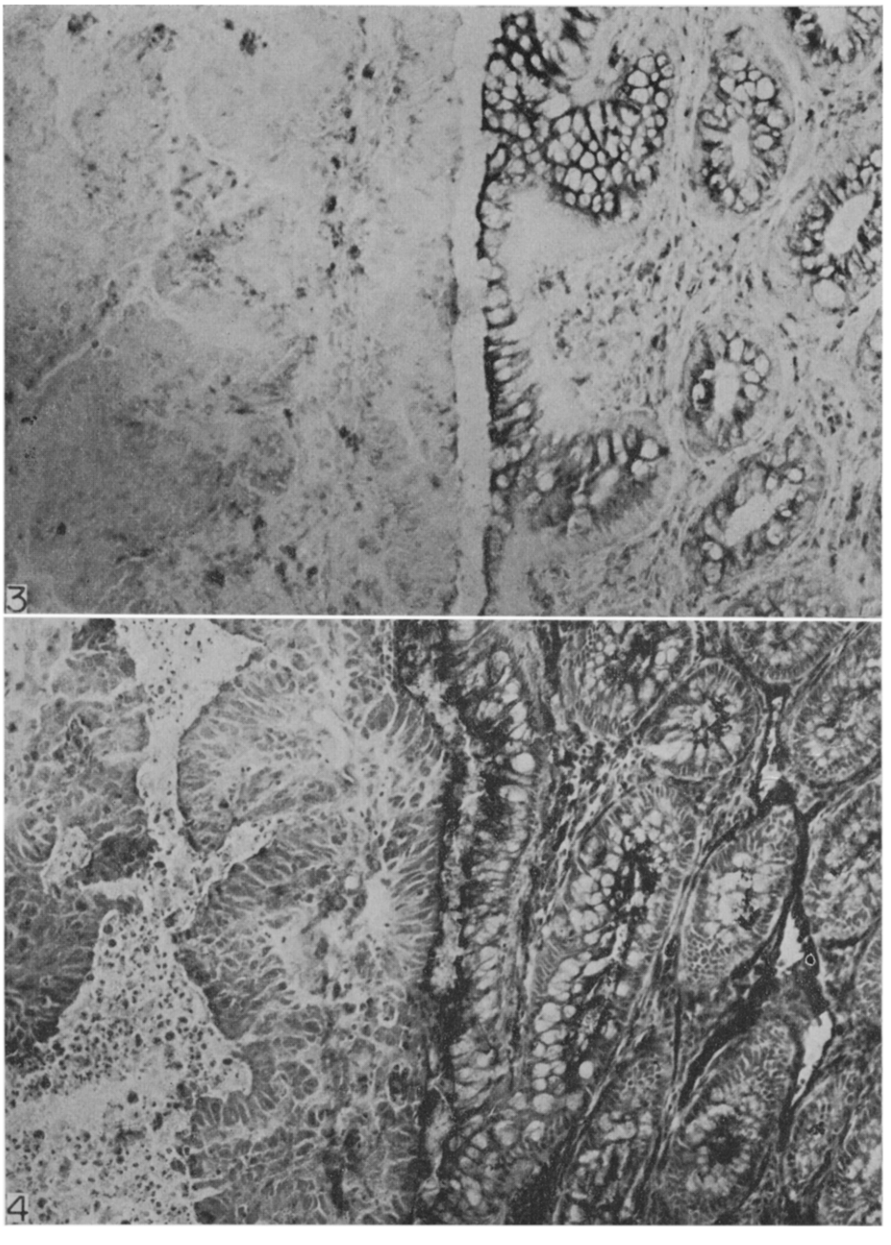


Fig. 3. *Acid phosphatase. Colon tissue.*
Right: the normally high actvity in comparable normal epithelium, especially on the apical side.
Left: tumour tissue showing almost no activity of this enzyme.

Fig. 4. *Adenosine triphosphatase. Colon tissue.*
Right: high activity on apical side of normal cells.
Left: tumour tissue with barely demonstrable activity.

(to face p. 108)

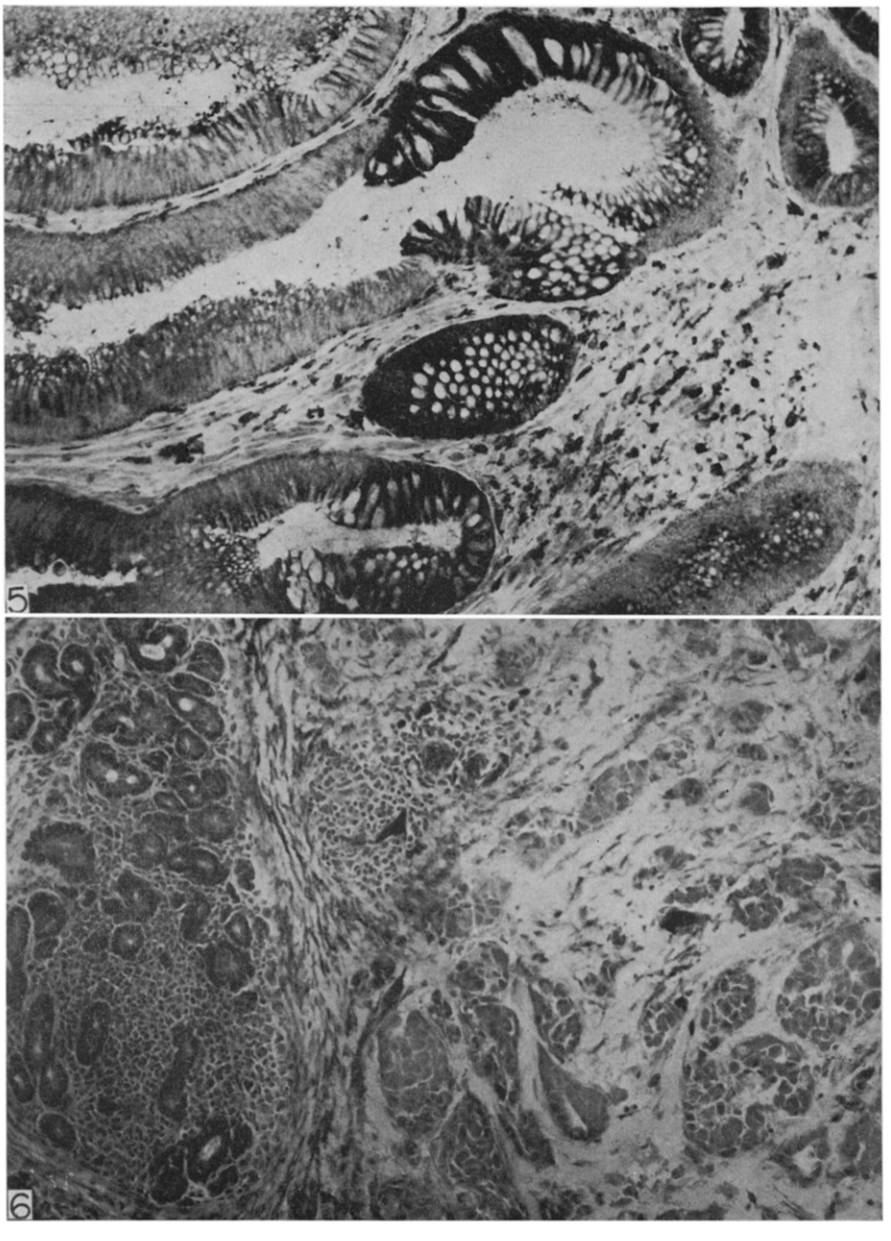


Fig. 5. *α-naphthylacetate esterase. Colon tissue.*
Acini in the transitional zone between neoplastic and normal epithelim.
The normally high activity of the enzyme decreases sharply in one and the same acinus.

Fig. 6. *Alkaline phosphatase. Breast tissue.*
Left: normal acinus with extremely active myo-epithelial cells.
Right: tumour tissue lacking activity of this enzyme.

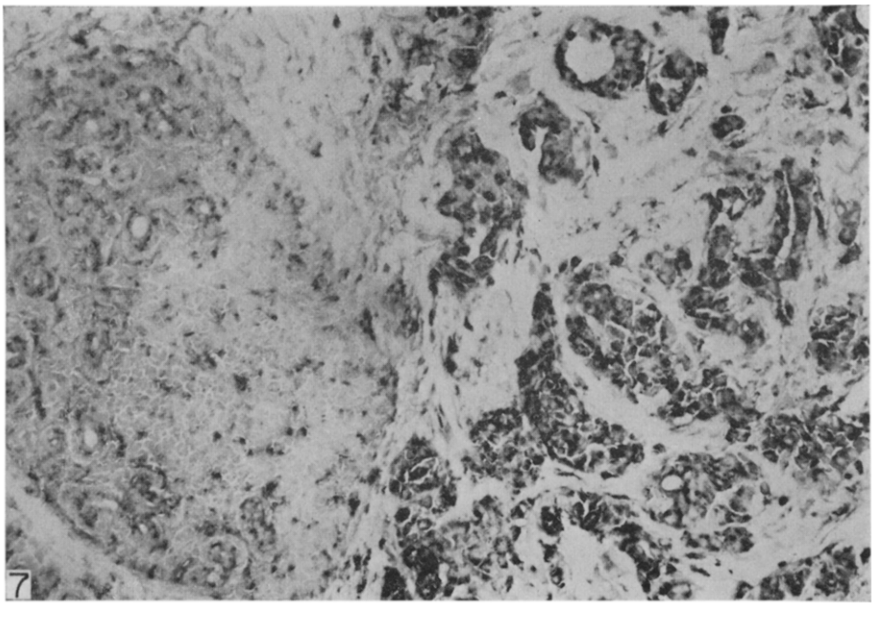


Fig. 7. Acid phosphatase. Breast tissue.
Left: some activity in acinus cells.
Right: neoplastic tissue with a greatly elevated activity of this enzyme.

7. M. M. NACHLAS, B. MONIS, D. ROSENBLATT and A. M. SELIGMAN, Improvement in the histochemical localization of leucine aminopeptidase with a new substrate, L-leucyl-4-methoxy-2-naphthylamide. *J. biophys. biochem. Cytol.* **7,** 261 (1960).
8. R. G. J. WILLIGHAGEN, Histochemisch onderzoek van de aktiviteit van alkalische en zure fosfatase in normaal en in pathologisch verandered menselijk weefsel. *Diss. Leiden* (1960).
9. B. CZERNOBILSKY and K. C. TSOU, Adenocarcinoma, adenomas and polyps of the colon. Histochemical study. *Cancer* **21,** 165 (1968).
10. M. I. FILIPE, Some aspects of the histochemical enzyme distribution in colon and rectum: normal and pathological. *Acta Histochem.* **9,** 333 (1971).
11. H. SCHMIDT-MATTIESEN, Adenosine triphosphatase, 5'nucleotidase und Alkalische phosphatase im Carcinombindegewebe. *Arch. Geschwulstforsch.* **14,** 256 (1959).
12. M. WACHSTEIN, Histochemistry of enzymes in tumours. Handbuch der Histochemie. Bd. VII, zweiter Teil. Gustav Fischer Stuttgart (1962).
13. J. M. DRENNAN, Alkaline phosphatase in breast carcinomas. *Brit. J. Surg.* **39,** 458 (1952).
14. H. FANGER and B. E. BARKER, Histochemistry of breast diseases. I. Phosphatases. *Arch. Path.* **67,** 293 (1959).
15. W. MEIER-RUGE, Die Diagnostische Bedeutung des Sexchromatigehaltes in Relation von Enzymhistochemie, DNS-Gehalt und Mitose index beim Mammacarcinom. *Verh. dtsch. Ges. Path.* **50,** 330 (1966).
16. M. CUTLER, *Tumours of the Breast.* Pitman, Lippincott Philadelphia (1961).
17. H. HAMPERL and E. LICHTENBERGER, Uber Vorkommen und Bedeutung von myo-epithelialen Zellen (Myothlien) in Tumoren. *Klin. Wschr.* **49,** 144 (1971).
18. A. B. NOVIKOFF, Correlated morphological and chemical studies in tumour cytochemistry. *Unio Internat. contra Concre Acta* **16,** 866 (1960).
19. A. J. CARR, S. W. B. EWEN and C. SKINNER, Histochemical study of aminopeptidase activity in the human breast. *Brit. J. Cancer* **22,** 714 (1968).
20. B. PAPLA and A. M. KRAKOW, Activity of distribution of leucine aminopeptidase in nodular lesions of the mammary gland. *Pat. pol.* 7 (1968).
21. P. J. MELNICK and K. BULLOCK, Histochemical studies of breast neoplasms. *Amer. J. Path.* **35,** 706 (1959).
22. H. KLUCK, Menselijke mamma. Normaal, proliferatieve lesies en tumoren. *Diss. Amsterdam* (1974).
23. JOH. KOUDSTAAL, B. MAKKINK and S. H. OVERDIEP, Enzyme histochemical patterns in human tumours. II. Oxidoreductases in carcinoma of the colon and the breast. *Europ. J. Cancer* In press (1974).
24. R. G. J. WILLIGHAGEN, Enzyme histochemistry of human tumours. *Beitr. path. Bd.* **141,** 280 (1970).
25. R. G. J. WILLIGHAGEN, Enzymhistochemie in der Diagnostik maligner Tumoren. *Verh. dtsch. Ges. Path.* **57,** 85 (1973).

Europ. J. Cancer Vol. 11, pp. 111–115. Pergamon Press 1975. Printed in Great Britain

Enzyme Histochemical Pattern in Human Tumours—II. Oxidoreductases in Carcinoma of the Colon and the Breast

JOHAN KOUDSTAAL, BOB MAKKINK and SIEBE H. OVERDIEP
Department of Pathology, "SSDZ", Stichting Samenwerking Delftse Ziekenhuizen, Reynier de Graefweg 7, Delft, Hollande

Abstract—*The activity of a number of oxidoreductases in carcinomas of the breast, colon and rectum was studied enzyme histochemically and compared with the activity in adjacent normal epithelium. In both mammary and colonic carcinoma, the activity of the NADPH–tetrazolium reductase system was found to be elevated. In colonic and rectal carcinoma there seems to be a strong tendency of the catabolic enzymes (NADH–tetrazolium reductase, succinic acid dehydrogenase, and α-glycerophosphate oxidase) towards decreased activity. Breast cancers, however, have a tendency toward an elevated catabolism.*

INTRODUCTION

ANOMALIES in the intermediary metabolism of the cancer cell were found and studied extensively in the 1920's by Warburg (1926) [1]. Since then, metabolism of the tumour cell has been the subject of many studies [2–5]. Enzyme histochemical methods can provide information concerning intermediary metabolism which may be a basis for further biochemical investigation. The present study on colonic–rectal and mammary carcinoma is an attempt to proceed in this direction.

MATERIAL AND METHODS

The mammary and colonic–rectal material used for the enzyme histochemical investigation of the hydrolytic enzymes [6], was also studied with enzyme histochemical methods for oxidoreductases. The following reactions were performed: reduced nicotinamide–adenine–dinucleotide (NADH) tetrazolium reductase and reduced nicotinamide–adenine–dinucleotide phosphate (NADPH) tetrazolium reductase [7, 8]; β-hydroxybutyric acid dehydrogenase [7]; α-glycerophosphate oxidase [9]; glucose-6-phosphate dehydrogenase and isocitric acid dehydrogenase [8]; phosphoglucose isomerase [10]; and succinic acid dehydrogenase [11]. β-Hydroxybutyric acid dehydrogenase can be demonstrated only if NADH–dehydrogenase is present in the cell. Glucose-6-phosphate dehydrogenase, isocitric acid dehydrogenase, and phosphohexose isomerase are dependent on NADPH and the presence of NADPH–tetrazolium reductase. It means that the biochemical significance of the results of the enzyme histochemical study is reduced.

Accepted 20 May 1974.

RESULTS

For the sake of orientation, the enzyme pattern of the normal secretory epithelium of the breast and colon are shown in Table 1. In the tissues studied, glucose-6-phosphate dehydrogenase and isocitric acid dehydrogenase had more or less the same activity. The enzyme activity of phosphoglucose isomerase was stronger, but the localization was the same as NADPH tetrazolium reductase, glucose-6-phosphate dehydrogenase and isocitric acid dehydrogenase. To avoid repetition, these

Table 1.

	Secretory epithelium of the breast		Colonic epithelium
	Ducts	Lobules	
NADH–tetrazolium reductase	++	++	++
β-hydroxybutyric acid dehydr.	+	+/±	+/±
Succinic acid dehydrogenase	++/+	++/+	++
α-glycerophosphate oxidase	++	++	++/+
NADPH–tetrazolium reductase	++	++	+
Isocitric acid dehydrogenase	±	+/±	±

± = trace activity; + = low activity; ++ = moderate activity.

enzymes are not mentioned separately in the following:

Figures 1 and 2 indicate the numbers of tumours investigated and whether the activity of the enzymes was found to be the same, higher, or lower than the activity in the adjacent normal epithelium. Two groups of mammary carcinoma with a divergent degree of differentiation (Fig. 3 and 4) are mentioned separately. Particularly in breast carcinomas, the activity of NADPH–tetrazolium reductase and the enzymes depending on it tend to be higher than in normal corresponding tissue (Fig. 5 and 6).

In most of the colonic neoplasms the activity of NADH–tetrazolium reductase, succinic acid dehydrogenase, (Fig. 7), β-hydroxybutyric acid dehydrogenase, and α-glycerophosphate oxidase is diminished (Fig. 8). In breast carcinoma, however, most of the enzymes tend to show increased activity, (Fig. 9 and 10) which is a striking divergence. Succinic acid dehydrogenase however, appears to be regularly less active in tumour tissue (Fig. 11). As two groups of breast carcinomas (C II, grades 2 and 3) proved to be large enough, the diagrams of the oxidoreductases were analysed to check whether there were differences that might reflect the degree of differentiation (Fig. 3 and 4).

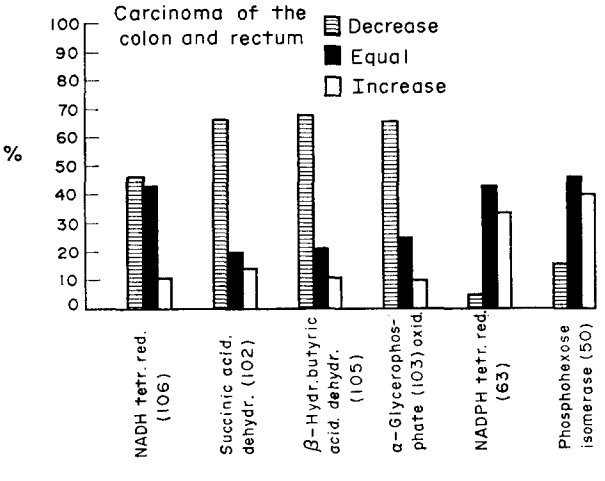


Fig. 1. *Diagram showing the enzyme pattern in colonic–rectal carcinoma. The left-hand column of each group indicates the percentage of the tumours in which enzyme activity was lower than in the adjacent epithelium; the middle column the percentage with the same activity; and the right-hand column the percentage with increased activity. The value under the name of the enzyme indicates the number of comparable tumours investigated.*

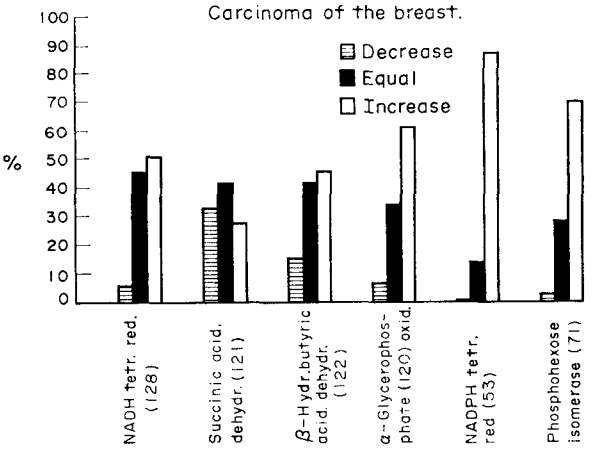


Fig. 2. *Enzyme pattern in breast carcinoma. (See legend to Fig.* 1.)

The results showed no significant differences. A large majority of the less differentiated tumours had a higher activity of most of the oxidoreductases than the better differentiated tumours. The same analysis of the colonic carcinomas showed hardly any, and no relevant, differences. In all cases of the colonic and rectal carcinomas with an elevated activity of α-glycerophosphate oxidase, there was no decrease of the NADH-tetrazolium reductase or succinic acid dehydrogenase, the activity of which was the same or higher than the level in the normal mucosa.

The activity of some of the breast carcinomas was lowered, whereas activity of the other enzymes was increased.

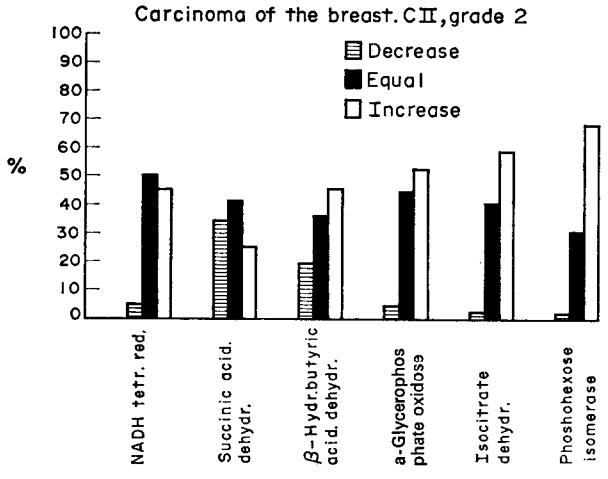


Fig. 3. *Breast carcinoma. Moderate differentiation.* (*See legend to Fig.* 1.)

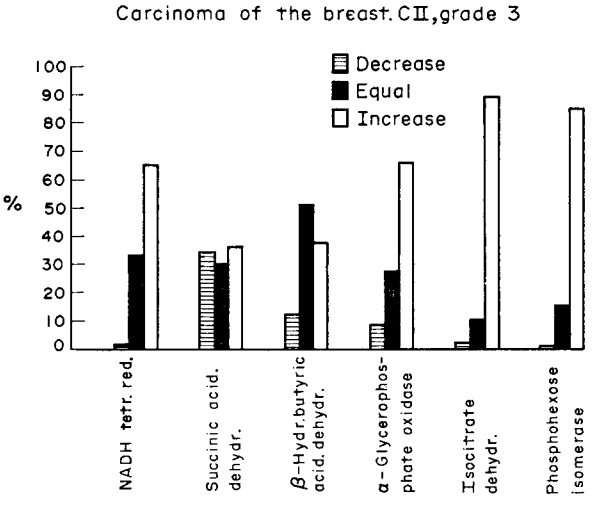


Fig. 4. *Breast carcinoma. Little differentiation.* (*See legend to Fig.* 1.)

In a few cases it was possible to study transitions between normal colonic mucosa, disturbed hyperplastic mucosa, and tumour tissue. The decreased activity of the hydrolytic enzymes and of NADH–tetrazolium reductase and the enzymes depending on it, already becomes apparent in the disturbed mucosa. In some cases there is a decreased and in others an increased activity of NADPH–tetrazolium reductase and the enzymes depending on it.

The transition from neoplastic to normal mucosa occasionally may be sharply visible even in a single acinus.

Although the stroma of colonic and rectal carcinomas and of mammary carcinomas shows fewer capillaries with alkaline phosphatase activity than the connective tissue of corresponding normal areas, a difference in vascularization does not seem to explain the differences observed in enzyme activity.

DISCUSSION

The intermediary metabolism consists of catabolic and anabolic processes. The catabolic process includes degradation of glycogen, fats, and proteins, resulting in energy-rich compounds. Data provided by experimental oncology lead to the conclusion that the catabolism of amino acids, proteins and nucleic acids is lowered and that tumour cells show accelerated synthesis of proteins and nucleic acids. The tumour cell has streamlined its metabolism in an attempt to simplify its growth and proliferation [5]. Our colonic and rectal material showed a tendency to a higher activity of the groups of enzymes which might reflect anabolism (NADPH–tetrazolium reductase and the enzymes dependent on it). Filipe [12] also noticed an increase of glucose-6-phosphate dehydrogenase in carcinomas of the colon. This effect is still more pronounced in mammary carcinomas. Cohen [13], Jensen [14], and Hilf *et al.* [15] found an elevated glucose-6-phosphate dehydrogenase activity in human mammary carcinoma. Pfleiderer [16] described the same phenomenon in women, endometrial carcinoma which has been found in prostatic cancer by Miller *et al.* [17].

Malignant ovarian tumours can show a higher activity of glucose-6-phosphate dehydrogenase than benign ovarian tumours [18]. An increased activity of this enzyme has also been reported in experimentally induced cervico-vaginal carcinomas [19–21], and mammary carcinoma [22, 23]. When, for instance, after ovariectomy, mammary carcinomas showed regression, the activity of this enzyme decreased. A rise in the activity of glucose-6-phosphate dehydrogenase or a related enzyme can be seen in benign proliferating tissue as well as in malignancies [13, 24].

The increased activity of NADPH–tetrazolium reductase and the enzymes depending on it points to an increase in anabolic activity for the benefit of the proliferative processes. The extent to which this increase in activity is correlated with the growth rate of the tumour cannot be deduced from the available data.

The mitochondrially localized enzymes (NADH–tetrazolium reductase, succinic acid

dehydrogenase, and α-glycerophosphate oxidase) show a distinct tendency towards lower activity in colonic and rectal carcinomas. In particular, the decreased activity of the first two of these enzymes points to a diminished respiratory capacity, whatever may be the cause of this inhibition. Wattenberg (1959) [25], demonstrated a decreased activity of α-glycerophosphate dehydrogenase in colonic carcinoma. In the present study, a high proportion of the colonic and rectal carcinomas showed decreased activity of α-glycerophosphate oxidase. All these data point to a reduced activity of the glycerol phosphate cycle.

The most striking of the biochemical findings of Shonck (1965) [26], was the sharply diminished activity of α-glycerophosphate dehydrogenase, which is in accordance with our enzyme histochemical findings. The decrease in the activity of β-hydroxybutyric acid dehydrogenase (in Fig. 1 equalling that of α-glycerophosphate oxidase) is indicative possibly of a reduction in the activity of a corresponding cycle.

In a number of experimental tumours of different types, Boxer and Shonk [27] found hardly any activity of α-glycerophosphate dehydrogenase either. The enzyme histochemical data obtained on colonic carcinoma show correlation with the biochemical data of Shonk [26], at least as regards the glycerophosphate cycle. This correlation does not, however, hold well for mammary carcinoma. The enzyme histochemical results indicate an increased activity of the glycerophosphate cycle (elevated α-glycerophosphate oxidase activity), whereas Hilf *et al.* [28], demonstrated a decrease of α-glycerophosphate dehydrogenase with biochemical methods. Our findings indicate that α-glycerophosphate oxidase may be elevated in mammary carcinoma. At the same time, another mitochondrially localized enzyme, i.e. succinic acid dehydrogenase, can show a decreased activity. This points to increased activity of the glycerophosphate cycle and a decreased respiration. Another indicator of respiration i.e. NADH–tetrazolium, may become simultaneously more active. The available biochemical data do not offer a simple explanation for this discrepancy.

Unlike other investigators [29], we were unable to demonstrate differences in enzyme pattern between tumours with a different degree of differentiation. Dehydrogenases of the citric acid cycle are claimed to be more active in differentiated than in undifferentiated tumours.

The morphological disturbances and the changes of activity of enzymes of the intermediary metabolism are not related in a simple way. The results of the enzyme histochemical investigation of colonic–rectal and mammary carcinomas, two distinctly different and frequently occurring human tumours, indicate some degree of resemblance as regards the anabolic processes and rather marked differences as far as several intramitochondrially localized enzymes are concerned. An explanation for the discrepancy between biochemical facts mentioned in literature and some results from our enzyme histochemical study cannot be given, but might be provided by a combined enzyme histochemical and biochemical investigation such as Jöbsis and Meijer [30] performed in muscle tissue.

As it has been found empirically that even non-specific cytologic and histologic morphologic changes can provide a basis for diagnosis, better knowledge of non-specific metabolic changes in carcinoma could be important for the refinement of diagnostic methods, providing a firm basis for treatment.

REFERENCES

1. O. Warburg, *Uber den Stoffwechsel der Tumoren.* Springer, Berlin (1926).
2. J. P. Greenstein, *Biochemistry of Cancer*, Academic Press, New York (1957).
3. W. E. Knox, The enzyme pattern of neoplastic tissue. In *Advances in Cancer Research.* Edited by A. Haddow and S. Weinhouse, Vol. 10, p. 117. Academic Press, New York (1967).
4. S. Weinhouse, Respiration, Glycolysis and Enzyme alterations in liver neoplasms. Homologies in enzymes and metabolic pathways. In *Metabolic Alterations in Cancer.* (Edited by W. J. Whelan and J. Schultz), North-Holland Amsterdam–London. (1970).
5. W. E. Criss, A review of isoenzymes in cancer. *Cancer Res.* **31,** 1523 (1971).
6. Joh. Koudstaal, B. Makkink and S. H. Overdiep, Enzyme histochemical pattern in human tumours. I. Hydrolytic enzymes in carcinoma of the colon and breast. *Europ. J. Cancer* In press (1974).
7. M. M. Nachlas, D. G. Walker and A. M. Seligman, A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase. *J. biophys. biochem. Cytol.* **4,** 29 (1958).

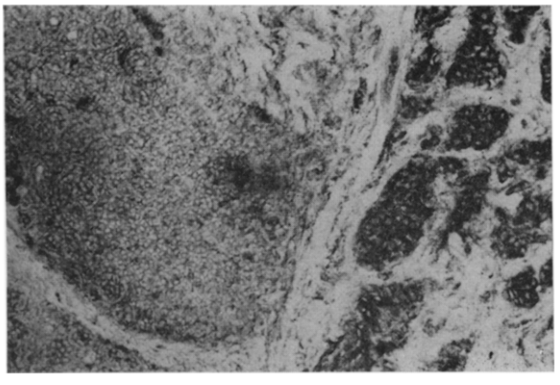


Fig. 5. *Isocitric acid dehydrogenase (NADP-dependent).*
Left: a normal mammary acinus.
Right: tumour tissue with unusual activity.



Fig. 7. *Succinic acid dehydrogenase.*
Left: normal colonic mucosa.
Right: carcinoma tissue with a low level of enzyme activity.



Fig. 6. *Isocitric acid dehydrogenase. (NADP-dependent).*
Left: the activity in normal colonic mucosa.
Right: markedly increased activity in carcinoma.

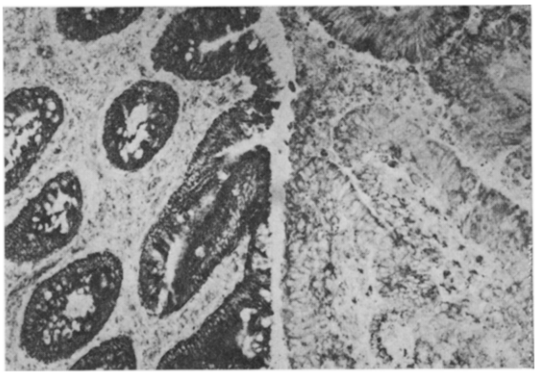


Fig. 8. *α-glycerophosphate oxidase.*
Left: normal colonic mucosa with rather strong enzyme activity.
Right: tumour tissue with only trace activity of the enzyme.

(to face p. 114)

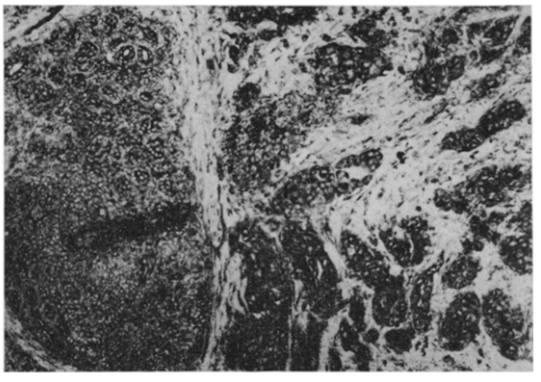


Fig. 9. *NADH-tetrazolium reductase.*
Left: normal mammary acinus.
Right: the tumour tissue shows a higher level of enzyme activity.

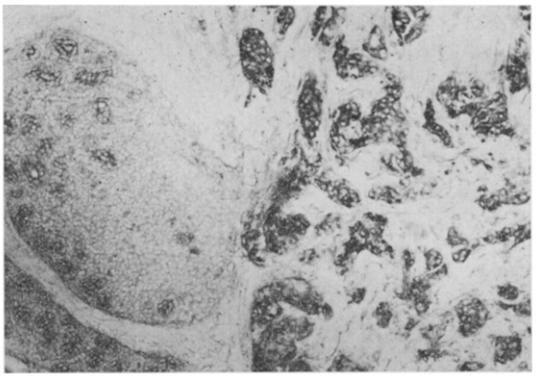


Fig. 10. *α-glycerophosphate oxidase.*
In tumour tissue (right) the level of activity of this enzyme is higher than in the epithelial cells of a normal acinus (left).

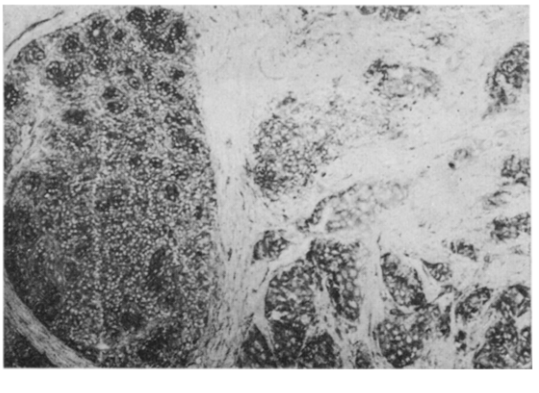


Fig. 11. *Succinic acid dehydrogenase.*
The tumour tissue on the right shows a lower activity of this enzyme than the tumour tissue on the left.

8. M. M. NACHLAS, D. G. WALKER and A. M. SELIGMAN, A histochemical method for the demonstration of triphosphopyridine nucleotide diaphorase. *J. biophys. biochem. Cytol.* **4,** 467 (1958).
9. L. W. WATTENBERG and J. L. LEONG, Effects of coenzyme 10 and menadione on succinic dehydrogenase activity as measured by tetrazolium salt reduction. *J. Histochem. Cytochem.* **8,** 296 (1960).
10. R. ORCHARDSON and J. MACGADEY, The histochemical demonstration of phosphoglucose isomerase. *Histochemic.* **22,** 136 (1970).
11. M. J. HARDONK, The use of phenazinemethosulfate as an electron carrier in the histochemical demonstration of dehydrogenase. *Histochemic.* **4,** 563 (1965).
12. M. I. FILIPE, Some aspects of the histochemical enzyme distribution in colon and rectum: normal and pathological. *Acta histochem.* **9,** 333 (1971).
13. R. B. COHEN, Glucose-6-phosphate dehydrogenase activity in hyperplastic and neoplastic lesion of the breast. *Cancer* **17,** 1067 (1964).
14. H. JENSEN, Fibroadenomatosis and breast carcinoma. Enzyme histochem. studies. *Acta path. microbiol. scand.* 421 (1970).
15. R. HILF, H. GOLDENBERG and J. MICHEL, Enzymes, nucleic acids and lipids in human breast cancer and normal breast tissue. *Cancer Res.* **30,** 1874 (1970).
16. A. PFLEIDERER, Enzymhistochemische Untersuchungen am Carcinom des Corpus Uteri. *Fortschr. Geburtsch Gynäk,* **36,** In *Bibl. gynaec.* **49,** S. Karger, Basel (1968).
17. H. C. MILLER, W. RECTOR and R. HILF, Preliminary enzymatic studies of human benign prostate hypertrophy and prostatic cancer. *Invest. Urol.* **10,** 1 (1972).
18. JOH. KOUDSTAAL, B. BOSSENBROEK and M. J. HARDONK, Ovarian tumours investigated by histochemical and enzyme histochemical methods. *Amer. J. Obstet. Gynec.* **102,** 1004 (1968).
19. D. G. SCARPELLI and E. VON HAAM, Experimental carcinoma of the uterine cervix. In *Progression Experimental Tumor Research.* (Edited by F. HOMBURGER) Vol. I, p. 179. Karger, Basel (1960).
20. M. THIERY, Bijdrage tot de kennis van het experimentele cervicovaginale carcinoom. *Arschia uitgaven N.V. Brussel* (1962).
21. JOH. KOUDSTAAL, M. J. HARDONK and H. N. HADDERS, The development of induced cervicovaginal carcinoma in intact and estrogen-treated castrated mice studied by histochemical and enzyme histochemical methods. *Cancer Res.* **26,** 1943 (1966).
22. R. HILF, H. GOLDENBERG, I. MICHEL, M. J. CARRINGTON, C. BELL, M. GRUENSTEIN, D. R. MERANZE and M. B. SHIMKIN, Biochemical characteristics of mammary glands and mammary tumours of rats induced by 3-Methylcholanthrene and 7-12-dimethylbenz(a)anthracene. *Cancer Res.* **29,** 977 (1969).
23. F. B. HERSHEY, G. JOHNSTON, S. M. MURPHY and M. SCHMITT, Pyridine nucleotidelinked dehydrogenase and isoenzymes of normal rat breast and growing and regressing cancers. *Cancer Res.* **26,** part I, 265 (1966).
24. J. CHAYEN, L. BITENSKY, E. K. AVES, C. R. N. JONES, A. A. SILCOX and G. J. CUNNINGHAM, Histochemical demonstration of 6-phosphogluconate dehydrogenase in proliferating and malignant cells. *Nature (Lond.)* **195,** 714 (1962).
25. L. W. WATTENBERG, A histochemical study of five oxidative enzymes in carcinoma of the large intestine. *Amer. J. Path.* **35,** 113 (1959).
26. C. E. SHONK, R. N. ARISON, B. J. KOVEN, H. MAJIMA and G. E. BOXER, Enzyme patterns in human tissues—III. Glycolytic enzymes in normal and malignant tissues of the colon and rectum. *Cancer Res.* **25,** 206 (1965).
27. G. E. BOXER and C. E. SHONK, Low levels of soluble DPN-linked-Glycerophosphate dehydrogenase in tumours. *Cancer Res.* **20,** 85 (1960).
28. R. HILF, H. GOLDENBERG, R. A. ORLANDO and F. L. ARCHER, Some biochemical characteristics of human breast cancer and nonmalignant breast lesions. *Proc. Soc. exp. Biol. (N.Y.)* **132,** 613 (1969).
29. R. BÄSSLER, Enzymhistochemische Befunde bei verscheidenen Funktionsphasen und bei Pathologischer Veränderungen. *Acta histochem. Supp.* **9,** 605 (1971).
30. A. C. JÖBSIS and A. E. F. H. MEIJER, Evaluation of enzyme histochemical observations for metabolic studies. A combined histochemical and biochemical investigation of experimentally induced skeletal muscle-changes. *Histochemie* **36,** 51 (1973).

Europ. J. Cancer Vol. 11, pp. 117–121. Pergamon Press 1975. Printed in Great Britain

Double Stranded Ribonucleic Acid in Human Leukemic Blast Cells*

UMBERTO TORELLI, GIUSEPPE TORELLI and RUGGERO CADOSSI
Institute of Medical Pathology, University of Modena, 41100 Modena, Italy

Abstract—*Labelled double-stranded RNA(dsRNA) has been extracted from human leukemic blast cells incubated* in vitro *with radioactive precursors. Purification of dsRNA has been obtained using selective nuclease digestion and chromatography on cellulose columns. The dsRNA molecules appear to be confined to the nucleus and carry, at least in part, polyadenylate sequences. Time course experiments show that the proportion of labelled dsRNA increases during* in vitro *incubation of leukemic blast cells.*

INTRODUCTION

DOUBLE-STRANDED RNA(dsRNA) has been observed recently in several types of animal cells [1–5]. So far, the function of these molecules in cellular RNA metabolism is unknown. However, the ability of very small amounts of dsRNA to inhibit initiation of protein synthesis in cell-free extracts [6] has led to the hypothesis that these molecules play a role in regulation of translation of messenger RNA in eukariotic cells [6].

At least in some types of cells, dsRNA is bound to polyadenylate (poly(A)) sequences [4], is associated with large nuclear RNA molecules, and has metabolic properties characteristics of heterogeneous nuclear RNA [4, 5].

The occurrence in leukemic blast cells of a proportion of poly(A)-bound nuclear RNA molecules larger than that observed in normal lymphocytes [7], led us to extend our investigations of the characteristics of nuclear RNA in human leukemic blast cells. We report here evidence that these cells do synthesize dsRNA. Some structural and metabolic characteristics are also reported.

MATERIAL AND METHODS

Cells and labelling conditions

Leukemic blast cells were obtained from the peripheral blood of four patients with acute myeloid leukemia having a high number of circulating leukocytes. Heparinized blood was sedimented by gravity at 37°C and the supernatant was diluted to a final concentration of 2×10^6 cells per ml in Eagle's medium with 20% autologous plasma. [^{3}H-5]-uridine and [^{3}H]-adenosine (Radiochemical Centre, Amersham) were added at the final concentration of 10 μCi/ml and the cells incubated for periods up to 9 hr.

RNA extraction

Small pellets of cells washed twice with saline were resuspended in 20 ml of buffer (Na acetate 0·01 M, NaEDTA 0·01 M and bentonite 0·05%). One millilitre of 10% sodium dodecyl sulfate was then added, followed 30 sec later by 20 ml of 90% solution of phenol-*m*-cresol (7·9:1 v/v) containing 0·1% hydroxyquinoline. The suspension was shaken vigorously for 3 min at 60°C in a water bath. The aqueous phase was separated by centrifuging for 10 min at 15,000 rev/min and the extraction was repeated twice. The final aqueous phase was brought to 2% in potassium acetate and 75% in ethanol and stored overnight at −20°C.

Isolation of double-stranded RNA

The precipitated nucleic acid was collected by centrifugation for 15 min at 2500 rev/min at 4°C, rinsed with ethanol 70% containing 2% potassium acetate and washed twice with 5 ml

Accepted 28 June 1974.

*Research supported by a grant from the Consiglio Nazionale delle Ricerche.

of 3 M sodium acetate. The pellet was then dissolved in 5 ml of buffer (50 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris–HCl, pH 7·4) and DNase (Worthington) was added at the final concentration of 20 μg/ml. After 30 min of incubation at room temperature, the solution was brought to 0·25 M NaCl by adding 5 M NaCl. Thirty microgrammes per millilitre of pancreatic RNase (Sigma) and 20 units/ml of T1 RNase (Worthington) were then added and the solution was incubated 30 min at 37°C. Fifty microgrammes of subtilisin were then added and the reaction was terminated 30 min later by extracting twice with phenol-*m*-cresol and precipitating the nucleic acid as described above.

In order to isolate and purify double-stranded RNA from single-stranded RNA, transfer RNA and DNA, we used chromatography on cellulose columns, according to the method of Franklin [8]. Nucleic acid was dissolved in 0·5 ml of buffer (0·1 M NaCl, 0·05 M Tris.HCl, 0·001 M NaEDTA, pH 6·9), brought to 35 % with ethanol. The sample was applied to a cellulose column (1·5 × 10 cm), and the column was washed with the same solution. Under these conditions, DNA and transfer RNA fail to remain on the column but can be eluted directly with buffer with 35 % ethanol. Single-stranded RNA remains on the column but can be eluted with buffer containing 15 % ethanol. Double-stranded RNA is finally removed with buffer alone. Six millilitre fractions were collected for a total 72 ml of each washing. For each fraction, acid precipitable radioactivity was determined, and representative fractions of the third peak were pooled and further characterized.

Table 1. *Synthesis of double-stranded RNA in leukemic blast cells incubated with* [^3H]*-uridine for* 3 *hr*

	Case			
	1	2	3	4
I Total RNA radioactivity treated with nucleases*	$4{\cdot}17 \times 10^5$	$2{\cdot}10 \times 10^5$	$1{\cdot}04 \times 10^6$	$2{\cdot}81 \times 10^5$
II RNA radioactivity after digestion placed on column	$2{\cdot}58 \times 10^4$	$5{\cdot}50 \times 10^3$	$1{\cdot}06 \times 10^5$	$2{\cdot}73 \times 10^4$
III RNA radioactivity in third chromatographic peak	$4{\cdot}76 \times 10^3$	$1{\cdot}44 \times 10^3$	$8{\cdot}40 \times 10^3$	$1{\cdot}70 \times 10^3$
As percentage of I	1	0·7	0·8	0·6

*Acid insoluble radioactivity, count/min.

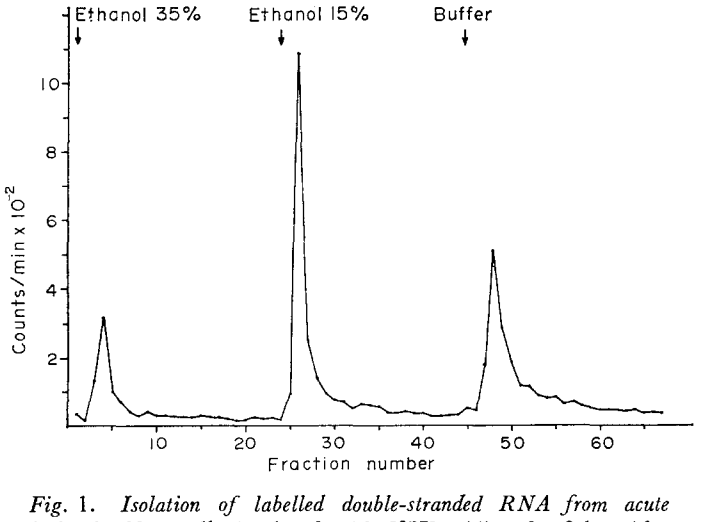


Fig. 1. *Isolation of labelled double-stranded RNA from acute leukemia blast cells incubated with* [^{3}H]*-uridine for* 3 *hr. After treatment with DNase and RNase, the total yield of reextraction of degraded nucleic acids was applied to a cellulose column. The radioactivity profile shows that a fairly large amount of labelled material eluted in buffer alone.*

Assay of poly(A)-bound double-stranded RNA sequences

The proportion of dsRNA molecules bound to poly(A) sequences was evaluated by measuring the proportion of labelled dsRNA bound to glass fiber filters on which poly(U) had been previously immobilized by the technique of Sheldon *et al.* [9].

To each fiberglass filter (Whatman GF/C, 2·4 cm), was added 0·15 ml of poly(U) solution (1 mg/ml in distilled water). The filters were then dried at 37°C and irradiated for 2·5 min on each side at a distance of 22 cm from a 30 W Sylvania germicidal lamp. Each filter was rinsed with 50 ml of distilled water to remove unbound poly(U). Four fractions from the third peak were pooled and filtered through each filter at 2 ml/min. The filters were washed with 20 ml of buffer, followed by 20 ml of ice cold 5 % thrichloroacetic acid and 10 ml of 95 % ethanol, then dried and counted. Input radioactivity was evaluated by drying on filters 0·5 ml of the pooled fractions.

Nuclei isolation

To isolate nuclei of leukemic blast cells, the cells were suspended in cold hypotonic solution (0·01 M Tris, 0·08 M sucrose, 0·003 M $CaCl_2$, pH 7·4) and gently shaken by hand for 15 min. Triton X100 was then added at the final concentration of 0·2 % and the suspension shaken again for 15 min and centrifuged at 2000 rev/min to separate nuclei.

RESULTS

As shown in Table 1, from 5 % to 15 % of the originally labelled RNA extracted from leukemic blast cells incubated with [^{3}H]-uridine for 3 hr remained TCA-precipitable after nuclease treatment. Most of the label could be eluted from the cellulose column with ethanol, 35 % or 15 %. However, as shown in Table 1, significant radiolabel left on the columns was eluted with buffer containing no ethanol (Fig. 1), as expected from double-stranded RNA [8]. The material eluting in the third peak was still 95 % TCA-precipitable after digestion for 30 min at 37°C with 20 μg/ml of pancreatic RNase in 0·25 M NaCl. Nonetheless, the substance was RNA, since it was hydrolized completely in 0·3 M NaOH. Furthermore, thermal denaturation experiments (Fig. 2) showed that

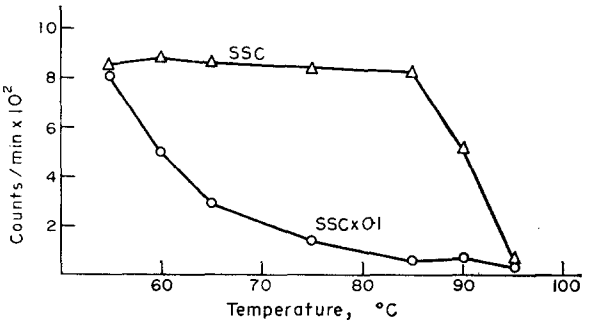


Fig. 2. Melting curves of ds-[^{3}H] RNA from leukemic blast cells incubated with [^{3}H]-uridine for 6 hr. Labelled RNA eluted in the third peak was precipitated in ethanol with tRNA as a carrier, and then dissolved in SSC x 0·1 or SSC. One millilitre aliquots were raised to various temperatures for 5 min, chilled rapidly in an ice-water bath, brought to 0·25 M total NaCl, and incubated 30 min at 37°C with 20 μg/ml of pancreatic RNase. Acid-insoluble radioactivity was then determined using albumin as a carrier.

the Tm is higher at higher ionic strength, as expected for a duplex polynucleotide. As shown in Table 2, heating for 3 min at 100°C followed by rapid chilling made the [^{3}H]-uridine labeled material completely sensitive to RNase, whereas the RNA labelled with [^{3}H]-adenosine after denaturation by heating, was still resistant to RNase in a proportion up to 28 %. The proportion of double-stranded RNA from leukemic cells carrying poly(A) sequences is shown in Table 3. Twenty-three per cent and twenty-seven per cent respectively, of third peak radioactivity from two RNA samples labelled with [^{3}H]-uridine was bound to poly(U)-impregnated fiberglass filters.

A marked increase in the proportion of labelled RNA in double-stranded form was observed in

Table 2. Effect of denaturation on ribonuclease resistance of labelled double-stranded RNA

	[^{3}H]-uridine-labelled RNA		[^{3}H]-adenosine-labelled RNA	
I Acid insoluble radioactivity from third chromatographic peak	1100	1860	550	2060
II Acid insoluble radioactivity after denaturation plus RNase	40	32	155	520
As percentage of I	3·6	1·7	28	25

some cases by increasing the period of incubation with the labelled precursor. As shown in Table 4, in one of our cases this proportion did not reach 1 % after 3 hr, but was up to 2·3 % after 9 hr of incubation.

Experiments with cell fractionation showed that dsRNA in leukemic blast cells is almost exclusively confined to the nucleus. As shown in Table 5, 83 % and 91 % respectively of the total dsRNA of a certain amount of cells comes from the correspondent nuclear fractions.

Table 3. *[^3H]-uridine labelled ds-RNA bound to poly (U)-impregnated fiberglass filters*

	Case	
	1	2
I Total RNA radioactivity from third chromatographic peak put on filter.	1550	2470
II RNA radioactivity bound to filter.	350	665
As percentage of I	23	27

DISCUSSION

The results of our experiments show that a significant proportion of the RNA synthesized in leukemic blast cells is in an RNase resistant form with the properties of dsRNA. Polyadenylate sequences would not be digested by pancreatic and T1 ribonucleases, but they do not cochromatograph with dsRNA. This has been shown by Kronenberg and Humphrey [4] and confirmed in experiments which have shown that the material eluting with 15 ethanol includes a large amount of RNase-resistant substance with poly(A) characteristics. It seems unlikely that the third chromatographic peak includes RNA-DNA hybrids since Nygaard and Hall [10] have shown that such hybrids do not survive DNase digestion employed in our experiments. Subtilisin digestion makes also unlikely the presence in the third peak of RNA-protein complexes.

The whole of our results suggests that the substance eluting in the third peak is a duplex polyribonucleotide associated with polyadenylate sequences. The significance of this association, which has also been observed in sea urchin embryos [4], is unknown. Polyadenylation had

Table 4. *Changes in proportion of labelled dsRNA after increasing periods of incubation with [^3H]-uridine*

Case		Hours of incubation		
		3	6	9
1	I Total RNA radioactivity treated with nucleases.	$2{\cdot}10 \times 10^5$	$2{\cdot}10 \times 10^5$	$3{\cdot}925 \times 10^4$
	II RNA radioactivity in third peak	1.44×10^3	$2{\cdot}73 \times 10^3$	$6{\cdot}67 \times 10^2$
	As percentage of I	0·7	1·3	1·7
2	I Total RNA radioactivity treated with nucleases.	$2{\cdot}81 \times 10^5$	$3{\cdot}29 \times 10^5$	$1{\cdot}511 \times 10^5$
	II RNA radioactivity in third peak	$1{\cdot}73 \times 10^3$	$6{\cdot}25 \times 10^3$	$3{\cdot}47 \times 10^3$
	As percentage of I	0·6	1·9	2·3

Table 5. *Occurrence of double-stranded RNA in nuclei of leukemic blast cells. A suspension of blast cells incubated for 2 hr with [^3H]-adenosine was divided into two equal parts. One of these parts was immediately extracted with phenol, while the other part was treated to fractionate the cells and separate the nuclei before RNA extraction*

	Whole cells		Nuclei	
I Total RNA radioactivity treated with nucleases.	$2{\cdot}737 \times 10^5$	$3{\cdot}865 \times 10^5$	$9{\cdot}528 \times 10^4$	$8{\cdot}36 \times 10^4$
II RNA radioactivity in third peak	$1{\cdot}99 \times 10^3$	$1{\cdot}93 \times 10^3$	$1{\cdot}65 \times 10^3$	$1{\cdot}75 \times 10^3$
As percentage of I	0·73	0·5	1·73	2·1

been considered originally an integral part of the processing mechanism by which the primary transcription products are converted to functional messenger RNA molecules [11, 12]. However, evidence has been presented recently to show that much of the nuclear polyadenylate sequences are degraded within the nucleus [13]. This is consistent with the view of Kronenberg and Humphrey [4] that poly(A)-bound dsRNA represents a portion of heterogeneous nuclear RNA which is degraded in the nucleus. This conclusion is also supported by their kinetic experiments, showing that the proportion of total labelled RNA with double-stranded characteristics decreases with increasing the period of exposure to the label, as would be expected for a component of heterogeneous nuclear RNA.

Although our results are at variance with those of the latter authors, one should not exclude the possibility that the dsRNA molecules which have been isolated in our experiments are part of the heterogeneous nuclear RNA of leukemic blast cells. We have shown previously that labelled heterogeneous, rapidly sedimenting RNA molecules accumulate in leukemic blast cells after several hr of labelling [14, 15]. This suggests that heterogeneous nuclear RNA of these cells does not behave kinetically as that of rapidly proliferating cells. A decrease in ribosomal RNA accumulation during the incubation period, accompanied by a reduced turnover rate of heterogeneous nuclear RNA, including double-stranded segments, might well explain our results. It must also be pointed out that an increase in the life span of dsRNA molecules, due to the ability of these molecules to inhibit initiation of protein synthesis, might reduce markedly the protein synthesis rate in leukemic blast cells.

REFERENCES

1. C. Colby and P. H. Duesberg, Double-stranded RNA in vaccinia virus infected cells. *Nature* **222,** 940 (1969).
2. R. Stern and R. Friedman, Double-stranded RNA synthesized in animal cells in the presence of actinomycin D. *Nature* **222,** 612 (1970).
3. L. Montagnier and L. Harel, Homology of double-stranded RNA from rat liver cells with cellular genome. *Nature, New Biology* **229,** 106 (1971).
4. L. H. Kronenberg and T. Humphreys, Double-stranded ribonucleic acid in sea urchin embryos. *Biochemistry* **111,** 2020 (1972).
5. W. Jelinek and J. E. Darnell, Double-stranded regions in heterogeneous nuclear RNA from HeLa cells. *Proc. nat. Acad. Sci. (Wash.)* **69,** 2537 (1972).
6. H. D. Robertson and M. B. Mathews, Double-stranded RNA as an inhibitor of protein synthesis and as a substrate for a nuclease in extracts of Krebs II ascites cells. *Proc. nat. Acad. Sci. (Wash.)* **70,** 225 (1973).
7. U. Torelli and G. Torelli, Poly(A)-containing molecules in heterogeneous nuclear RNA of normal PHA-stimulated lymphocytes and acute leukemia blast cells. *Nature, New Biology* **244,** 134 (1973).
8. R. M. Franklin, Purification and properties of the replicative intermediate of the RNA bacteriophage R17. *Proc. nat. Acad. Sci. (Wash.)* **55,** 1504 (1966).
9. R. Sheldon, C. Jurale and J. Kates, Detection of polyadenylic acid sequences in viral and eukaryotic RNA. *Proc. nat. Acad. Sci. (Wash.)* **69,** 417 (1972).
10. H. P. Nygaard and B. D. Hall, A method for the detection of RNA-DNA complexes. *Biochem. biophys. Res. Commun.* **12,** 98 (1963).
11. J. E. Darnell, L. Philipson, R. Wall and M. Adesnik, Polyadenylic acid sequences: role in conversion of nuclear RNA into messenger RNA. *Science* **174,** 507 (1971).
12. M. Edmonds, M. H. Vaughan and H. Nakazato, Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labelled polyribosomal RNA of HeLa cells: possible evidence for a precursor relationship. *Proc. nat. Acad. Sci. (Wash.)* **68,** 1336 (1971).
13. R. P. Perry, D. E. Kelly and J. La Torre, Synthesis and turnover of nuclear and cytoplasmic polyadenylic acid in mouse L cells. *J. molec. Biol.* **82,** 315 (1974).
14. U. Torelli, G. Torelli and C. Mauri, Competition hybridization studies on rapidly sedimenting RNA of acute leukemia blast cells. *Europ. J. Cancer* **8,** 653 (1972).
15. U. Torelli and G. Torelli, Patterns of macromolecular RNA metabolism in normal human lymphocytes and in acute leukemia blast cells. In *Erythrocytes, Thrombocytes and Leukocytes.* (Edited by E. Gerlach, K. Moser, E. Deutsch and W. Wilmanns) p. 372. Georg Thieme Publ., Stuttgart (1973).

Europ. J. Cancer Vol. 11, pp. 123–126. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Differences in Marrow and Spleen Cell Karyotype in Early Chronic Myeloid Leukaemia

A. ZACCARIA, M. BACCARANI*, E. BARBIERI and S. TURA
Division of Haematology, St. Orsola University Hospital, Bologna, Italy

CYTOGENETIC studies indicate that the evolution of chronic myeloid leukaemia (CML) towards blastic crisis (BC) is characterized by an increasing frequency of abnormal cell variants [1, 2], but the underlying mechanisms are unknown. Investigations aiming to clarify such mechanisms are relevant both to the understanding of leukaemogenesis, and to the therapeutical management of CML. Recently, we have focused our attention on the possible role of the spleen [3, 4], and a number of clinical trials are in progress in order to evaluate the therapeutical effect of early splenectomy [4–7]. Spiers and Baikie [8] have shown that spleen myeloid cells also carry the Philadelphia chromosome (Ph^1), but no information is available as to whether the production or the defective elimination of abnormal variants [1, 2] follows the same pattern in the spleen as in marrow. Therefore we have analyzed simultaneously the karyotype of marrow and spleen myeloid cells in nine patients with CML, splenectomized at the onset of the disease.

Three patients were splenectomized without any prior treatment, within 8–16 days from diagnosis. The other patients were treated immediately after diagnosis either by Hydroxyurea (HU) or by Arabinosyl Cytosine (ARA-C), and all of them underwent splenectomy within 5 to 14 days from the end of the treatment, except for one who was splenectomized 43 days after stopping HU. All patients but one were Ph^1 positive.

Accepted 17 October 1974.

*Supported in part by Foundation Roberto Gatti.

Marrow was aspirated when the patient was already under general anaesthesia, and direct chromosome preparation was performed according to a slight modification of the method described by Tjio and Whang [9]. Immediately after surgical removal, spleen cell suspension was prepared under sterile conditions, and adjusted to a final concentration ranging between 10 and 20×10^6 cells/ml. From this suspension, metaphases were prepared either directly, as described above for marrow cells, or after 16 hr of incubation according to the method described by Spiers and Baikie [10] (Patients No. 1, 8, and 9). The karyotype of 146 marrow cells, and of 89 spleen cells were analysed under direct observation in the microscope.

The distribution of the metaphases according to chromosome number is tabulated in Tables 1 and 2. Spleen cells were more frequently hypodiploid, pseudodiploid, and hyperdiploid. Pooling together all the metaphases carrying one or more extrachromosomes (pseudodiploid plus hyperdiploid), the difference is even more striking: 22·4% in the spleen vs 11·5% in the marrow. Patient No. 8 (N.M.) contributed most to this difference, as a good number of her marrow cells were hypodiploid, in constrast with spleen cells that were more frequently pseudodiploid and hyperdiploid.

In order to better assess the frequency of the metaphases bearing two Ph^1, which are quite rare at the onset of the disease, additional metaphases were scored for the Ph^1 wherever possible (in cases No. 1, 6, and 8) up to a total number of 200 in the marrow as well as in the spleen. Patient No. 9 (C.R.) was taken out of the study because his cells were all Ph^1 negative.

Results are reported in Tables 3 and 4; in case No. 8, cells bearing 2 Ph^1 were observed in the marrow and in the spleen, while in two other patients (No. 1 and No. 6) they were discovered only in the spleen.

Table 5 gives the overall frequency of chromosome gain and loss. Spleen cells show a more frequent loss of C, and possibly of D chromosomes, and a more frequent gain of F, and possibly of G chromosomes, as compared to marrow.

The cytogenetic patterns of the evolution of CML towards BC have been summarized as follows [1, 2]: increasing aneuploidy, emergence

Table 1. *Myeloid marrow cells karyotype*

Case	Treatment	No. of metaphases analyzed	No. of metaphases with chromosome number 43–45	46 normal	46 pseudo	47–48
1. T. E.	None	19	7	10	1	1
2. L. A.	None	20	4	16	0	0
3. D. P. C.	None	19	3	15	1	0
4. L. M. L.	HU	6	1	3	2	0
5. A. F.	HU	4	0	4	0	0
6. M. A.	HU	20	3	13	4	0
7. G. E.	ARA-C	20	6	14	0	0
8. N. M.	ARA-C	19	8	11	0	0
9. C. R.	ARA-C	20*	1	11	5	3
Total		147	33	97	13	4
Percentage			22·4	66·1	8·8	2·7

*All metaphases Ph^1 negative.

Table 2. *Myeloid spleen cells karyotype*

Case	Treatment	No. of metaphases analyzed	No. of metaphases with chromosome number 43–45	46 normal	46 pseudo	47–48
1. T. E.	None	20	11	6	1	2
2. L. A.	None	3	2	1	0	0
3. D. P. C.	None	2	2	0	0	0
4. L. M. L.	HU	2	1	0	1	0
5. A. F.	HU	9	1	7	1	0
6. M. A.	HU	20	6	13	1	0
7. G. E.	ARA-C	6	3	2	0	1
8. N. M.	ARA-C	23	3	7	9	4
9. C. R.	ARA-C	4*	2	2	0	0
Total		89	31	38	13	7
Percentage			34·8	42·8	14·6	7·8

*All metaphases Ph^1 negative.

Table 3. *Frequency of* 2 Ph^1 *metaphases in marrow myeloid cells*

Case	Treatment	No. of metaphases scored for Ph^1	No. of metaphases with 1 Ph^1	with 2 Ph^1
1. T. E.	None	40	40	0
2. L. A.	None	20	20	0
3. D. P. C.	None	19	19	0
4. L. M. L.	HU	6	6	0
5. A. F.	HU	4	4	0
6. M. A.	HU	37	37	0
7. G. E.	ARA-C	20	20	0
8. N. M.	ARA-C	54*	51	2
Total		200*	197	2

*1 metaphase without Ph^1.

Table 4. Frequency of 2 Ph¹ metaphases in spleen myeloid cells

Case	Treatment	No. of metaphases scored for Ph¹	No. of metaphases with 1 Ph¹	with 2 Ph¹
1. T. E.	None	64	62	2
2. L. A.	None	3	3	0
3. D. P. C.	None	2	2	0
4. L. M. L.	HU	2	2	0
5. A. F.	HU	9	9	0
6. M. A.	HU	46*	43	2
7. G. E.	ARA-C	6	6	0
8. N. M.	ARA-C	68	65	3
Total		200*	192	7

*1 metaphase without Ph¹.

Table 5. Frequency of chromosome gain (+) and loss (−) (percentage of all the metaphases in all the 9 patients pooled together)

Chromosome group	A_1		A_2		A_3		B		C		D	
	+	−	+	−	+	−	+	−	+	−	+	−
Marrow	/	/	/	1·3	/	2·7	/	1·3	5·6	16·5	/	6·8
Spleen	/	/	/	2·2	/	3·3	/	/	7·8	31·4	3·3	11·2

Chromosome group	E_{16}		E_{17-18}		F		G	
	+	−	+	−	+	−	+	−
Marrow	/	2·7	1·3	4·1	1·3	2·0	1·3	4·8
Spleen	/	2·2	3·3	5·5	11·2	2·2	4·4	4·4

of hyperdiploid variants, and progressive gain of C, F, and G (Ph¹) chromosomes. Our patients were studied at diagnosis, probably far from BC. However the data, though not conclusive, are consistent in part with the hypothesis that the spleen plays a role in the development of BC, and indicate that combined marrow and spleen chromosome investigations, performed later during the course of CML, could be useful in order to better define the role of the spleen and to assess its importance.

Whether the spleen is operating as a direct source of more malignant haemopoietic stem cells, or as an abnormal haemopoietic microenvironment, remains a matter of speculation [4], as does the actual meaning of the observed increase of T lymphocytes [11]. However, it must be acknowledged that there are also other factors responsible for the evolution of CML towards BC. Splenectomy, followed by HU and leukopheresis, was unable to prevent the development of BC in a group of 18 patients [12]. A combination of splenectomy with a more intensive, acute myeloid leukaemia-like chemotherapeutic attack, could prove to be more successfull [4, 5, 7].

REFERENCES

1. B. Pedersen, The karyotype evolution in chronic granulocytic leukaemia—I. The chromosomes gained and lost during initiation of the evolution. *Europ. J. Cancer* **9,** 503 (1973).
2. B. Pedersen, The karyotype evolution in chronic granulocytic leukaemia—II. The chromosome and karyotype pattern of advanced evolution. *Europ. J. Cancer* **9,** 509 (1973).
3. S. Tura, New trends in the management of chronic myeloid leukaemia. *Haematologica* **57,** 741 (1972).
4. S. Tura and M. Baccarani, Progression, prevention and therapy of the blastic crisis of chronic myeloid leukemia. *Acute Leukemias, First Intern. Meeting of Therapy, Rome.* In press (1973)
5. M. D. Dowling, S. Hopfan, W. H. Knapper, T. Vaartaja, T. Gee, M. Haghbin and B. D. Clarkson, Attempt to induce true remission in chronic myelogenous leukemia (CML) Houston, March 27–28, 1974. *Proc. Amer. Soc. clin. Oncol.* In press.

6. A. S. D. Spiers, Personal communication.
7. E.O.R.T.C. Leukaemia and Haematosarcoma Cooperative Study Group.
8. A. S. D. Spiers and A. G. Baikie, Chronic granulocytic leukaemia: demonstration of the Philadelphia chromosome in cultures of spleen cells. *Nature (Lond.)* **208,** 497 (1965).
9. J. H. Tjio and J. Whang, Chromosome preparation of bone marrow cells without prior *in vitro* or *in vivo* colcemide administration. *Stain Technol.* **37,** 17 (1962).
10. A. S. D. Spiers and A. G. Baikie, Cytogenetic studies in the malignant lymphomas. *Lancet* **I,** 506 (1966).
11. J. Kaur, D. Catovsky, A. S. D. Spiers and D. A. G. Galton, Increase of T lymphocytes in the spleen in chronic granulocytic leukaemia. *Lancet* **I,** 834 (1974).
12. L. Schwarzenberg, G. Mathe, P. Pouillart, R. Weiner, J. Lacour, J. Genin, M. Schneider, F. De Vassal, M. Hayat, J. L. Amiel, J. R. Schlumberger, C. Jasmin and C. Rosenfeld, Chemotherapy with hydroxyurea, leucopheresis and splenectomy in the treatment of chronic myeloid leukaemia at the problastic phase. *Haematologica* **57,** 601 (1972).

Europ. J. Cancer Vol. 11, pp. 127–130. Pergamon Press 1975. Printed in Great Britain

The Metabolism of ^{3}H-estradiol-17β in Human Breast Cancer in Organ Culture*

A. GEIER,† H. HORN, I. S. LEVIJ,‡ E. LICHTSHTEIN and M. FINKELSTEIN

Department of Endocrinology, Hebrew University-Hadassah Medical School, Jerusalem and ‡Department of Pathology, Hadassah University Hospital, Jerusalem, Israel

Abstract—*The metabolism of ^{3}H-estradiol-17β was studied in twelve tissue samples of human female breast grown in organ culture. Five samples were from carcinomas, three were from uninvolved tissue from the cancer-bearing breast, two were from fibroadenomas and two from cystic mastitis. In all cases, estradiol-17β was metabolized to estrone. The non-cancerous tissue, fibroadenomas and cystic mastitis showed greater conversion of estradiol-17β into estrone than the cancerous tissues. No indication was obtained for conversion of estradiol-17β into estriol.*

INTRODUCTION

WE REPORTED previously on the metabolism of 4-^{14}C-testosterone by benign and malignant human breast tissue grown in organ culture [1]. In all cases of cultured samples, *e.g.*, carcinomas, uninvolved regions of cancer-bearing breasts, and fibroadenomas, 4-^{14}C-testosterone was metabolized to androstenedione, androsterone, dihydrotestosterone, androstanedione and androstanediol. However, the retention, uptake and metabolism of 4-^{14}C-testosterone were invariably lower by the cancerous tissue than by the uninvolved tissue of the same patient. The differential response of the cancerous and of the uninvolved tissue towards testosterone could reflect differences in activity of testosterone-17β-hydroxy-dehydrogenase in the two types of tissue.

Whether the difference in 17β-hydroxy-dehydrogenase activity between the cancerous and uninvolved tissue was limited to testosterone or whether it was of a more general nature is the topic of the present study. In this paper we used 6,7^{3}H estradiol-17β as a substrate to study its metabolism by cancerous and non-cancerous human breast tissue grown in organ culture.

MATERIAL AND METHODS

Twelve tissue samples from nine patients were studied. Five patients had breast carcinoma, two had fibroadenoma and two had cystic mastitis. From three breast cancer patients, uninvolved tissue which was found to be free of malignant cells on macroscopic and microscopic examination, was included in the study. Four of the cancer patients (B.V., C.A., S.E., T.C.) had normal menstrual cycles and the fifth (M.V.) was post-menopausal. Both patients with fibroadenomas and the two patients with cystic mastitis had regular menstrual cycles.

The methods used for organ culture were the same as reported previously [1]. Tissue samples were obtained within 10 min of excision under aseptic conditions and were washed with artificial medium M-199 containing, per

Accepted 29 November 1974.

*This investigation was supported by grants from the Steiner Family Endowment Fund, Vancouver, Canada, and from Chief Scientist's Office, Israel Ministry of Health.

†This work is part of a thesis to be submitted by A. Geier to the Hebrew University in partial fulfilment of the requirements for the Ph.D. degree.

liter, 0·2 mg nipasol, 0·2 mg nipagin, 100 mg penicillin and 270 mg streptomycin (M.A.I., Jerusalem, Israel). The tissue was dissected free of adipose and of necrotic tissue and cut into fragments of approximately 2 mm^3. Fifteen to twenty explants were put on each stainless steel grid which was covered with lens paper and placed on a watch glass containing 2 ml medium (80% M-199 with antibiotics and 20% calf serum). Each watch glass was placed in a closed Petri dish and the Petri dishes were incubated at 37°, in a humid atmosphere, in a chamber perfused with 95% O_2 and 5% CO_2. After 72 hr of culture, the transplants were transferred to 1·5 ml of fresh medium with added 0·25 μCi 6,7-^{3}H-estradiol-17β (S.A. 50Ci/mM, NEN, Boston, Mass.) and were cultured for an additional 48 hr.

Immediately following the final culturing, the tissue was examined microscopically, on randomly selected samples, to ensure its vitality and absence of autolysis. Other criteria for the vitality of the tissue grown in organ culture were the incorporation of ^{3}H-thymidine and ^{3}H-uridine (both from Nuclear Research Centre, Negev, Israel) into DNA and RNA respectively, and of ^{14}C L-amino acids (NEN, Boston, Mass.) into protein, during the last 48 hr of organ culture.

At the end of the culturing period, the contents of 4 Petri dishes were pooled and processed as one sample. The transplants were separated from the medium and washed with at least 3 portions of 10 ml buffer (0·15 M KCl, 0·003 M $NaHCO_3$, 0·006 M EDTA), until no radioactivity was detected in the washings. Carrier estradiol-17β, estrone and estriol (Ikapharm, Israel), 200 μg each, were added to the medium and to the tissue. Then the tissue was homogenized 3 times with 3 ml of acetone and ethanol (20:3) and the homogenate left overnight at 4°C. The precipitate was then separated and used for estimation of the protein content according to Lowry [2]. The supernatant was used for the metabolism studies.

The metabolism of ^{3}H-estradiol-17β was investigated both in the "medium" and "tissue" fractions. They were partitioned, separately, into the neutral and phenolic fractions. Since in no case was radioactivity detected in the neutral fraction, it was discarded. The phenolic fraction was partitioned between benzene, petrol–ether and water to separate estriol from estrone and estradiol-17β [3].

The estriol fraction was chromatographed for 48 hr on paper (Whatman No. 1, impregnated with formamide/methanol) with chloroform saturated with formamide and the fraction containing estradiol-17β and estrone was chromatographed with orthodichlorobenzene saturated with formamide. In the latter system, estrone is separated from estradiol-17β within 4 hr.

The estrone and estradiol-17β zones were eluted and each was chromatographed on TLC in the system benzene/methanol 9:1. The radioactive metabolites were located by scanning the chromatograms ("Berthold" Wildbad, Germany, Model LB 2723). The identity of the radioactive metabolites was established by their mobility in different chromatographic systems and by crystallization of an aliquot with 20 mg of an appropriate carrier. The estrone fraction was repeatedly crystallized from ethanol or aqueous methanol and the estradiol-17β fraction was crystallized from aqueous methanol or acetone-n-hexane. Correction for procedural losses during extraction and chromatography was made by quantitation of the respective carrier steroids by fluorometry [3]. Radioactive measurements were carried out in a Packard tricarb liquid scintillation spectrometer (model 3380) employing PPO-POPOP dissolved in toluene. The samples were counted to at least 1000 dis/min with s.d. of 2–5% or less.

RESULTS AND DISCUSSION

The uptake and metabolism of ^{3}H-estradiol-17β was studied in twelve tissue samples of human female breast grown in organ culture. In all cultured samples (5 carcinoma, 3 uninvolved breast tissue, 2 fibroadenoma and 2 cystic mastitis) the tissue remained vital according to the following criteria: (*a*) histological examination, and (*b*) incorporation of ^{3}H-thymidine into DNA, of ^{3}H-uridine into RNA and of ^{14}C-amino acids into protein [1].

In control experiments where ^{3}H-estradiol-17β was added to the medium and incubated without tissue, 99% or more was recovered as unchanged estradiol-17β. Therefore, labelled estrone which was found in the medium of the respective organ cultures was derived from ^{3}H-estradiol-17β which was metabolized by the tissue.

In all investigated tissue samples, estradiol-17β was metabolized to estrone which was identified both in the tissue and in the medium (Table 1). The percentage of conversion of estradiol-17β to estrone by the cancerous tissue was always lower (2·3–26·9%) than by the uninvolved tissue (33·9–44·8%) or by the benign tissue (30·5–33·9%). The percentage

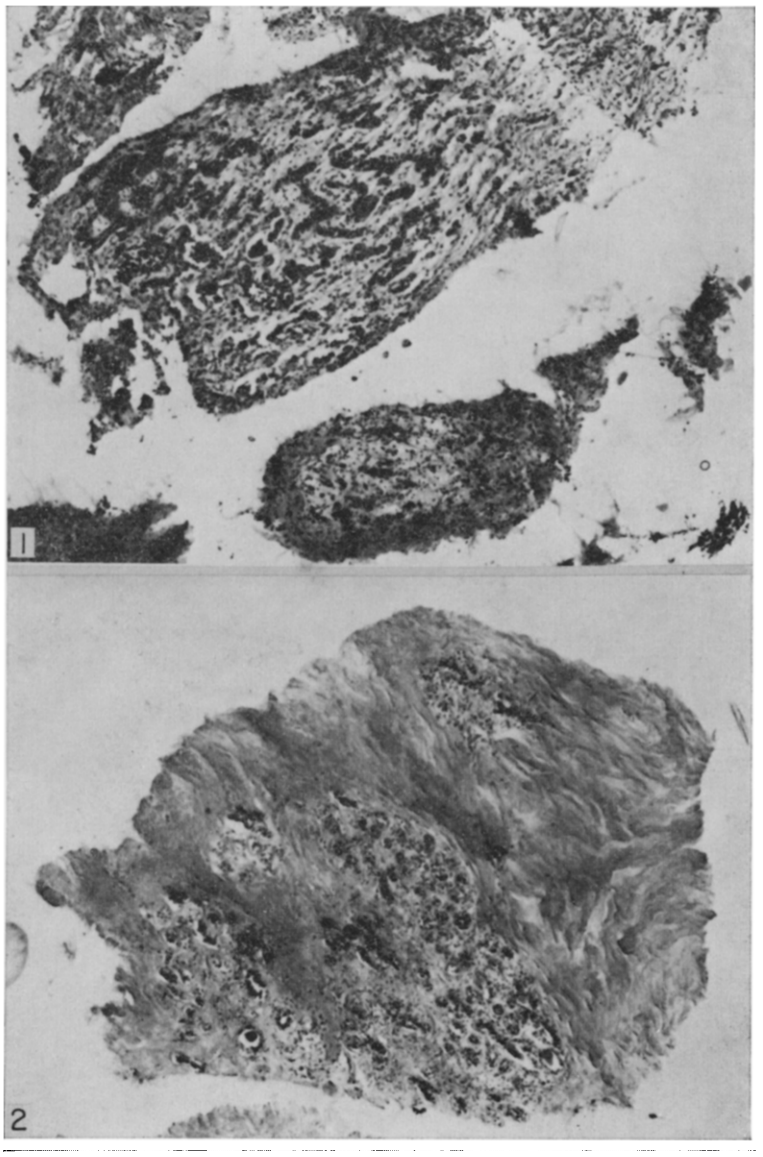


Fig. 1. *Tissue sample of breast carcinoma before culturing. There are strands of malignant cells in a fibrous stroma with slight lymphoytic infiltration. Note absence of fat cells.* (*Hematoxylin and eosin,* × 75).

Fig. 2. *Tissue sample of uninvolved breast tissue adjacent to carcinoma, before culturing. There are lobules of breast tissue in a hyaline fibrous stroma. Note absence of fat cells.* (*Hematoxylin and eosin,* × 75).

(to face page 128)

Table 1. Percentage of radioactivity recovered as estrone and as estradiol-17β from tissue and medium of breast tissue grown in organ culture with 1 μCi ^{3}H-estradiol-17β

Name and age		Tissue	Radioactivity extracted (dis/min mg^{-1} protein)	Distribution of radioactivity (%)				
				E_1*	(tiss + med)	E_2	(tiss + med)	$E_1 + E_2$
B.V.	44	Invasive breast cancer	234,060	2·3	(1·2 + 1·1)	70·8	(4·5 + 66·3)	73·1
		Uninvolved tissue	247,370	44·8	(8·2 + 36·6)	38·5	(6·6 + 31·9)	84·3
C.A.	42	Carcinoma of breast	192,060	16·1	(4·4 + 11·7)	65·9	(3·8 + 62·1)	82·0
		Uninvolved tissue	184,610	33·9	(5·5 + 28·4)	49·2	(13·0 + 36·2)	83·1
S.E.	45	Solid adenocarcinoma	197,660	19·7	(3·2 + 17·5)	62·2	(5·0 + 57·2)	81·9
		Uninvolved tissue	332,890	37·0	(2·5 + 34·5)	40·3	(11·1 + 29·2)	77·3
T.C.	44	Carcinoma simplex	187,560	26·9	(4·1 + 22·8)	54·7	(4·9 + 49·8)	81·6
M.V.	72	Poorly differentiated carcinoma	188,470	18·8	(2·6 + 16·2)	68·7	(6·5 + 62·2)	87·5
A.M.	25	Fibroadenoma	402,000	33·9	(1·0 + 32·9)	49·9	(1·1 + 48·8)	83·8
K.C.	22	Fibroadenoma	465,330	32·6	(1·0 + 31·6)	49·7	(1·1 + 48·6)	82·3
R.T.	42	Cystic mastitis	445,850	30·5	(6·5 + 24·0)	52·5	(9·5 + 43·0)	83·0
J.D.	27	Cystic mastitis	494,230	33·1	(5·7 + 27·4)	49·6	(11·2 + 38·4)	82·7

*Abbreviations: E_1—estrone; E_2—estradiol-17β; tiss—tissue; med—medium.

of unchanged estradiol-17β was 54·7–70·8% in the cancerous, 38·5–49·2% in the uninvolved, and 49·6–52·5% in the benign tissue. The total recovery of radioactivity (as ^{3}H-estrone + ^{3}H-estradiol-17β) was between 73·1 and 87·5%. No indication was obtained for conversion of estradiol-17β to estriol by any of the tissue samples.

Our results are in agreement with those of James *et al.* [4] who infused ^{3}H-estradiol-17β into breast cancer patients and identified in the cancerous tissue 3·0–50·2% of extractable tritium as estrone and 45·0–103·5% as estradiol-17β; no radioactivity was found attached to estriol.

The conversion of estradiol-17β to estrone by the cancerous tissue in organ culture relative to the non-cancerous tissue was not due to the fat contents of the respective tissue, since adipose tissue was virtually absent in all our cultured tissue samples. On microscopic examination of the explants, fat cells were found to be equally rare in the cancerous and the noncancerous tissue (Fig. 1 and 2). Moreover, Bleau *et al.* [5] reported that the percentage of conversion of estradiol-17β to estrone by human adipose tissue was only 0·46–4·8%.

Comparison of the metabolism of labelled estradiol-17β and of labelled testosterone by cancerous and uninvolved tissue shows that in two patients both estradiol-17β and testosterone were metabolized more effectively by the uninvolved breast tissue (Table 2). In a third patient (S.E.), although estradiol-17β was also

*Table 2. 17β-ol-steroid dehydrogenase activities in human breast tissue grown in organ culture**

Name and age		Tissue	E_2 metabolized into E_1	T metabolized into 17KS†
			(%)	
B.V.	44	Invasive breast cancer	2·3	19·6
		Uninvolved tissue	44·8	33·8
C.A.	42	Carcinoma of breast	16·1	18·8
		Uninvolved tissue	33·9	45·7
S.E.	45	Solid adenocarcinoma	19·7	8·4
		Uninvolved tissue	37·0	8·3
A.M.	25	Fibroadenoma	33·9	8·8
K.C.	22	Fibroadenoma	32·6	33·7

*Abbreviations: E_2—estradiol-17β; E_1—estrone; T—testosterone; 17KS—17-ketosteroids.

†From Geier *et al.* (1974).

more effectively metabolized by the uninvolved tissue, there was no significant difference in testosterone metabolism between the uninvolved and cancerous tissue.

The metabolism of estradiol-17β by cultures of fibroadenoma in the two examined cases was uniform and in the range found for the uninvolved tissue of cancerous breast. The metabolism of testosterone was, however, less uniform (Table 2): in one case (patient K.C.) it was in the range observed for the uninvolved tissue of two cancerous patients (B.V. and C.A.) and in the second case (Patient A.M.) it was conspicuously low and similar to that observed on the above-mentioned patient S.E. with breast cancer.

The quantitative differences between the dehydrogenation at C-17 of estradiol-17β and of testosterone by the individual samples of breast tissue may be explained by assuming the presence of several types of 17β-hydroxysteroid-dehydrogenases. One type, which oxydized to an equal extent both estradiol-17β and testosterone (Patient C.A.) would be similar to the 17β-hydroxysteroid dehydrogenase from Pseudomonas testosteroni [6]. The other type which preferentially oxydizes estradiol-17β (Patient S.E.) would be rather similar to the 17β-hydroxysteroid-dehydrogenase of the human placenta, which has affinity only for estradiol-17β [7]. A third type would be a 17β-hydroxysteroid-dehydrogenase with an affinity directed mainly to testosterone (cancerous tissue of Patient B.V.). Such a type, to our best knowledge, has not been described.

Should the relatively low activity of 17β-hydroxy-steroid-dehydrogenase found in the cultures of cancerous breast reflect the situation *in vivo*, then it is possible that the cancerous portion of the breast is exposed to a higher level of either testosterone or estradiol-17β than the uninvolved tissue.

REFERENCES

1. A. Geier, H. Horn, E. Lichtshtein, I. S. Levij and M. Finkelstein, The uptake and metabolism of 4-^{14}C-testosterone in human breast cancer grown in organ culture. *Acta endocrinol. (Kbh.)* **75,** 195 (1974).
2. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
3. S. Ladany and M. Finkelstein, Isolation of estrone, estradiol-17β and estriol from female human urine. *Steriods* **2,** 297 (1963).
4. F. James, H. Braunsberg, W. T. Irvine and V. H. T. James, Radioactivity in human breast tumours after infusion of ^{3}H-oestradiol: effect of androgen treatment. *Steroids* **15,** 669 (1969).
5. G. Bleau, K. D. Roberts and A. Chapdelaine, *In vivo* and *in vitro* uptake and metabolism of steroids in human adipose tissue. *J. clin. Endocr.* **39,** 236 (1974).
6. P. Talalay and P. I. Marcus, Specificity kinetics and inhibition of α and β-hydroxysteroid dehydrogenases. *J. biol. Chem.* **218,** 675 (1965).
7. L. J. Langer, J. A. Alexander and L. L. Engel, Human placental estradiol-17β dehydrogenase. *J. biol. Chem.* **234,** 2609 (1959).

Europ. J. Cancer Vol. 11, pp. 131–138. Pergamon Press 1975. Printed in Great Britain

Biochemical Properties and Replication of Murine Intracisternal A Particles During Early Embryogenesis*

STRINGNER S. YANG,† PATRICIA G. CALARCO‡ and NELSON A. WIVEL†

†*Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland* 20014, *U.S.A.*

‡*Department of Anatomy, University of California, School of Medicine, San Francisco, California* 94143, *U.S.A.*

Abstract—*Intracisternal A particles exhibit features which are characteristic of oncogenic RNA viruses and features which are reminiscent of fetal antigen expression. Evidence has been developed by ultrastructural study which points toward a vertical transmission of these particles, a situation analogous to known RNA tumor viruses. Intracisternal A particles are readily found in certain preimplantation mouse embryos; they are found occasionally in a variety of neoplasms. Biochemical properties of highly purified intracisternal A particles isolated from murine neuroblastoma cells or plasma cell tumor include high molecular weight* 60–70*S RNA and an RNA-dependent DNA polymerase. The enzymatic characterization of the A particle DNA polymerase is reported. The enzyme is found analogous to other C-type viral reverse transcriptase by virtue of its preference for* Mn^{2+} *activation and certain DNA-RNA hybrid templates, oligo* $dT^{12}\cdot poly\ rA$ ($dT^{12}\cdot rA^{n}$) *and oligo* $dG^{12}\cdot poly\ rC$ ($dG^{12}\cdot rC^{n}$), *for the stimulation of its activity. The endogenous reaction of the A particle RNA-dependent DNA polymerase produces a DNA transcript of its intrinsic RNA with base complementarity as demonstrated by the results of hybridization studies. The control of the expression of this gene entity and its relevance to malignant transformation constitute a problem worthy of further investigation.*

INTRODUCTION

NEOPLASTIC transformation has been viewed as a departure from the normal process of differentiation. Frequently the reappearance of fetal characteristics accompanies the onset of the neoplastic state; for example, proteins like serum alpha-fetoprotein and carcinoembryonic antigen can serve as diagnostic markers for hepatocellular carcinoma [1] and carcinoma of the digestive tract [2] respectively. Such phenomena have been attributed to the reactivation of the fetal genes during neoplastic transformation of the cell. Intracisternal A particles (IAP), known primarily for their association with tumors in mice [3–6], gerbils [7], rats [8], and guinea pigs [9], are most probably vertically transmitted from maternal oocyte to fertilized embryo [10, 11]. Their appearance in large numbers during murine preimplantation development has been reported recently [11–13]. In the cell, IAP consist of two concentric shells surrounding a relatively electron lucent core; they measure between 70 and 100 nm in outer diameter when appearing singly (Fig. 1). They are confined exclusively within the cisternae of the endoplasmic reticulum and are formed by budding at the reticulum membrane [14]. The purposes of this communication are two-fold, 1, to detail the mode of appearance and subsequent fate

Accepted 19 November 1974.

*This work was presented earlier at the *Third Conference on Embryonic and Fetal Antigens in Cancer*, Nov. 4–7, 1973, Oakridge, Tenn., U.S.A.

of IAP in preimplantation mouse embryos, and 2, to enumerate nucleic acid and DNA polymerase characteristics which indicate that IAP are an expression of an RNA tumor virus genome.

MATERIAL AND METHODS

Electron microscopy

Oocytes were obtained from CF-1 female mice. Preimplantation stages of development were collected from Swiss Webster, ICR, BALB/c, CF-1, New Zealand Black and AKR strain females. Preparative techniques were as outlined previously [11].

Sources for IAP

A mouse plasma cell tumor (MOPC-104E) was maintained by s.c. transplantation in BALB/c mice in this laboratory. Neuroblastoma N-18, a continuous tumor cell line described previously [15], was cultured in Dulbecco's modified Eagle medium supplemented with 10% heat inactivated fetal calf serum, penicillin and streptomycin as described earlier. All tissue culture materials were obtained from GIBCO, Grand Island, N.Y., U.S.A.

Isolation and purification of IAP

Isolation and purification of IAP have been well documented [14–15]. Briefly, a pellet of mitochondria and microsomes was prepared by centrifugation of cytoplasmic extracts obtained from tumor homogenates at 13,000 $\times g$ for 20 min. Intracisternal A particles were then sheared from the microsomal vesicles in the presence of Triton X-100. The freed A particles were then purified by sucessive discontinuous and continuous linear sucrose gradient centrifugations for three or more times. The methods employed allow for the preparation of a highly homogenous concentrate of IAP. Appropriate control experiments established that these purified isolates were free of any C-type particle contamination [15]; therefore the demonstrated biochemical properties can be considered as intrinsic to IAP alone. It should also be noted that no comparable preparation could be isolated from normal tissues uninfected with intracisternal A particles.

DNA polymerase assays

Details of DNA polymerase assays have been described earlier [16–18]. Some minor variations were allowed to optimize the assay conditions. Briefly, the assay mixture contained Tris–HCl, pH 8·1, 50 mM; Mg acetate, 10 mM or $MnCl_2$, 0·8 mM; dithiothreitol (DTT), 8 mM; KCl, 75 mM; and specified amount of purified IAP protein treated with 0·018% Triton X102 for 10 min at 0°. For assaying the endogenous reaction, cold dCTP, dGTP, and dATP at a final concentration of 8×10^{-5} M were added along with 5 μCi of ^{3}H-TTP (New England Nuclear) in a 50 μl reaction was carried out at 37°. In assays with synthetic templates, the appropriate substrates as specified in the Table were used; the reaction was carried out at 30°. The reaction of DNA polymerase was terminated with additions of 2 ml of ice cold 0·08 M sodium pyrophosphate in 12·5% trichloroacetic acid (TCA) and 20 μg of yeast nucleic acid as carrier. The radioactive precipitates were collected on Millipore filters (Millipore Filter Corp., Mass.), washed extensively with 5% TCA, then 80% ethanol, dried and counted by a liquid scintillation method.

All chemicals were obtained from Calbiochem, Calif. and Fischer Scientific Co., New Jersey. Triton X-100 or X-102 detergents were obtained from Sigma, Mo. All isotopes were purchased from New England Nuclear, Mass. Bovine pancreatic RNase, at 0·25 mg/ml and boiled prior to use, was obtained from Worthington, Germany.

Virus purification

Avian myeloblastosis virus (AMV) and Rauscher leukemia virus (RLV) at 10^{11} particles/ml were generously supplied by Drs. J. W. Beard and D. P. Bolognesi and Electronucleonics, Md. respectively via the Special Cancer Virus Program of the National Cancer Institute. Further purification of these viruses was described [19]. Materials banded at the 1·16 g/ml area after isopycnic sucrose density gradient centrifugation were used for RNA extraction.

RNA extraction and purification

High molecular weight, 60–70S, RNA was extracted from AMV and RLV and purified by velocity gradient centrifugation prior to being used as standards and templates in DNA polymerase assays. Details of the extraction and purification procedures were described elsewhere [15]. Purified IAP from N-18 neuroblastoma cells, labelled with all four ^{3}H-ribonucleosides at 5 μCi/ml of media for 48 hr,

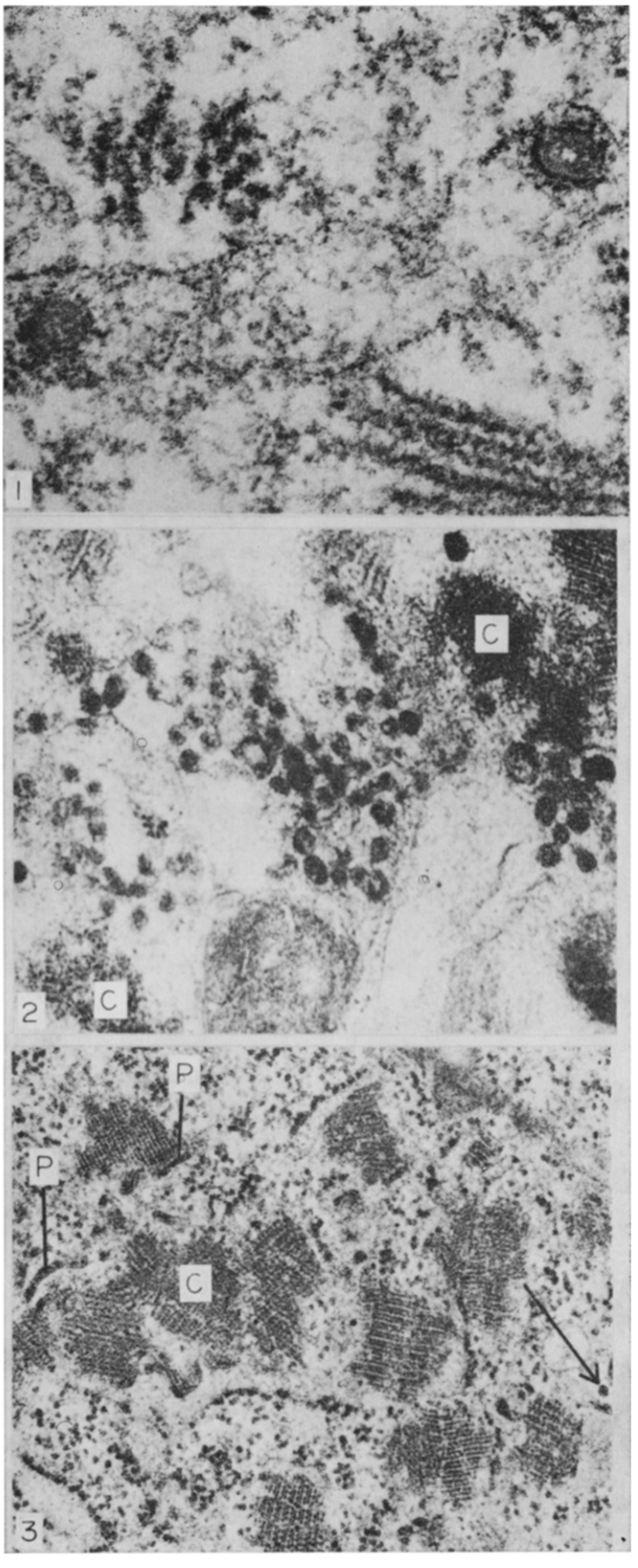


Fig. 1. *Intracisternal A particles in a CF-*1 *mouse oocyte.* × 106,000.

Fig. 2. *Intracisternal A particles in a Balb/c* 4 *cell stage. Note associated crystalloid materials* (*C*). × 45,500.

Fig. 3. *Blastocyst of Swiss–Webster mouse embryo. Arrow denotes small particle which appears to be intracisternal, C, crystalloid materials ; P, plaques along endoplasmic reticulum.* × 39,000.

(to face p. 132)

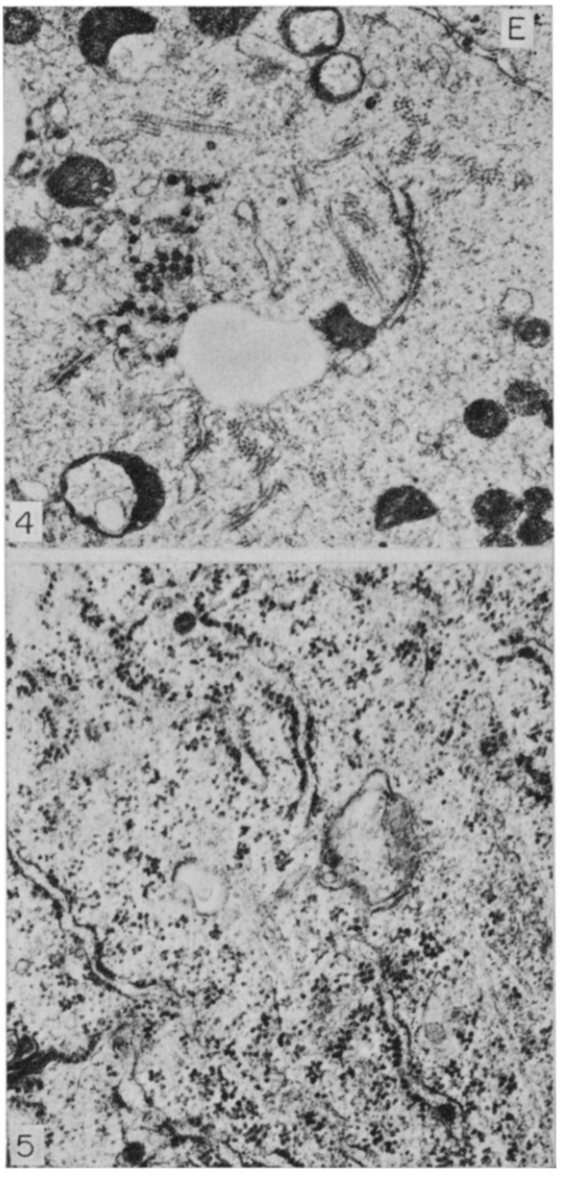


Fig. 4. *Second polar body from an ICR blastocyst. Note the intracisternal A particles, fibrous lined ER cisternae and the lack of ribosomes. E. embryo proper.* × 19,700.

Fig. 5. *Intracisternal A particles and budding intracisternal A particles in cell from mouse embryo placed in culture as a blastocyst and grown for* 4 *days.* × 28,000.

were subjected to RNA extraction and purification by the same method [15]. The purified ^{3}H-RNA from IAP was then analyzed by velocity glycerol gradient along with standard 18S rRNA and 70S RNA in a SW 40 rotor at 40,000 rev/min for 90 min.

Templates

All natural templates were purchased from Miles Laboratory, Ill. Activated salmon sperm DNA was prepared as described by Schlabach *et al.* [20]. Copolymers and homopolymers of various synthetic oligo-nucleotides with specified chain lengths were obtained from either Miles Laboratory or Collaborative Research Corp., Mass.

Purification and analysis of the DNA products

The radioactive DNA products from an endogenous RNA-dependent DNA polymerase reaction of IAP were purified by sequential extractions with phenol-SDS and ether, and then precipitated in ethanol–NaCl at – 20°. The radioactive DNA precipitates were redissolved in 0·3 M Na-acetate and further purified by gel filtration in a sulphopropyl Sephadex C-50 column in the same buffer. The radioactive fractions at the void volume were pooled and reprecipitated in ethanol–NaCl prior to cesium sulfate isopycnic centrifugation analysis at 40,000 rev/min for 60 hr at 4° in a SW 50 rotor. At the end of the equilibrium run, the sample was fractionated. Density was determined by direct weighing and the samples were precipitated in 12·5% TCA and processed for radioactive determination as described. Purified DNA product was also hybridized back to IAP 60–70S RNA by the 50% formamide method at 37° for 18 hr. At the end of the hybridization the reaction mixture was adjusted for S-1 exonuclease digestion according to the method described earlier [21].

S-1 Exonuclease

The preparation of S-1 exonuclease from *Aspergillus oryzae* crude α-amylase (Sigma, Mo.) has been described earlier [21].

Protein determination

Protein was determined by the Lowry method [22]. Samples were first precipitated and washed with 10% perchloric acid prior to alkali digestion.

RESULTS

Replication of intracisternal A particles during early embryogenesis

IAP are seen in small numbers in dictyate oocytes immediately after their release from ovarian follicles. They are always enclosed within agranular portions of the endoplasmic reticulum (Fig. 1). IAP are only rarely observed in oocytes after break-down of the germinal vesicle has begun. None were seen in oocytes which reached metaphase II either *in vitro* or *in vivo*, nor were any such particles observed in fertilized eggs. At approximately the 4 cell stage, large numbers of complete IAP reappear within the endoplasmic reticulum (Fig. 2).

Quantitation of IAP at different stages of development was not feasible, but the number of particles seems to peak at the 4–8 cell stage. By the 16 cell stage (morula) and the 32 + cell stage (blastocyst), A particles are only rarely encountered within the now granular encoplasmic reticulum, and no cytoplasmic densities or budding particles are seen (Table 1).

In tracing the morphologic sequence of events preceding the development of IAP, the following observations were made. Crystalloid aggregates (C) composed of units about 40 nm wide, 300 nm long, with a cross striation every 12 nm [23], and usually contiguous with the endoplasmic reticulum, appear first in the fertilized egg (Fig. 3). These structures seem to increase in number and size until the morula stage. During the blastocyst stage crystalloid units often form densely staining plaques (P) along cisternae of encoplasmic reticulum (Fig. 3). The very small particles seen occasionally within the endoplasmic reticulum at this stage may be cross sections of these plaques (Fig. 3).

Stages in IAP formation have also been observed within the first and second polar bodies. Polar bodies possess most of the organelles of the embryo but are notably deficient in ribosomes. Crystalloid formation often proceeds in the second polar body until one or a few very large crystalloids are formed; these fill much of the polar body and are easily visible in sections cut for the light microscope. Interestingly, at the blastocyst stage when IAP are rare within the embryo proper, IAP as well as cisternae of endoplasmic reticulum lined with fibrous material can still be observed in the surviving second polar body (Fig. 4).

When mouse embryos are cultured beyond blastulation they hatch from their zonas, attach to the substrate, and cell proliferation

begins, *i.e.*, the outgrowth stage begins. These proliferating cells show an occasional budding or complete IAP (Fig. 5) but crystalloid material is notably absent.

The time of appearance and approximate frequency of occurrence of crystalloids and IAP from fertilized egg to the outgrowth stage is summarized in Table 1.

Table 1. *Occurrence of intracisternal A particles and associated structures in preimplantation mouse embryos*

Stage	Crystal-loids	Budding A particles	Complete A particles
Fertilized egg*	+	+	0
2 cell (early)	+	+	0
2 cell (late)	+	+ +	+
4 cell	+ +	+ + +	+ + + +
8 cell	+ + +	+	+ + + +
Morula	+ +	0	+
Early blastocyst	+ +	0	+
Expanded blastocyst	+	0	+
Outgrowth	0	+	+

*Examined at the stage just prior to the first cleavage, approximately 24 hr after fertilization.

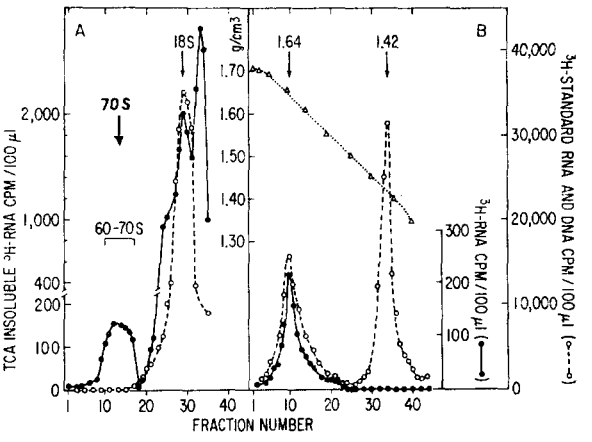


Fig. 6. *Profiles of high molecular weight RNA* (60–70*S*) *associated with intracisternal A particles purified from N-*18 *neuroblastoma cells.* 6*A. Velocity glycerol* (10–35%) *gradient analysis of a typical preparation of* ^{3}*H-RNA of intracisternal A particles,* ●—●—●. *Standard* ^{3}*H-*18*S ribosomal RNA was from Miles Lab.,* – –○– –○– –, *and standard* 70*S RNA from RLV (arrow) was run in an accompanying gradient simultaneously.* 6*B. Cesium sulfate isopycnic centrifugation analysis of the* 60-70*S* ^{3}*H-RNA of intracisternal A particles from the bracketed fractions in* 8*A,* ●—●—●. *Standards are* ^{3}*H-*18*S rRNA and* ^{3}*H-DNA purified from* 3*T*3 *cells,* ○– –○– –○, *ran simultaneously in another tube. Density was designated by* △ . . △ . . △.

Table 2. *Characteristics of an RNA-dependent DNA polymerase activity associated with intracisternal A particles*

Conditions	TCA-insoluble tritium incorporation (Counts/min per 30 min/50 µl)	^{3}H-substrate used
Complete with Mg^{2+}, 10 mM	864	^{3}H-TTP
Complete with Mn^{2+}, 0·8 mM	1478	
Minus Mg^{2+} or Mn^{2+}	25	
Minus DTT	18	
Minus Na^+ or K^+	560	
Minus dA, dG, dC, TP	42	
Minus Triton X-102 treatment	228	
Preincubation with RNase at 37°/2 hr	23	
Preincubation at 37°/2 hr	1150	
Stimulation by synthetic RNA, DNA and RNA–DNA		
dT^{12-18}·poly rA (50 µg/ml)	8825	^{3}H-TTP
dT^{12-18}·poly dA (50 µg/ml)	940	
poly dT·poly rA (50 µg/ml)	5075	
poly rA·poly rU (50 µg/ml)	812	
Endogenous—none	410	^{3}H-dGTP
dG^{12-18}·poly rC (50 µg/ml)	2050	

All assay conditions were described in the text. Complete condition referred to inclusions of all critical requirements such as K^+ or Na^+, Mn^{2+} or Mg^{2+}, DTT, all four deoxyribonucleoside triphosphates, and 0·018% Triton X-102 treatment, at 37°. Each assay contained 12·5 µg of IAP protein from plasma cell tumor (MOPC-104E). Preincubation with RNase (25 µg/ml) was carried out in 0·15 M NaCl and the subsequent assays were in the presence of Mn^{2+}. Assays with synthetic templates were carried out with 100 µCi/ml of the specified substrate and in the presence of Mn^{2+}, at 30°. ^{3}H-TTP was 13,000 counts/min per pmole and ^{3}H-dGTP was 2560 counts/min per pmole.

High molecular weight RNA as intrinsic nucleic acid of IAP

IAP are observed occasionally in normal differentiated murine tissue. Upon neoplastic transformation however, these viral particles can be recovered in sufficient amounts from such tissues as the plasma cell tumor or neuroblastoma so that biochemical analyses may be carried out. It has been established earlier that IAP purified from N-18 neuroblastoma cells showed the ability to incorporated tritiated ribonucleosides into intrinsic RNA [15]. This intrinsic RNA has a sedimentation value of 60–70S (Fig. 6A) since it co-sedimented with RLV 70S RNA, and it exhibits a density of 1·64 g/cm^3 in cesium sulfate isopycnic centrifugation analysis (Fig. 6B), typical of an RNA.

DNA polymerase activities associated with IAP

Purified IAP exhibited an endogenous RNA-dependent DNA polymerase that can be stimulated to much greater activity by the addition of exogenous templates. Table 2 summarizes the basic requirements for maximal endogenous activity of the A particle polymerase; they included the presence of DTT, Mn^{2+} or Mg^{2+}, Na^+ or K^+, and all four deoxyribonucleoside triphosphates. Analogous to other C-type viral reverse transcriptase, the RNA-dependent DNA polymerase of IAP was sensitive to digestion with a low concentration of RNase even at a high concentration of NaCl, and it preferred Mn^{2+} over Mg^{2+} for its divalent cation requirement [24–26]. It is interesting to note that preincubation of the IAP in the same condition as that of RNase digestion resulted in a minor loss of RNA-dependent DNA polymerase activity suggesting that the enzyme is rather unstable at 37°.

The stimulatory effects of a variety of natural and synthetic templates on the activity of the RNA-dependent DNA polymerase of IAP have also been investigated. Among the natural RNA templates tested at 50 μg/ml, which included AMV 70S RNA, RLV 70S RNA. MS-2* RNA, VSV† RNA, Influenza viral RNA and QB‡ RNA, only AMV showed some stimulation. Others either did not stimulate or showed slight inhibitory effect. Likewise, the natural DNA templates, T_2 phage DNA and activated salmon sperm DNA at 50 μg/ml, showed little or no stimulatory effect on the A particle RNA-dependent DNA polymerase.

*MS-2 phage; †VSV is vesicular stomatitis virus; ‡QB phage.

Among the synthetic templates studied (Table 2), $dT^{12}\cdot rA^n$ exhibited the greatest stimulatory effect on A particle DNA polymerase activity, but not $dT^{12}\cdot dA^n$. When the activities of the polymerase stimulated by these two templates were compared, a characteristic ratio of $dT^{12}\cdot rA^n/dT^{12}\cdot dA^n$ greater than 8·5 was observed. Poly dT·poly rA, the universal template that stimulates both viral and cellular DNA polymerases [20, 27] also stimulated the IAP DNA polymerase. But the DNA copolymer, poly d(A-T), which was used efficiently by cellular DNA polymerase [20] did not stimulate A particle DNA polymerase. The diagnostic synthetic hybrid template oligo dG^{12}·poly rC [28] also stimulated A particle DNA polymerase.

Kinetics and product analyses of the endogenous reaction of the RNA-dependent DNA polymerase of IAP

Kinetics of the endogenous RNA-dependent DNA polymerase of IAP differed in the presence of actinomycin D. The endogenous reaction of a representative preparation of IAP was generally linear up to 50–60 min under the optimal assaying conditions described above (Fig. 7A). In the presence of 5 μg/ml of actinomycin D, the initial rate of the endogenous reaction became slightly slower although its linearity extended up to 120 min before it slowed down (Fig. 7B).

The DNA products labelled in tritium synthesized at different points during the course

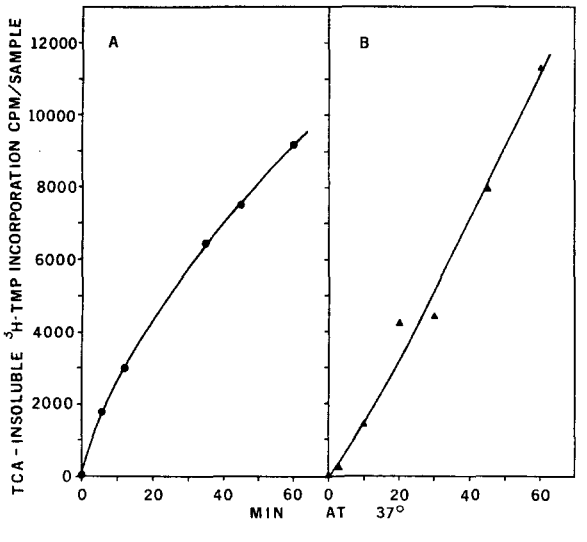


Fig. 7. Kinetics of the endogenous reaction of the RNA-dependent DNA polymerase of intracisternal A particles from MOPC-104E plasma cell tumor, ●—●—● (A), as compared with the same in the presence of 5 μg/ml of actinomycin D, ▲– –▲– –▲ (B).

of the endogenous reaction were examined by cesium sulfate isopycnic gradient centrifugation analysis. It was found that the DNA products obtained at short-term reaction (5 min) were primarily linked to the endogenous RNA of the A particles (Fig. 8) and hence sedimented at the areas of both the RNA (1·65 g/cm^3) and the RNA–DNA hybrid (1·52–1·58 g/cm^3). At the 15 min point, the distribution of the newly synthesized DNA was at all three regions, the RNA area, the RNA–DNA hybrid area, and the DNA area (1·42–1·44 g/cm^3), although the majority of the DNA was now at the hybrid

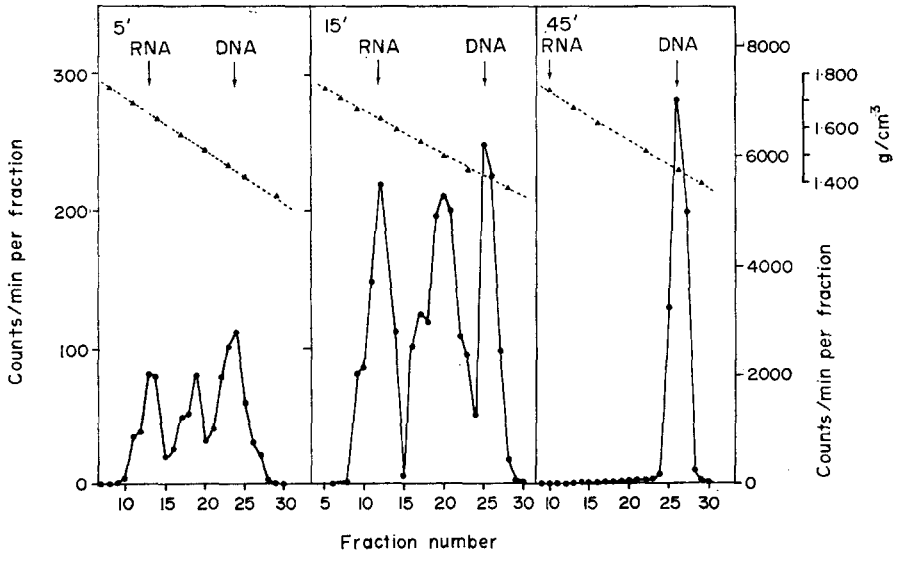


Fig. 8. Cesium sulfate isopycnic gradient centrifugation analysis of the DNA products of the endogenous reaction of the RNA-dependent DNA polymerase of intracisternal A particles. Approximately 2·5 mg of highly purified intracisternal A particle protein was assayed as described in the text at 37° with 100 μCi of ^{3}H-dA, dC, dG, T, TP at equal molar ratio and adjusted to the same specific activity, 5 mCi/0·05 mg, in a final volume of 1·0ml. Samples of 200 μl were withdrawn at 5 min and at 15 min, and the remaining 600 μl at 45 min. The reaction was terminated by adjustment to 0·3 M Na-acetate and phenol-SDS extraction, followed by extensive purification of the ^{3}H-DNA products as described in the text. ^{3}H-DNA distribution was designated by ●—●—● and density was designated by ▲---▲---▲.

and DNA areas (Fig. 8). At the end of a reaction of 45 min, the newly synthesized DNA sedimented totally at the DNA area (Fig. 8). The sedimentation pattern of the newly synthesized DNA, dependent on the time of the reaction in this case, also resembled those for other oncornaviruses [29–32].

When the long-term DNA product was purified exhaustively and hybridized back to the 35S and 60–70S RNAs of IAP in 50% formamide at 37° for 18 hr and the percentages of hybridization was analyzed by the *Aspergillus* S-1 exonuclease digestion method [21], a 70–90% efficiency was observed, using approximately 3500 counts/min of input ^{3}H-DNA product (equivalent to about $6{\cdot}4 \times 10^{-4}$ μg) and 2·0 μg of A particle RNA. There was no hybridization of the ^{3}H-DNA product of IAP endogenous DNA polymerase reaction with other heterologous RNA such as QB RNA. Details of this study will be reported elsewhere. These results indicated that the newly synthesized DNA in an endogenous RNA-dependent DNA polymerase reaction of IAP represented a faithful transcript of the intrinsic RNAs of the A particles as reflected by their base complementarity in the hybridization results.

DISCUSSION

The presence of intracisternal A particles in oocytes, their disappearance during maturation, and their reappearance at the 2–4 cell stage suggests a vertical mode of transmission. Furthermore, the observation of A particle formation in the first polar body at the late 2 cell stage suggests that information for gene expression was present in the maternal ovum.

The time and place of appearance of crystalloid material accumulated during preimplantation period suggests a possible relationship with intracisternal A particle formation; how-

ever, the proof of this relationship remains to be developed.

Complete intracisternal A particles, when isolated in quantity from tumors, possess biochemical properties analogous to RNA tumor viruses, such as 60–70S RNA and an RNA-dependent DNA polymerase that synthesizes a DNA transcript of the intrinsic RNA with base complementarity.

Therefore, in intracisternal A particles, there is an experimental system which provides some fundamental aspects of viral gene expression during embryogenesis on both the ultrastructural and biochemical levels, and which is quite possibly related to the problem of neoplastic transformation. Whether intracisternal A particles, similar to that of fetal antigen, represent a particular gene entity accidentally co-expressed with the other molecular anomalies in tumor development, or whether they might themselves be carcinogenic remains to be resolved. The relationship between the expression of this gene entity and malignant transformation constitutes a problem worthy of further investigation. Biochemical properties of intracisternal A particles, when better understood, might lead to development of diagnostic tools such as labelled DNA probe for early detection of viral synthesis and a more accurate tracing of events during tumor growth in an animal model system.

Acknowledgement—We thank Mr. R. Steinberg for his excellent assistance in photography.

REFERENCES

1. K. R. McIntire, C. L. Vogel, G. L. Princler and I. R. Patel, Serum Alpha-fetoprotein as a biochemical marker for hepatocellular carcinoma. *Cancer Res.* **32,** 1941 (1972).
2. E. D. Holyoke, G. Raynoso and T. Chu, Carcinoembryonic antigen in patients with carcinoma of the digestive tract. In *Embryonic and Fetal Antigens in Cancer*, p. 215–220. Oakridge National Laboratory (1972).
3. A. J. Dalton and M. D. Felix, The electron microscopy of normal and malignant cells. *N.Y. Acad. Sci.* **63,** 1117 (1956).
4. A. J. Dalton, M. Potter and R. M. Merwin, Some ultrastructural characteristics of a series of primary and transplanted plasma-cell tumors of the mouse. *J. nat. Cancer Inst.* **26,** 1221 (1961).
5. E. DeHarven and C. Friend, Electron microscopy study of a cell-free induced leukemia of the mouse. *J. biophys. biochem. Cytol* **4,** 151 (1958).
6. N. A., Wivel and G. H. Smith, Distribution of intracisternal A particles in a variety of normal and neoplastic mouse tissues. *Int. J. Cancer* **7,** 167, (1971).
7. J. J. Tumilowicz and J. J. Cholon, Intracisternal type A particles and properties of a continouus cell line originating from a gerbil fibroma. *Proc. Soc. exp. Biol.* **136,** 1107 (1971).
8. A. J. Novikoff and L. Biempica, Cytochemical and electron microscopic examination of Morris 5123 and Reuber H-35 Hepatomas after several years of transplantation. *Gann* **1,** 65 (1966).
9. E. W. Nadel, W. Banfield, S. Burstein and A. J. Tousimis, Virus particles associated with strain 2 guinea pig leukemia (L2c/N-B). *J. nat. Cancer Inst.* **38,** 979 (1967).
10. W. Biczysko, D. Solter, C. Graham and H. Koprowski, Synthesis of endogenous type-A virus particles in pathenogenetically stimulated mouse eggs. *J. nat. Cancer Inst.* **52,** 483 (1974).
11. P. G. Calarco, and D. Szollosi, Intracisternal A particles in ova and preimplantation stages of the mouse. *Nature New Biol.* **243,** 91 (1973).
12. W. Biczysko, M. Pienkowski, D. Solter and H. Koprowski, Virus particles in early mouse embryos. *J. nat. Cancer Inst.* **51,** 1041 (1973).
13. D. G. Chase and L. Pikó, Expression of A- and C-type particles in early mouse embryos. *J. nat. Cancer Inst.* **51,** 1971 (1973).
14. E. L. Kuff, N. A. Wivel and K. K. Leuders, The extraction of intracisternal A particles from a mouse plasma-cell tumor. *Cancer Res.* **28,** 2137 (1968).
15. S. S. Yang and N. A. Wivel, Analysis of high molecular weight ribonucleic acid associated with intracisternal A particles. *J. Virol.* **11,** 287 (1973).
16. D. Baltimore, Viral RNA-dependent DNA polymerase. *Nature (Lond.)* **226,** 1209 (1970).

17. H. M. Temin and S. Mizutani, RNA-dependent DNA polymerase in virions of Rous Sarcoma Virus. *Nature (Lond.)* **226,** 1211 (1970).
18. S. S. Yang, F. M. Herrera, R. G. Smith, M. S. Reitz, G. Lancini, R. C. Ting and R. C. Gallo, Rifamycin antibiotics: inhibitors of Rauscher murine leukemia virus reverse transcriptase and of purified DNA polymerases from human normal and leukemic lymphoblasts. *J. nat. Cancer Inst.* **49,** 7 (1972).
19. D. P. Bolognesi and H. Bauer, Polypeptides of avian RNA tumor viruses. I. Isolation and Physical and Chemical Analysis. *Virology* **42,** 1097 (1970).
20. A. Schlabach, B. Friedlander, A. Bolder and A. Weissbach, DNA-dependent DNA polymerase from HeLa cell nuclei. II. Template and substrate utilization. *Biochem. Biophys. Res. Commun.* **44,** 879 (1971).
21. R. E. Benveniste and E. Scolnick, RNA in mammalian sarcoma virus transformed non-producer cells homologous to murine leukemia virus RNA. *Virology* **51,** 370 (1973).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin-phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
23. A. C. Enders and S. J. Schlafke, The Fine structure of the blastocyst: some comparative studies. In *Preimplantation Stages of Pregnancy.* (Edited by G. E. W. Wolstenholme and M. O'Connor), Brown and Co., Boston (1965).
24. M. Fry and A. Weissbach, The utilization of synthetic ribonucleic acid template by a new deoxyribonucleic acid polymerase from cultured murine cells. *J. biol. Chem.* **248,** 2678 (1973).
25. M. Green, M. Rokutanda, K. Fujinaga, R. K. Ray, H. Rokutanda and C. Gurgo, Mechanism of carcinogenesis by RNA tumor viruses. I. An RNA-dependent DNA polymerase in murine sarcoma viruses. *Proc. nat. Acad. Sci. (Wash.)* **67,** 385 (1970).
26. E. Scolnick, E. Rands, S. A. Aaronson and G. J. Todaro, RNA-dependent DNA polymerase activity in five RNA viruses: divalent cation requirements. *Proc. nat. Acad. Sci. (Wash.)* **67,** 1789 (1970).
27. S. Spiegelman, A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek and K. Watson, Synthetic DNA–RNA hybrids and RNA–DNA duplexes as templates for the polymerases of the oncogenic RNA viruses. *Nature (Lond.)* **228,** 430 (1970).
28. D. M. Scolnick, W. P. Parks, G. J. Todaro and S. A. Aaronson, Immunological characterization of primate C-type virus reverse transcriptase. *Nature New Biol.* **235,** 35 (1972).
29. K. Fujinaga, J. T. Parsons, J. W. Beard, D. Beard and M. Green, Mechanism of carcinogenesis by RNA tumor viruses. III. Formation of RNA·DNA complex and duplex DNA molecules by the DNA polymerases of avian myeloblastosis virus. *Proc. nat. Acad. Sci. (Wash.)* **67,** 1432 (1970).
30. A. C. Garapin, J. P. McDonnell, W. Levinson, N. Quintrell, L. Fanshier and J. M. Bishop, Deoxyribonucleic acid polymerase associated with Rous sarcoma virus and avian myeloblastosis virus: properties of the enzyme and its product. *J. Virol.* **6,** 589 (1970).
31. K. F. Manly, D. F. Smoler, E. Bromfeld and D. Baltimore, Forms of deoxyribonucleic acid produced by virions of the ribonucleic acid tumor viruses. *J. Virol.* **7,** 106 (1971).
32. S. Spiegelman, A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek and K. Watson, Characterization of the products of RNA-directed DNA polymerases in oncogenic RNA viruses. *Nature (Lond.)* **227,** 563 (1970).

Europ. J. Cancer Vol. 11, pp. 139–144. Pergamon Press 1975. Printed in Great Britain

The Relationship of Cellular Metabolism of Benzo(a)pyrene to the Induction of Cytotoxicity and Cell Transformation in Fetal Hamster Cells *In Vitro**

R. A. LUBET†, M. TURNER-LUBET† and G. L. WHITSON
Institute of Radiation Biology and Department of Zoology, University of Tennessee, Knoxville, Tennessee 37916, *U.S.A.*

Abstract—*Employing inhibitors of benzo(a)pyrene metabolism, the relationship between cellular metabolism of benzo(a)pyrene and benzo(a)pyrene-induced toxicity and transformation in hamster embryo cells has been investigated. Benzo(a)pyrene-induced cytoxicity closely paralleled cellular metabolism of benzo(a)pyrene. Benzo(a)-pyrene-induced transformation, however, was not inhibited by significant inhibition of cellular metabolism of benzo(a)pyrene, implying that benzo(a)pyrene-induced transformation in hamster embryo cell cultures is not closely linked to overall metabolism of benzo(a)pyrene.*

INTRODUCTION

CERTAIN polycyclic hydrocarbons, including benzo(a)pyrene [B(a)P], when added to hamster embryo cells (HEC) produce both cytotoxic and transforming effects [1]. Cellular metabolism of the parent hydrocarbons leads to their conversion into toxic metabolites [2]. Thus, Diamond *et al.* [3] found that inhibition of hydrocarbon metabolism inhibits hydrocarbon-induced toxicity, while Gelboin *et al.* [4] have shown a clearcut relationship between a cell's ability to metabolize the parent hydrocarbon and susceptibility of that cell to hydrocarbon-induced toxicity.

The role of metabolism in B(a)P mediated carcinogenesis is less clear. Gelboin and Kinoshita [5, 6] working with dimethylbenzanthracene (DMBA)-induced ephithilial carcinomas have found that decreased aryl hydrocarbon hydroxylase (AHH) activity results in decreased tumor formation. This implies that the AHH enzyme system results in the metabolism of the parent hydrocarbon to some proximate carcinogenic entity. On the other hand, several other investigators, [7, 8] working with DMBA and B(a)P induced pulmonary adenomas and mammary gland tumors have found that pretreatment of mice with substances that enhance AHH activity results in a decreased incidence of both types of tumors. This has been interpreted to imply that the metabolism of the parent hydrocarbon results in less carcinogenic entities [8].

Recently, two groups of investigators have administered inducers or inhibitors of AHH activity together with B(a)P treatment in order to investigate the role of metabolism in mediating the carcinogenic effects of B(a)P. DiPaolo *et al.* [9] using an *in vitro* hamster embryo transforming system showed that pretreatment of cells with 7,8-benzoflavone or benzanthracene resulted in an enhancement of B(a)P induced transformation in this system.

Accepted 1 October 1974.

*This research was supported in part by ACS Grant IN-89D.

†Present address: Southwestern Medical School, Dallas, Texas. Correspondence should be sent here.

Similarly, Gelboin and Kinoshita [5] using a B(a)P-croton oil mediated system of skin tumorigenesis have shown that cotreatment with 7,8-benzoflavone did not decrease B(a)P induced carcinogenesis.

Since *in vitro* transformation systems lend themselves to quantitative studies of carcinogenesis without the obfuscating systemic effects found during *in vivo* investigations, we have investigated quantitatively both cellular metabolism of B(a)P and the toxic and carcinogenic effects of B(a)P in cell culture, in order to establish the relationship between these phenomena. We have cotreated cells with B(a)P and either benzanthracene or 7,8-benzoflavone and looked at their effect on cellular metabolism of B(a)P induced toxicity and carcinogenicity. Our data imply that B(a)P induced toxicity and overall metabolism of B(a)P are closely linked phenomena, since a decrease in metabolism of B(a)P causes a concomitant decrease in B(a)P mediated toxicity. In the case of transformation, however, overall metabolism and the carcinogenic process do not seem to be integrated phenomena, since significant variations in overall metabolism do not lead to similar variations in *in vitro* transformation.

MATERIAL AND METHODS

Measurement of B(a)P induced cytotoxicity and B(a)P induced transformation in vitro

Two to four day old secondary cultures of Syrian golden hamster fetuses were the source of all cells. Cells were collected by trypsinization, enumerated by the eosin exclusion test, and adjusted to 800–1600 cells per ml of Eagle's Miminual Essential Media (EMEM) supplemented with 10% Fetal Calf Serum (FCS) and antibiotics. One milliliter of cell suspension was added per 60 mm plastic petri dish which also contained 3 ml of the same media and a feeder layer of 6×10^4 lethally irradiated V-79-4* cells that had been incubated at 37°C and 5% CO_2 for 24 hr. The hamster fetal cells were incubated for 24 hr to allow the cells to attach and then 0·01 or 0·02 ml of B(a)P in dimethylformamide or B(a)P plus inhibitor was added with a 0·1 ml pipette. Benzanthracene (10 μg/ml) or 7,8-benzoflavone (5 μg/ml) was added to the cells in a similar manner. The B(a)P (5 μg/ml) was allowed to remain in contact with the cells for the duration of the experiment. After 8–10 days when macroscopic colonies became observable, the colonies were washed with Hank's balanced salt solution (HBSS), fixed for 5 min in absolute methanol, and stained with dilute Giemsa for twenty min. Plates were washed with distilled water and air dried. For toxicity measurements, every macroscopically visible clone was enumerated. For transformation assays, colonies were located at 10 × magnification and final decision on clonal morphology, using the criteria for transformation described by Berwald and Sachs [11], was determined at 40 × magnification. The transformation frequency is defined to be the ratio of the number of transformed foci to total number of colonies that could be unambiguously characterized.

Metabolism of B(a)P in hamster embryo cultures

Cells derived from hamster fetuses were placed in 60 mm Petri dishes in 4 ml of media containing EMEM, 10% FCS and antibiotics. At zero time, when a Petri dish had approximately $1–2 \times 10^5$ cells, B(a)P and H^3-B(a)P [H^3-B(a)P obtained from Amersham Searle, Rockport, Ill. (S.A. 8 Ci/mmole) final concentration of H^3-B(a)P 12·5 μCi/ml] in dimethylformamide were added to the cells. In those Petri plates that received treatment with either benzanthracene or 7,8-benzoflavone, these chemicals were added either 18 hr prior to or concomitant with the addition of B(a)P and H^3-B(a)P. At the indicated times, the media were removed from the Petri dishes and 2 ml of the media were added to 2 ml of cold acetone. These were mixed for 5 sec on a Vortex mixer. Next, 5 ml of hexane were added and the resulting mixture was shaken for 10 min and subsequently centrifuged to separate the organic-acetone from the aqueous-acetone phases. The organic-acetone phase was disposed of and the cold hexane step was repeated. From the resulting aqueous-acetone phase, 0·5 ml was removed and the amount of metabolites produced was determined in a Packard LS 200 liquid scintillation counter using a Toulene plus BBOT (4 g/l) as a scintillator fluid. The absolute radioactivity was calculated from the aqueous phase, corrected for quenching, and expressed in picograms/viable cell by calculating the per cent of metabolized versus unmetabolized H^3-B(a)P and using this value as the per cent of metabolized unlabelled B(a)P. Aqueous-acetone

*V-79 cells were used as a feeder layer instead of embryonic or other freshly explanted cells, since they do not metabolize B(a)P [10] and therefore do not contribute to the metabolism and subsequent carcinogenic properties of B(a)P.

counts obtainable from 24 hr cultures of Petri dishes containing B(a)P and H^3-B(a)P in the absence of cells represented less than 6% of the counts obtained during a 6-hr incubation in the presence of cells. This finding argues against autooxidation or contamination of the H^3-B(a)P itself playing a significant role in the determined hydrocarbon metabolism.

RESULTS

B(a)P metabolism in mass culture

The overall metabolism on a per cell basis shows a steady increase in B(a)P treated cells during a 48 hr period (Fig. 1). 7,8-benzoflavone

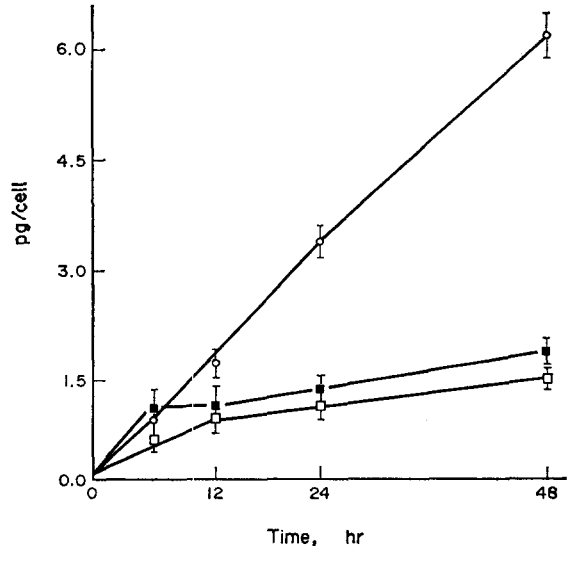


Fig. 1. The effects of 7,8-benzoflavone on the production of aqueous soluble metabolites of benzo(a)pyrene by fetal hamster cells in vitro.

○ *Cells continually exposed to B(a)P added at 0 hr.*

□ *Cells continually exposed to B(a)P and BF both added at 0 hr.*

■ *Cells exposed to B(a)P and BF, in which the B(a)P was added at 0 hr, and the BF 18 hr prior at—18 hr.*

Each experiment was performed in duplicate, and the ranges are given. The experiment was performed three times and the degree of inhibition ranged between 68–80%

either added previously to or concomitantly with administration of B(a)P markedly reduced the overall metabolism of B(a)P. When measured 48 hr post addition of B(a)P, simultaneous or prior administration of equivalent amounts of 7,8-benzoflavone (5 μg/ml) caused a greater than 70% decrease of B(a)P metabolism (Fig. 1). Concomitant administration of benzanthracene (10 μg/ml) showed an inhibitory effect on B(a)P metabolism (Fig. 2) at 48 hr, while at shorter time periods it resulted in metabolism similar to that of the control cells receiving B(a)P alone. Prior administration of benzanthracene elicits an enhanced metabolism of B(a)P at earlier time intervals, while causing a decrease in B(a)P metabolism at 48 hr.

Mediation of B(a)P induced toxicity with benzanthracene or 7,8-benzoflavone

Five μg/ml B(a)P results maximally in about 55% toxicity (Table 1). Administration of either benzanthracene or 7,8-benzoflavone, either 18 hr prior to or concomitant with the administration of B(a)P, is found to decrease the cytotoxic effect of this compound (Table 1). Benzanthracene reduces toxicity by about 25–35%, while 7,8-benzoflavone can reduce this toxicity 65–70%. 7,8-benzoflavone at this

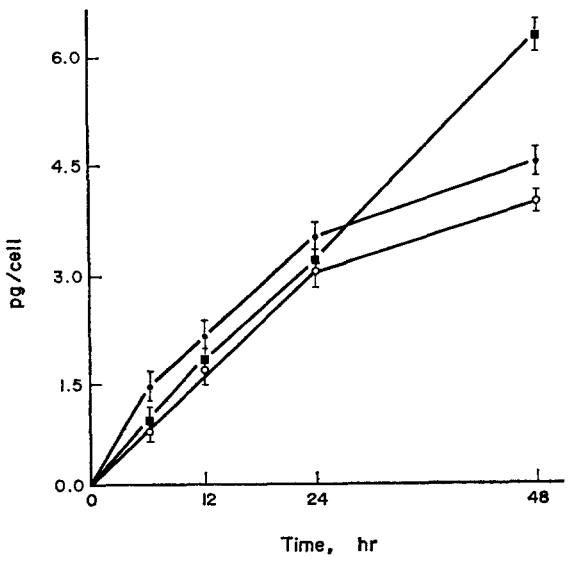


Fig. 2. The effects of benzanthracene on the production of aqueous soluble metabolites of benzo(a)pyrene by fetal hamster cells in vitro.

■ *Cells continually exposed to B(a)P added at 0 hr.*

○ *Cells continually exposed to B(a)P and BA both added at 0 hr.*

● *Cells exposed to B(a)P and BA, in which the B(a)P was added at 0 hr, and the BA 18 hr prior at—18 hr.*

Each experiment was performed in duplicate, and the ranges are given. The experiment was performed three times and the degree of inhibition ranged between 27–41%

concentration itself is somewhat toxic when compared to the dimethylformamide control, and in calculating the effect of 7,8-benzoflavone on B(a)P induced toxicity, comparisons were made with the 7,8-benzoflavone treated control cells.

B(a)P induced transformation in vitro

At concentration of 5 μg B(a)P/ml, we find that approximately 0·16% of the plated cells grow into colonies which show neoplastic

transformation, using the criteria of lack of contact inhibition, random growth, etc. [11]. Addition of either benzanthracene or benzoflavone either prior to or in conjunction with B(a)P results in no significant ($P > 0{\cdot}05$) enhancement in the percentage of morphologically transformed cells (Table 1).

DISCUSSION

Both benzanthracene and 7,8-benzoflavone reduce the toxicity of B(a)P (Table 1). This reduction in toxicity bears a close relationship to the ability of these compounds to decrease the metabolism of B(a)P by the treated cells (Figs. 1, 2). The inhibition of metabolism of B(a)P by benzoflavone is probably due to the fact that it is a potent inhibitor of AHH activity [12], whereas the inhibitory effect of benzanthracene is due to its ability to serve as a competitive substrate with B(a)P [13]. Thus, benzanthracene reduces the toxicity of B(a)P by some 25–30% (Table 1), while reducing the overall metabolism of B(a)P by 35–40% at 48 hr (Fig. 2). 7,8-benzoflavone reduces B(a)P mediated toxicity some 65–75% while causing a 70–80% decrease in overall B(a)P metabolism (Fig. 1). These findings support the view that the toxicity of B(a)P parallels the ability of the treated cells to metabolize it.

The results with respect to the role of overall metabolism in B(a)P induced transformation are less clear. Thus, 5 μg B(a)P/ml in the media results in up to 4% (0·16% of the plated cells) of the colonies that do grow out exhibiting morphological transformation. Concomitant or prior adminstration of either benzanthracene or 7,8-benzoflavone have no significant effects on the transforming ability of B(a)P. Prior administration of benzanthracene, which enhances overall B(a)P induced metabolism for the first 12 hr *in vitro* (Fig. 2), does not effect subsequent B(a)P induced transformation. This observation together with the fact that administration of 7,8-benzoflavone or concomitant administration of benzanthracene, which markedly inhibits B(a)P metabolism within 24 hr, implies that transformation does not directly relate to overall metabolism of B(a)P. These findings are similar to those of Gelboin and Kinoshita using a croton-oil mediated system of skin tumorigenesis [5] and DiPaolo *et al.* using an *in vitro* system similar to ours [9]. DiPaolo *et al.* reached a similar qualitative conclusion, showing that the toxic and carcinogenic metabolites of B(a)P are different entities. Quantitatively however, our results do differ significantly from those of DiPaolo *et al.* [9], who found a marked enhancement in the amount of transformation. Such differences might be due to alterations in methodology or to differences in the ages of the fetuses used, but this probably needs further investigation.

Although the rather striking reduction in B(a)P metabolism in cells treated with 7,8-benzoflavone brought no parallel decrease in carcinogenesis, this does not preclude the theory that metabolic activation is necessary for hydrocarbon induced transformation. Such a theory would seem to be supported by the findings that most of the parent hydrocarbons are relatively unreactive [14], while many of their epoxide derivatives, which have been shown recently to be metabolites of a number of hydrocarbons [15, 16], have proven to be a great deal more active than the parent com-

Table 1. *The effects of benzanthracene (BA) and 7,8-benzoflavone (BF) on toxicity and cell transformation in fetal cells exposed to benzo(a)pyrene [B(a)P]*

Treatment	No. of colonies	Plating efficiency(%)	Toxicity(%)	Transformants per plated Cell (%)
Control	572	8·9(0·48)*	0	0·031
BF (5 μg/ml)	392	6·1(0·18)	32(0)†	0·062
BA (10 μg/ml)	485	8·7(0·29)	3	0·060
B(a)P (5 μg/ml)	808	4·2(0·16)	53	0·155
B(a)P+BA‡	660	5·1(0·19)	43	0·177
B(a)P−BA§	781	5·6(0·21)	38	0·180
B(a)P+BF‡	633	5·2(0·22)	42(16)†	0·20
B(a)P−BF§	602	5·0(0·20)	44(19)†	0·21

*Number in parenthesis; Standard Deviation.
†Toxicity relative to BF treatment alone.
‡BA or BF added simultaneously with B(a)P.
§BA or BF added 18 hr prior to B(a)P.

pounds both as mutagens [17] and carcinogens [18, 19] in cell culture. In contradistinction to our findings, investigators using established cell lines [20] have found previously that inhibition of DBA or MCA metabolism inhibits hydrocarbon-induced carcinogenesis. The direct relationship of these findings to our own is difficult to ascertain since many of these established cell lines show only one tenth the amount of hydrocarbon metabolizing abilities exhibited by low passage hamster embryo cell cultures [21, 22].

In interpreting these results with respect to metabolism of B(a)P and subsequent cellular transformation, one must envisage either (1) differential inhibition of metabolism, thus if the carcinogenic metabolites are precursors to the toxic metabolites one might significantly inhibit the breakdown of the carcinogenic metabolites, or if the carcinogenic and toxic metabolites are derived from metabolism in different regions of the molecule, *i.e.*, K-region epoxide *vs* M-region phenol, one might preferentially inhibit metabolism of one region over another; or (2) effect of the inhibitor itself, or one of its metabolites on the interaction of the cell with the carcinogenic metabolites of B(a)P, *i.e.*, effects of repair synthesis of DNA. In conclusion, it appears that the toxic properties of B(a)P are closely linked to its overall metabolism while its carcinogenic properties and their relationship to overall metabolism in hamster embryo cell cultures are still undetermined.

REFERENCES

1. J. DiPaolo, P. Donovan and R. Nelson, Quantitative studies of *in vitro* transformation by chemical carcinogens. *J. nat. Cancer Inst.* **42,** 867 (1969).
2. R. A. Lubet, D. Q. Brown, R. E. Kouri, The role of 3-OH benzo(a)pyrene in mediating benzo(a)pyrene induced toxicity and transformation in cell culture. *Res. Commun. chem. Pathol. Pharmacol.* **6,** 929 (1973).
3. L. Diamond, R. McFall, R. Miller and H. V. Gelboin, The effects of two isomeric benzoflavones on AHH and the toxicity and carcinogenicity of polycyclic hydrocarbons. *Cancer Res.* **32,** 731 (1972).
4. H. V. Gelboin, E. Huberman and L. Sachs, Enzymatic hydroxylation of B(a)P and its relationship to cytotoxicity. *Proc. nat. Acad. Sci. (Wash.).* **64,** 1188 (1969).
5. N Kinoshita and H V Gelboin, Aryl hydrocarbon hydroxylase and polycyclic hydrocarbon: Effect of the enzyme inhibitor 7,8-benzoflavone on tumorigenesis and molecular binding. *Proc. nat. Acad. Sci. (Wash.).* **69,** 824 (1972).
6. N. Kinoshita and H. V. Gelboin, The role of aryl hydrocarbon hydroxylase in 7,12 DMBA skin tumorigenesis: On the mechanism of 7,8-benzoflavone inhibition of tumorigenesis. *Cancer Res.* **32,** 1329 (1972).
7. C. Huggins, L. Grand and R. Fukinishi, Aromatic azo derivatives preventing mammary cancer and adrenal injury from 7,12 DMBA. *Proc. nat. Acad. Sci. (Wash.).* **53,** 791 (1965).
8. L. S. Wattenberg and J. L. Leong, Inhibition of the carcinogenic action of B(a)P by flavones. *Cancer Res.* **30,** 1922 (1970).
9. J. DiPaolo, P. J. Donovan and R. L. Nelson, Transformation of hamster embryo cells *in vitro* by polycyclic hydrocarbons without cytoxicity. *Proc. nat. Acad. Sci. (Wash.).* **68,** 2958 (1971).
10. R. E. Kouri, R. A. Lubet and D. Q. Brown, Quantitation of aryl hydrocarbon hydroxylase activity in individual fetal hamster cells *in vitro*. *J. nat. Cancer Inst.* **49,** 993 (1972).
11. Y. Berwald and L. Sachs, *In vitro* transformation of normal cells to tumor cells by carcinogenic hydrocarbons. *J. nat. Cancer Inst.* **35,** 641 (1965).
12. L. Diamond and H. V. Gelboin, Alpha napthol flavone: An inhibitor of hydrocarbon cytotoxicity and microsomal hydroxylase. *Science* **166,** 1023 (1969).
13. W. F. Benedict, J. E. Geilen and D. W. Nebert, Polycyclic hydrocarbon produced toxicity, transformation and chromosomal abberations as a function of AHH activity in cell cultures. *Int. J. Cancer* **9,** 435 (1972).
14. M. J. Cookson, P. Sims and P. L. Grover, Mutagenicity of expoxided polycyclic hydrocarbons correlates with carcinogenicity of the parent hydrocarbons. *Nature New Biol.* **234,** 186 (1971).

15. P. L. Grover, A. Hewer and P. Sims, Epoxides as microsomal metabolites of polycyclic hydrocarbons. *Fed. Letters* **18,** 76 (1971).
16. I. Wang, R. W. Rasmussen and R. R. Croker, Isolation and characterization of an active DNA binding metabolite of benzpyrene from hamster liver microsomal incubation systems. *Biochem biophys Res. Commun.* **49,** 1142 (1972).
17. E. Huberman, S. Aspiras, C. Heidelberger, P. Grover and P. Sims, Mutagenicity to mammalian cells of epoxides and other derivatives of polycyclic hydrocarbons. *Proc. nat. Acad. Sci. (Wash.).* **68,** 3195 (1971).
18. P. Grover, E. Huberman, H. Marquardt, T. Kuroki and C. Heidelberger, *In vitro* transformation of rodent cells by K-region epoxides of polycyclic hydrocarbons. *Proc. nat. Acad. Sci. (Wash.).* **68,** 1097 (1971).
19. H. Marquardt, T. Kuroki, E. Huberman, J. Selkirk, C. Heidelberger and P. Sims, Malignant transformation of cells derived from mouse prostate by epoxides and other derivatives of polycyclic hydrocarbons. *Cancer Res.* **32,** 716 (1972).
20. H. Marquardt and C. Heidelberger, Influence of feeder layers and inducers and inhibitors of microsomal mixed function oxidases on hydrocarbon-induced malignant transformation of cells derived from C3H mouse prostate. *Cancer Res.* **32,** 721 (1972).
21. E. Huberman, J. K. Selkirk and C. Heidelberger, Metabolism of polycyclic aromatic hydrocarbons in cell culture. *Cancer Res.* **31,** 2161 (1971).
22. R. E. Kouri, R. Kiefer and E. M. Zimmerman, Hydrocarbon metabolizing ability of various mammalian cell lines in culture. *In Vitro.* **10,** 18 (1974).

Europ. J. Cancer Vol. 11, pp. 145–154. Pergamon Press 1975. Printed in Great Britain

Foci of altered Liver Cells Induced by a Single Dose of Diethylnitrosamine and Partial Hepatectomy: Their Contribution to Hepatocarcinogenesis in the Rat*

E. SCHERER and P. EMMELOT
Department of Biochemistry, Antoni van Leeuwenhoek-Huis, The Netherlands
Cancer Institute, 108 Sarphatistraat, Amsterdam, The Netherlands

Abstract—*Previous findings showed that a single dose of 10–20 mg diethylnitrosamine/kg rat induced foci of atypical liver cells (islands) which might qualify as tumor cell precursors since they closely resembled the preneoplastic lesions resulting from the repeated application of other liver carcinogens. The number of these islands was increased substantially by prior two-thirds hepatectomy, whereas number of cells per island increased autonomously with time.*

Since partial hepatectomy followed 24 hr later by 20 mg diethylnitrosamine/kg ("priming treatment") did not produce liver carcinoma, the contribution of this treatment to the carcinogenic process was investigated in the present study. To this end, the amounts of DENA required to arrive at liver carcinoma following daily administration of relatively non-toxic doses of the carcinogen (2·4 mg/kg) started at various times after the priming treatment, were measured. It was found that the contribution of the initial 20 mg/kg dose of diethylnitrosamine to the carcinogenic process was enhanced significantly by prior partial hepatectomy as well as by the time elapsed between the priming treatment and the subsequent instalment of 2·4 mg diethylnitrosamine/kg·day. The positive correlation between the amount of island tissue induced by 20 mg diethylnitrosamine/kg and the latter's contribution to the carcinogenic process furnishes further support for the view that the island cells induced by single application of diethylnitrosamine represent a causal stage in the hepatocarcinogenic process. More generally the present observations bear on the "memory effect" and the "time effect" in chemical carcinogenesis.

INTRODUCTION

IT HAS been demonstrated previously that a single dose of 10–20 mg diethylnitrosamine (DENA)/kg rat persistently changed foci of liver cells in adenosine triphosphatase, glucose-6-phosphatase, cytoplasmic basophilia, glycogen storage, and morphology [1]. These foci, or islands, are very probably of clonal origin [2]. The number of islands was from 5- to 10-fold increased by two-thirds hepatectomy performed 24 hr prior to DENA application. The number of cells per island increased with time, and 8 months after induction by 10 mg DENA/kg following two-thirds hepatectomy, about 4% of the liver consisted of island tissue [1].

Apart from size, islands resemble the hyperplastic alterations produced by the repeated application of many hepatocarcinogens [1].

Accepted 21 October 1974.

*Partly supported by a grant from the research fund of W.A.R.G. (Scientific Advisory Committee on Smoking and Health); this fund has been established by the Stichting Sigarettenindustrie (Dutch Cigarette Industry Foundation).

The latter lesions are considered to represent or harbor the precursor of the liver cell carcinoma, and to yield, either spontaneously or by further carcinogen application, true liver carcinoma. In this connection it is of interest that all hepatocarcinogens tested by us in single dose induced islands similar to those obtained with DENA. Therefore it was suggested that island cells induced by a single subcarcinogenic dose may represent the first morphologically altered cell stage in liver carcinogenesis and serve as precursor in the development of the tumor cell.

Two-thirds hepatectomy followed by 20 mg DENA/kg rat 20–24 hr later does not produce liver carcinomas in the Sprague–Dawley rats used in these experiments. Under these conditions the carcinogenic process does not progress far enough to yield carcinoma and to arrive at the latter further DENA administration is needed. From the data obtained in our previous study [1] two minimal requirements can be postulated for the islands to satisfy the criterion of representing a causal stage in liver carcinogenesis. First, the single application of 20 mg DENA/kg 24 hr following partial hepatectomy, designated as the priming treatment, should add significantly more to the carcinogenic effect of subsequently administered DENA (started at a fixed time after priming) than does the initial DENA application to intact rats. This postulate is based on the observation that partial hepatectomy profoundly increases the number of islands induced by a single dose of DENA. Secondly, the longer the time elapsed between the induction of islands by the priming treatment and the subsequent daily administration of DENA, the greater the contribution should be of the priming treatment to carcinoma formation. The rationale here is that island growth is kind of autonomous in that the number of cells per island continues to increase for a considerable period of time in the absence of any further treatment. The assumption in both cases is that island cells are tumor cell precursors by serving as target for further carcinogenic action, and therefore an increase in their number will increase the probability of tumor cell formation.

In the present paper evidence is presented that both these requirements are met. In the experiments, 20 mg DENA/kg with or without a preceding two-thirds hepatectomy was used for the initial induction of islands, whereas a dose of 2·4 mg/kg was used for subsequent daily applications to measure the amount of DENA required for completion of the carcinogenic process. The time interval between the initial induction of islands and daily application of DENA was varied from 29 to 312 days. The low daily dose, which was established as relatively non-toxic, was applied in order to minimize destruction of potential tumour cells or tumour cell precursors, and to prevent stimulation of cell proliferation. Death from liver carcinomas served as end point of the measurements.

MATERIAL AND METHODS

Female Sprague–Dawley rats (from either our own colony or the Zentralinstitut für Versuchstierzucht, Hannover, Germany, specific pathogen free) weighing 200–250 g, were fed a standard diet (Hope Farms, Woerden, Holland) and given water *ad libitum*.

Two-thirds hepatectomy, handling of liver specimens, histochemical staining and measurement of island number and size were performed as described previously [1]. (^3H–CH_3)-thymidine (The Radiochemical Centre, Amersham England), of specific activity 5 or 15 Ci/mmole was injected i.p. Autoradiography of cryostate sections (10 μm) fixed in ethanol–ether 1:1 and washed twice with ice-cold 0·1 M perchloric acid, was performed with Emulsion K5 (Ilford). Time of exposure was 70 days for determination of grain counts, and 20 days for that of labelling indices. Nuclear grain counts were measured on 8 areas of 100×100 μm per section, labelling indices on 3 areas of 1000×1000 μm per animal corresponding to about 15,000 nuclei.

For continuous administration, single doses of DENA (amounts indicated in the text) were given per stomach tube 5 or 6 days per week; the doses mentioned in the text being the average dose based on 7 days. All doses were adapted every two weeks during the experiments to the actual body weight of the animals. The daily 2·4 mg DENA/kg dose did not affect body weight. The schemes of DENA application are recorded in Fig. 3.

The number of tumours per liver was determined by microscopic evaluation of 5–10 cm^2 of liver sections containing pieces of each liver lobe per animal. The number of tumours per cm^2 of liver section was multiplied by 1/mean diameter of tumours (cm) and the liver weight to get a measure for the number of tumours per liver. Tumours were separated into well differentiated, weakly differentiated and anaplastic ones.

The significance of a difference between two samples was tested by Student's t test [3].

RESULTS

Establishment of a relatively non-toxic daily dose of DENA

(*a*) Liver cells were labelled by 2 injections of ^{3}H-thymidine (100 μCi each) 24 and 30 hr following two-thirds hepatectomy. Two weeks later, the rats received DENA in daily doses varying from 0·8 to 24 mg DENA/kg for 1–6 weeks. The elimination of radioactivity from the liver as a function of dose and time, based

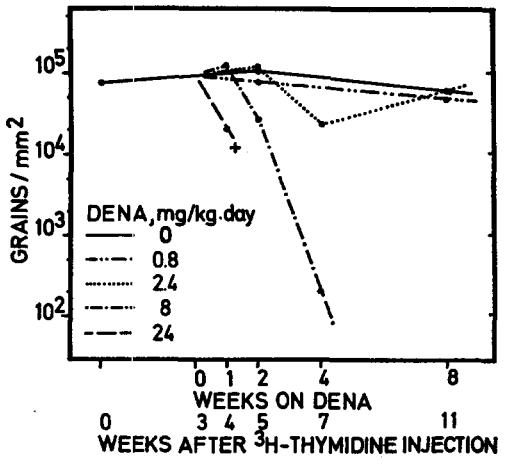


Fig. 1. *Grain count of liver cell nuclei prelabelled by ^{3}H-thymidine as a function of time and dose of continuous DENA administration. Two injections of (^{3}H-CH$_3$)-thymidine (each 100 μCi) were given 24 and 30 hr following two-thirds hepatectomy. After 2 weeks, the rats received from 0·8 to 24 mg DENA/kg/day for various periods of time. One week after terminating carcinogen application, autoradiography of the liver was performed. Each point represents the mean of measurements on 2 livers*

on the nuclear grain count per mm^2 liver prepared for autoradiography, is illustrated in Fig. 1. The highest daily dose of 24 mg DENA/kg caused the death of the rats within 9 days. About 30% of the radioactivity was eliminated daily from the livers of rats fed 8 mg DENA/kg day for 2 weeks. By the same criterion, 4·3 mg DENA/kg day was still somewhat toxic since it produced a daily loss of 5% of the label in another experiment [4]. In contrast, 0·8 and 2·4 mg DENA/kg·day did not (significantly) decrease the grain count.

Table 1. *Labelling with ^{3}H-thymidine following application of various daily doses of DENA for 4 weeks. After carcinogen feeding, rats received 4 injections of (^{3}H-CH$_3$)-thymidine each 200 μCi/kg with 6 hr intervals. Two hours after the last injection, rats were killed and nuclear labelling determined autoradiographically*

	Daily dose of DENA (mg/kg)				
	0·0	1·2	2·4	4·8	9·6
No. of rats	6	4	4	4	4
Labelled nuclei per 1 mm^2 liver section	17	22	28	35	121
± s.d.	8	11	10	11	50

(*b*) Whereas in the former experiment cell destruction was measured mainly, cell proliferation resulting from compensatory growth induced by DENA was also determined. To this end, rats received from 1·2 to 9·6 mg DENA/kg·day for 4 weeks followed by 4 injections of ^{3}H-thymidine (200 μCi/kg each). Autoradiographs were evaluated for labelling indices. As shown in Table 1, 9·6 mg DENA/kg/day produced a highly increased labelling index, whereas 1·2, 2·4 and 4·8 mg DENA/kg·day had only a minor effect, thus substantiating the results obtained in the former experiment.

On this account, 2·4 mg DENA/kg was used as a relatively non-toxic daily dose for the induction of liver carcinoma.

Effect of partial hepatectomy plus 20 mg DENA/kg on liver carcinogenesis by subsequently administered low daily doses of DENA

(*a*) In a preliminary experiment, daily doses of 2·4 mg DENA/kg were given 4 weeks after the priming treatment, which consisted of a single dose of 20 mg DENA/kg administered 24 hr after two-thirds hepatectomy (group A). Serving as controls were two groups of rats which had received either partial hepatectomy (group C) or the initial dose of 20 mg DENA/kg alone (group B) followed by 2·4 mg DENA/kg·day after 4 weeks. Results in this and the following experiments are expressed as the total amount of DENA ingested as continuous daily doses of 2·4 mg/kg leading to the deaths of 50% of the rats from liver carcinoma (D_{50}).

All rats died or were moribund (and killed) from liver carcinoma after having received from 300 to 600 mg DENA/kg (Fig. 2). Group A rats, having been subjected initially to the priming treatment, died first, the LD_{50} amounting to 365 ± 50 mg DENA/kg. The animals of the control groups B and C died from liver carcinoma after a substantially longer period, the D_{50} being 460 ± 50 and 465 ± 50 mg DENA/kg ($P_{A,\,B\,or\,C} < 0{\cdot}001$). When the numerical values of this experiment are compared with those of the following experiments some discrepancy is observed. One apparent reason is the use of rats from different breeding colonies, conventional Amsterdam bred Sprague–Dawley rats served in the preliminary experiment, and S.P.F. rats of the Sprague–Dawley strain bred in Hannover in the following experiments 1 and 2. A close correspondence between data in the latter experiments was

obtained. The design of these experiments is recorded in Fig. 3.

(*b*) In experiment 1, as in the former experiment, the effect of two-thirds hepatectomy on the carcinogenic effect of 20 mg DENA/kg applied 24 hr later was determined. Continuous DENA treatment (2·4 mg/kg) was started at a time at which the livers of the partially hepatectomized rats had been completely restored (73 days after the operation). This condition, combined with the finding that

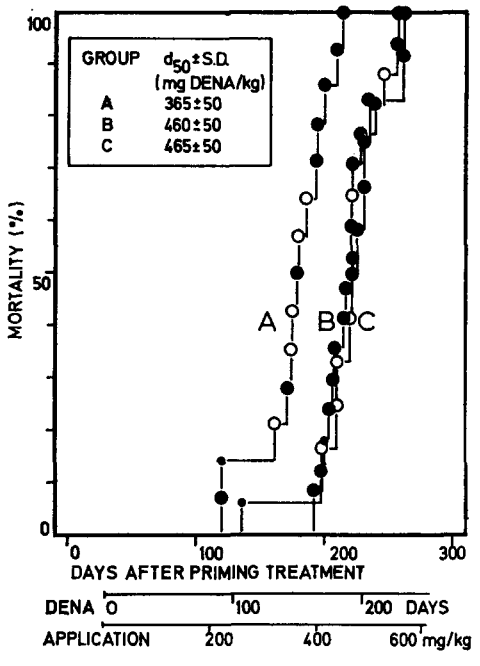


Fig. 2. Effect of the priming treatment on death rates from liver carcinoma following continuous application of DENA. Curve A, rats receiving on day 0 a single dose of 20 mg DENA/kg 24 hr after two-thirds hepatectomy (priming treatment); curve B rats receiving on day 0 a single dose of 20 mg DENA/kg; curve C, rats subjected to two-thirds hepatectomy at day −1. At day 29 administration of 2·4 mg DENA/kg·day to all animals was started until death resulted. ●, died from liver carcinoma; ○, killed moribund from liver carcinoma; •, died or moribund without liver carcinoma.

the relation of liver weight to body weight at the start of continuous DENA feeding, was independent of prior treatment whereas the relation was practically independent of the age of the rats (liver weight amounting to 3·3% of the body weight at 2 months and to 3·0% at 10 months after starting the experiments), allowed the carcinogen to be administered and the data to be expressed on the basis of the individual body weight of the rats. Thus, daily doses of 2·4 mg DENA/kg were installed 72 days after the initial treatment of partially hepatectomized or intact rats with 20 mg DENA/kg (groups A and B, respectively, in Fig. 3, exp. 1). Groups of rats which had not been pretreated (group C) or subjected to partial hepatectomy only (group D) served as controls. All rats died or were moribund from liver tumours (see below) after having received

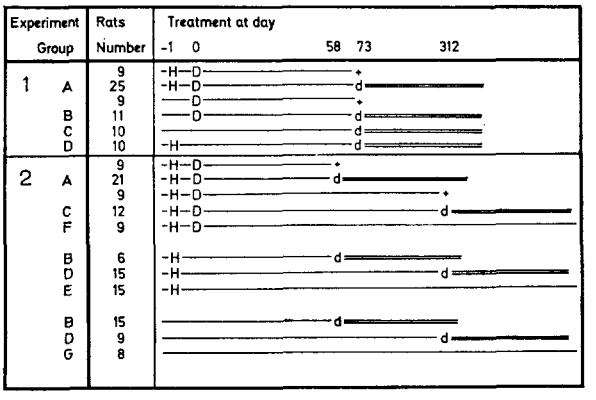


Fig. 3. Schemes of DENA application. Experiment 1, A: The priming was applied 73 days prior to continuous application of 2·4 mg DENA/kg·day; B, C and D: controls. The death rates are illustrated in Fig. 4. Experiment 2, A and C: The time allowed to proceed between the priming treatment and the instalment of 2·4 mg DENA/kg·day was varied; B, D–G: controls. The death rates are illustrated in Fig. 5. Abbreviations: H, two-thirds hepatectomy; D, single dose of 20 mg DENA/kg (H+D=priming treatment); d, daily application of 2·4 mg DENA/kg until death; +, killed for microscopic evaluation of number and size of adenosine triphosphatase deficient islands in the liver

from 350 to 600 mg DENA/kg (Fig. 4). The D_{50} of groups A, B, C and D amounted to, respectively, 440±40, 515±50, 525±50 and 518±50 mg DENA/kg. The results show that after the priming treatment (group A), a

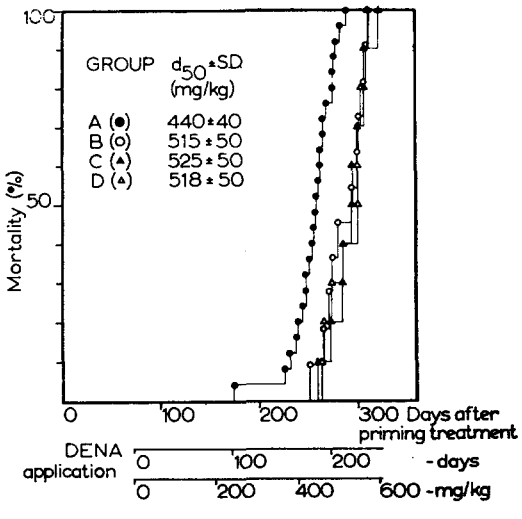


Fig. 4. Effect of priming treatment on death rates from liver carcinoma following continuous application of DENA. Curve A, rats receiving on day 0 a single dose of 20 mg DENA/kg 24 hr after two-thirds hepatectomy; curve B, rats receiving on day 0 a single dose of 20 mg DENA/kg; curve C, untreated rats; curve D, rats subjected on day −1 to two-thirds hepatectomy. In all cases 2·4 mg DENA/kg·day was installed at day 73 until death followed (see Fig. 3, exp. 1).

significantly smaller amount of DENA ($P_{A, B} < 0{\cdot}001$) was needed to complete carcinogenesis than in the case of other three groups, thus substantiating the result of the preliminary experiment. This means that the contribution of the initial single dose of 20 mg DENA/kg to the carcinogenic process is enhanced by the partial hepatectomy performed 24 hr previously. Since the number of islands induced by 20 mg DENA/kg is also enhanced by partial hepatectomy (Table 2 illustrating the number of islands per cm^3 liver at the moment of starting daily administration of the low DENA doses), the results support the view that the islands stand in a causal relation to the carcinogenic process. Taking into account a difference of 85 mg DENA/kg between groups A and B to reach lethal liver carcinoma and a recorded number of, respectively, 1820 and 205 islands/cm^3 liver, it follows that an effect of the initial dose of 20 mg DENA/kg without prior partial hepatectomy (group B) as compared with groups C and D (respectively, < 5 and 10 islands/cm^3) could not have scored in this system. Parenthetically, it does not seem very likely that the priming treatment would create altered systemic, *e.g.*, immunological conditions, promotory to liver cancer development, as compared with the single application of DENA alone.

Effect of time elapsed between the priming treatment and the subsequent instalment of daily low doses of DENA on liver carcinogenesis

At 58 and 312 days after the priming treatment, daily administration of 2·4 mg DENA/kg was started until death of the animals from liver carcinoma followed (Fig. 3, exp. 2—groups A and C, respectively). Serving as controls were untreated rats and rats which had been subjected to two-thirds hepatectomy only, receiving 2·4 mg DENA/kg·day at the corresponding time scale. Groups B and D each consisted of these two types of rats and since the two conditions yielded similar results —as in exp. 1—curves B and D in Fig. 5 were constructed from the cumulated data of both types of rats in each group.

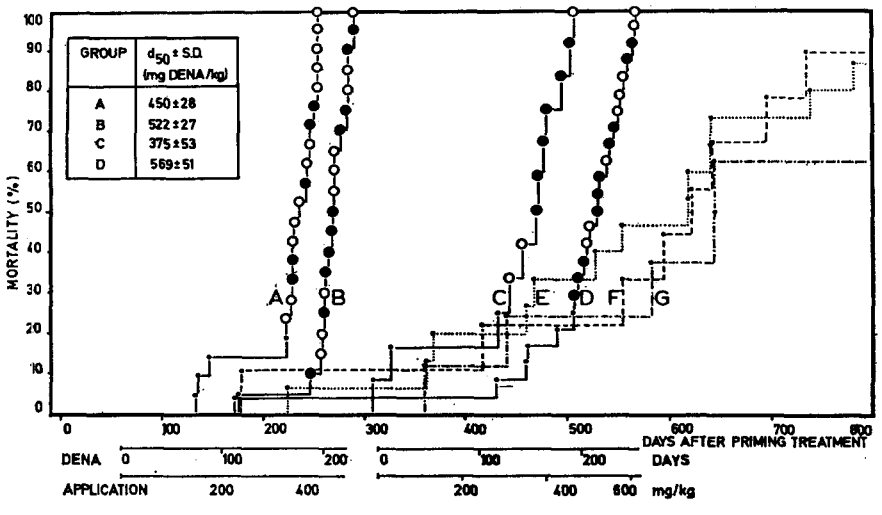


Fig. 5. Effect of time interval between the priming treatment and the instalment of 2·4 mg DENA/kg·day on death rates. Curves A and C, rats subjected at day 0 to the priming treatment (a single dose of 20 mg DENA/kg 24 hr after two-thirds hepatectomy). Curves B and D, cumulated results from rats without pretreatment and rats subjected at day −1 to two-thirds hepatectomy. Two-point-four milligrammes DENA/kg·day was administered from day 58 (curves A and B), and from day 312 (curves C and D). Curves E, F and G are further controls (see Fig. 3, exp. 2). ●, died from liver carcinoma; ○, killed moribund from liver carcinoma; •, died or moribund without liver carcinoma

Rats which had received the priming treatment before instalment of continuous DENA application died earlier than did the controls (Fig. 5). The priming treatment *per se* did not yield liver carcinoma (group F); these rats died at about the same time as did untreated intact and partially hepatectomized rats (groups E and G, respectively). Death in groups E, F and G resulted mostly from "spontaneous" mammary tumours, which is a common cause of death in the female Sprague–Dawley rat.

Ingestion of 2·4 mg DENA/kg·day started 58 days after the priming treatment (group A) yielded a D_{50} of 450±28 mg DENA/kg, the corresponding value for the control group B amounted to 522±27 mg DENA/kg ($P_{A,B} < 0{\cdot}001$).

A similar treatment started 312 days after the priming treatment (group C) led to a D_{50} of 375±53 mg DENA/kg, whereas the D_{50} for the corresponding control group D amounted to 569±51 mg DENA/kg ($P_{C,D} < 0{\cdot}001$).

The results of exp. 2 show that the longer the time allowed between the priming treatment and the subsequent feeding of the low daily doses of DENA, the greater the contribution of the priming treatment to the process of lethal liver carcinogenesis ($P_{A,C} < 0{\cdot}001$). Since the amount of island tissue increases with time after induction (Table 2, the volumetric fraction occupied by island tissue amounting to 0·2% at 58 days, and to 6·2% at 312 days after the priming treatment) the increased contribution of the priming treatment with time to carcinogenesis is in agreement with the role of island cells as tumour cell precursors.

Liver carcinoma at the time of death

Since for the interpretation of the results it is essential that the development of liver carcinomas at the time of death was identical in the various primed and non-primed groups receiving DENA continuously, the livers of all rats were studied microscopically. Control animals (exp. 2, groups E, F and G—Fig. 3) not receiving continuous DENA application died or were moribund from mammary carcinoma mainly. Untreated rats (group G) showed no liver alterations, whereas in partially hepatectomized (group E) some bile cysts were observed. The livers of the rats subjected to partial hepatectomy and 20 mg DENA/kg not followed by continuous DENA (group F) contained many large ATPase-deficient islands. Liver tumours were not observed in any of these control groups.

All rats of the groups receiving continuous application of DENA in the various experiments had many liver tumours at the time of death. These tumours, which resembled closely those described adequately elsewhere [5–7], were classified histologically as hepatocellular carcinomas of differing degrees of differentiation and anaplastic neoplasms. Common for these tumours was their lack of ATPase and often glucose-6-phosphatase staining. Cytoplasmic basophilia and glycogen content of the well differentiated hepatomas varied strongly between different tumours, in weakly differentiated carcinomas, however, the cytoplasm was strongly basophilic and generally contained no glycogen. The growth pattern was trabecular, sometimes with regions of adenomatous appearance. Anaplastic neoplasms varied in appearance from tumours containing a

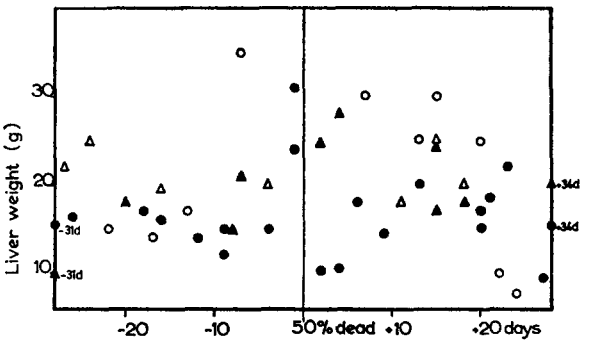


Fig. 6. Relation between the liver weight at death and the time of death within the experimental groups of experiment 1. Symbols and experimental design see Figs. 2 and 4.

hemorrhagic core (1–5 mm dia) in which clusters of anaplastic tumour cells were embedded, to large blood spaces lined by only a thin rim of tumour cells. The cells varied in size and showed moderate to strong basophilia (metachromatic shift toward reddish where stained by cresyl violet, pH 4, cryostat section) evenly distributed over the cytoplasm. Sarcomatous stroma was occasionally present between cords of anaplastic tumour cells.

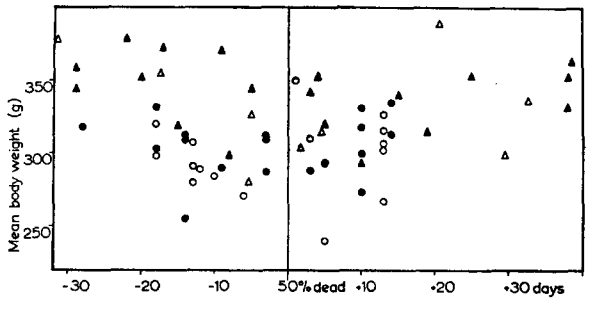


Fig. 7. Relation between the mean individual body weight during the continuous application of 2·4 mg DENA/kg·day and the time of death within the experimental groups of experiment 2. Experimental design see Fig. 2. ○, group A; ●, group B; △, group C; ▲, group D.

Death had been due mainly to bleeding from liver ruptures resulting in abdominal hemorrhage which was observed also in the majority of the rats killed moribund (Table 3). In all groups, the lethal bleedings originated from anaplastic neoplasms and poorly differentiated carcinomas. Lung metastases which were

Table 2. Number and size distribution of adenosine triphosphatase deficient islands in experiments 1 and 2, 73 respectively 58 and 312 days after a single dose of 20 mg DENA/kg and/or partial hepatectomy

Exp.	Initial treatment	Time after DENA application days	Islands per cm³ liver (range: ± s.d.)	Distribution (% of total) over size groups of island diameter (μm)										Volumetric fraction occupied by island tissue (%)
				80	115	160	225	320	450	640	900	1280	1800	
1	2/3 hep. DENA	73	1820 (210–2730)	23	47	26	4	0·3	0·3	0	0	0	0	0·2
	DENA	73	205 (128–325)	18	51	29	2	0·3	0·1	0·1	0	0	0	0·02
2	2/3 hep. DENA	58	1969 (1271–3048)	14	56	28	1·5	0·2	0·1	0·1	0	0	0	0·2
	2/3 hep. DENA	312	2524 (2128–2992)		8	33	28	18	9	2·5	1	0·2	0·1	6·2

Table 3. Summary of macroscopic and quantitative microscopic evaluation (exp. 2)

Exp. group	Initial treatment	Start of daily doses of DENA (days after initial treatment)	Liver weight at death (g)	Fraction of animals with abdominal hemorrhage	Fraction of animals with lung metastases	Average number and size of liver neoplasms: Well differentiated Number per liver	Well differentiated Mean diameter (cm)	Weakly to undifferentiated Number per liver	Weakly to undifferentiated Mean diameter (cm)	Anaplastic Number per liver	Anaplastic Mean diameter (cm)
2 A	2/3 hep, DENA	58	16 (7–35)	15/18	6/17	14 (3–18)	0·4 (0·1–1)	7 (1–14)	0·3 (0·1–0·5)	6 (1–13)	1 (0·1–2)
2 B	2/3 hep	58	16 (10–40)	11/15	6/15	21 (10–48)	0·5 (0·1–1)	8 (3–19)	0·5 (0·1–0·8)	5 (2–15)	0·7 (0·2–1·6)
2 C	2/3 hep, DENA	312	17 (14–25)	7/10	3/7	8 (2–15)	0·6 (0·1–1·7)	4 (2–10)	0·4 (0·2–0·6)	3 (2–10)	1·1 (0·5–2)
2 D	2/3 hep	312	14·5 (8–26)	16/20	4/17	9 (1–16)	0·5 (0·2–0·9)	3 (1–8)	0·6 (0·1–1)	4 (1–10)	0·9 (0·5–1·3)

mainly of the anaplastic type were observed in about 35% of rats (Table 3). The liver weight (blood clumps removed) at death differed largely between individual animals, but no difference was observed between the various experimental groups. (Table 3; for individual rats of exp. 1, Fig. 6). Time of death in each group was not related to either body weight during the continuous carcinogen treatment (Fig. 7) or liver weight at the time of death (Fig. 6). Parenthetically, these findings confirm that application of DENA, and expression of the data on a body weight basis are correct procedures.

The question of the cellular origin of the liver tumours in the rat has been discussed by Steward and Shell [5] and by Butler [7]. A hepatocellular origin was concluded for the carcinomas of trabecular or adenomatous structure [5], and was indicated for the anaplastic, hemorrhagic neoplasms [7]. We have examined closely 65 of such tumours and obtained the following results which were compatible with the view that these anaplastic neoplasms are of hepatocellular rather than endothelial origin.

(*a*) In 10 of these anaplastic tumours a gradual change in cytological properties of the cells from poorly differentiated hepatocellular to anaplastic could be observed.

(*b*) Only a small amount of reticulum was present between tumour cells as compared with tumours of endothelial origin [8] (as demonstrated by Gömöry silver reticulin stain [9]).

(*c*) In 8 cases the hemorrhagic tumours were clearly associated with well differentiated hepatocellular carcinomas, suggesting that the latter tumours served as precursor of the anaplastic tumour cells.

(*d*) The cells of two hemorrhagic and typically anaplastic tumours with metastases in the lung contained glycogen as demonstrated by PAS staining and diastase.

The incidence of liver tumours at the time of death was quantitated microscopically in the three categories, well differentiated, weakly to undifferentiated and anaplastic tumours. Results are presented for exp. 2, groups A–D in Table 3; results for exp. 1 were similar to those of groups A and B of exp. 2. The average number and size of the tumours per liver and per tumour type were similar for groups 2A and 2B on the one hand, and for groups 2C and 2D on the other. Although the rats of groups 2C and 2D tended to have less tumours at the time of death than did those of groups 2A and 2B (size of tumours being, however, similar for all 4 groups), the relative incidence of the three tumour types were the same for all groups. Adjustment of the parameters (incidence and size of the various tumours) measured for individual rats in four cohorts (of 15 days) according to their time of death within the groups did not reveal any particular correlation other than did the average illustrated in Table 3. The finding that the rats of groups 2C and 2D, in which continuous DENA treatment had been started at day 312, died from a smaller burden of liver tumours than did the younger rats of groups 2A and 2B, may suggest that the older rats were somewhat more sensitive to liver tumours in terms of a lethal reaction. Note also that the older control rats of group 2D did not require less, but rather more carcinogen than did the younger control rats of group 2B to die from a lesser burden of liver tumours. Hence, carcinogenic action did not increase with age *per se*. Accordingly, in all groups, the relative incidence and absolute size, and in comparable age groups the absolute incidence of all three tumour types were the same at the time of death. In view of the similar development of the various tumour processes at the time of death, the latter parameter appears to be a correct endpoint for establishing relative carcinogenic effects.

DISCUSSION

Arguments for a precursor–product relationship between cells involved in tumour development are commonly based on the criteria of time, place and cell characteristics, primarily morphological. The preneoplastic cell population precedes frank tumour cells in appearance. The tumour cells arise amidst or close to the preneoplastic population. The preneoplastic cells behave atypically, *e.g.*, are active mitotically, and may resemble morphologically or biochemically the tumour cells (see Farber [10], for references on liver). However, in our opinion a causal relation between cell types in chemical carcinogenesis has never been demonstrated satisfactorily. For instance, the experimentally induced preneoplastic liver lesions studied until now either sooner or later automatically give rise to tumours due to the relatively large doses of carcinogen used for their induction, or, if not, their contribution to liver carcinogenesis has not been established beyond doubt. A case in point are the hyperplastic nodules induced in rat liver by 2-acetylaminofluorene [11, 12]. Here tumour cells are apparently initiated already and the development of the nodules could parallel the

process of liver carcinogenesis and not be directly related to that process [13]. Thus, what is actually needed for establishing a causal link between precursor and tumour is a preneoplastic cell type that does not automatically lead to tumour cells and then to show that this cell type behaves as precursor of the tumour cell.

In the morphologically and histochemically altered foci of liver cells (islands) induced by a single dose of DENA, a cell stage is present that (*a*) might qualify as tumour cell precursor in the latter sense, and (*b*) may be studied quantitatively in relation to tumour formation. It is possible to modify experimentally not only the number of islands induced by a given dose of DENA, *e.g.*, by prior two-thirds hepatectomy (without changing cell numbers per island [1]), but also the number of cells per island since these cells continue to proliferate for considerable periods of time after induction in the absence of any further treatment ([1] and Table 2). These two conditions have enabled us in the present experiments to demonstrate that island cell tissue, in proportion to its amount, potentiates the carcinogenic action of subsequent low doses of DENA, and therefore may be substrate for further carcinogenic action.

In these experiments, death from liver carcinoma served as an "end point" for measuring the rate of liver carcinoma formation. From the qualitative and quantitative microscopic survey of the tumour-bearing livers, it was concluded that death was a reliable parameter of liver carcinoma formation, even though the parenchymal origin of the most lethal liver tumours, although very likely, could not be established definitely. The problem here is that in order to have causal significance, the precursor–product relation should be one between the precancerous island cells derived from parenchymal cells and liver tumours of parenchymal origin. However, since the development of the more and less differentiated types of liver carcinomas (and the anaplastic lethal tumours) was similar in the various experimental groups at the time of death of the rats, such a relationship has been established for at least the former tumour types.

It should be pointed out that the present method of administering the daily low doses of DENA until death or the moribund stage was not ideal since it provided relative data only, whereas the minimal amounts of carcinogen actually required for liver carcinoma formation were not established. Accordingly, far too much carcinogen may have been applied in the present experiments, and the relative differences between groups may therefore be larger than those derived here. Also, higher total doses of carcinogen could favour the formation of the malignant anaplastic tumours. We have been investigating very recently these points by reducing the administration of the daily doses of DENA to primed and non-primed rats to 4 weeks and sacrificing the animals after another 8 weeks. Enough data already have been collected to state that the aforementioned expectations are fully borne out even to the point that with low daily doses of DENA (2·8–5·0 mg/kg), all or none effects are obtained, *i.e.*, hepatomas in primed rats, and only islands in non-primed rats, whereas with daily doses up to 10 mg/kg, a manifold increase in liver tumours is obtained in primed as compared with non-primed rats.

It is concluded that the ATPase-deficient island cells induced by a single application of a subcarcinogenic dose of DENA serve as target for further carcinogenic action and thus may be considered as precursor cells in the process of liver tumour formation. The results further suggest that these island cells represent the first morphologically identifiable stage in the hepatocarcinogenic process. The "memory effect" of a subcarcinogenic dose appears to be based on the induction and persistance of a heritably altered cell type of precursor character, the island cell. A positive effect of time ("time factor") in liver carcinogensis had been noted first by Druckrey [14]; it is now interpreted in terms of the proliferation of the island cells with time. Proliferation of tumour cell precursors could have general significance for the process of chemical carcinogenesis, especially in man.

Acknowledgements—We wish to thank Miss B. Mülder and Mrs. M. van der Steen-Kamerbeek for the competent and careful technical assistance and Drs. W. H. Butler (MRC Toxicology Unit, Carshalton, Surrey, England), C. H. Hollander, C. Zurcher (Institute for Experimental Gerontology, Rijswijk, Holland) and W. Misdorp (The Netherlands Cancer Institute) for their comments on the morphology of the tumours.

REFERENCES

1. E. Scherer, M. Hoffmann, P. Emmelot and H. Friedrich-Freksa, Quantitative study on foci of altered liver cells induced in the rat by a single dose of diethylnitrosamine and partial hepatectomy. *J. nat. Cancer Inst.* **49,** 93 (1972).
2. E. Scherer and M. Hoffmann, Probable clonal genesis of cellular islands induced in rat liver by diethylnitrosamine. *Europ. J. Cancer* **7,** 369 (1971).
3. G. M. Clarke, Statistics and experimental design, In *Contemporary Biology.* E. Arnold, London (1969).
4. E. Scherer and H. Friedrich-Freksa, Zur Zentralvene gerichtete Wanderung von Leberzellen der Ratte nach partieller Hepatektomie und nach Verabfolgung von Diäthylnitrosamin. *Z. Naturforsch.* (B) **25,** 637 (1970).
5. H. L. Stewart and K. C. Snell, The histopathology of experimental tumors of the liver of the rat. *Acta Un. int. Cancer.* **13,** 770 (1957).
6. M. D. Reuber, Histopathology of transplantable hepatic carcinomas induced by chemical carcinogens in rats. *Gann Monograph* **1,** 43 (1966).
7. W. H. Butler, Pathology of liver cancer in experimental animals. *IARC scientific Publ.* **1,** 30 (1971).
8. A. P. Stout and R. Lattes, Tumors of the soft tissues, In *Atlas of Tumor Pathology,* 2nd series, fasc. 1. Armed Forces Inst. Pathology, Washington (1967).
9. G. Gömöri, Silver impregnation of reticulum in paraffin sections. *Amer. J. Path.* **13,** 993 (1937).
10. E. Farber, Hyperplastic liver nodules. *Meth. Cancer. Res.* **7,** 345 (1973).
11. M. D. Reuber, Development of preneoplastic and neoplastic lesions of the liver in male rats given 0·025 per cent N-2-fluorenyldiacetamide. *J. nat. Cancer Inst.* **34,** 697 (1965).
12. S. Epstein, N. Ito, L. Merkow and E. Farber, Cellular analysis of liver carcinogenesis: The induction of large hyperplastic nodules in the liver with 2-fluorenylacetamide or ethionine and some aspects of their morphology and glycogen metabolism. *Cancer Res.* **27,** 1702 (1967).
13. E. Farber, Chemical carcinogenesis, In *Current Research in Oncology* (Edited by C. B. Anfinsen, H. Potter and A. N. Schechter) p. 95. Academic Press, New York (1973).
14. H. Druckrey, Quantitative aspects in chemical carcinogenesis, In Potential Carcinogenic Hazards from Drugs *UICC Monograph Series,* Vol. 7 (Edited by Réne Truhaut) Springer-Verlag, Berlin (1967).

Europ. J. Cancer Vol. 11 pp 155–158. Pergamon Press 1975. Printed in Great Britain

Specific G2 Block in HeLa-S3 Cells by Neocarzinostatin*

T. EBINA, K. OHTSUKI, M. SETO and N. ISHIDA

Department of Bacteriology, Tohoku University School of Medicine, 2–1 Seiryo-machi, Sendai 980, *Japan*

Abstract—*The effects of an antibiotic, neocarzinostatin (NCS), on cell division and DNA synthesis in synchronized and random culture of HeLa-S3 cells were examined by using different methods of localizing the site of NCS-induced block of cell progress in their life cycles. It was found that the first site of action of NCS was the inhibition of DNA synthesis at early stage of S phase and the second site was the block at G2 phase, almost one hr ahead of the block point of vinblastine sulfate. The block at G2 phase by NCS was not dependent upon the inhibition of DNA synthesis by the antibiotic.*

The possibility that NCS affects microtubular proteins, resulting in both early S block and G2 block in HeLa-S3 cells is discussed.

INTRODUCTION

NEOCARZINOSTATIN (NCS), an antitumor antibiotic obtained from the culture filtrate of *Streptomyces carzinostatics*, is an acidic single-chain protein with a molecular weight of 10,700, cross-linked by two disulfide bridges [1, 2]. A marked antitumor activity of NCS is known in various mouse and rat ascites tumors [3, 4]. Recently, a clinical application of NCS has been shown to be effective against an acute myelogenous leukemia [5].

In a previous paper [6], it has been reported that NCS causes dual action in synchronized culture of HeLa-S3 cells; the inhibition of DNA synthesis and the prevention of cell division. The purpose of this paper is to determine in detail the sensitive site of action of NCS, which induces the immediate inhibition of DNA synthesis and that of cell mitosis induced by NCS.

Accepted 26 September 1974.

*This work was supported in part by the grant from the Ministry of Education of Japan.

MATERIAL AND METHODS

Tissue culture cells

A clonal line of HeLa-S3 cells was grown in a medium consisting of 0·1% yeast extract and 0·5% lactalbumin hydrolysate in Earle's solution with 0·45% glucose (YLE) supplemented with 10% bovine serum.

Neocarzinostatin

The antibiotic was a gift from the Kayaku Antibiotics Research Laboratory (Tokyo, Japan).

Synchronous culture

To obtain synchronized cells on S phase, an amethopterin method was used [7]. HeLa-S3 cells which had been cultured at a concentration of 3×10^5 cells per ml for 48 hr at 37°C were incubated with medium containing 10^{-6}M amethopterin and 5×10^{-5}M adenosine for 16 hr. The cultures were washed rapidly with prewarmed Hanks' solution 4 times and received fresh medium containing 4×10^{-6}M unlabeled thymidine. The time when unlabeled

thymidine was added to the culture was taken as time 0.

Mitotic index determination

Monolayer coverslip cultures of HeLa-S3 cells treated with NCS were stained with May–Grünwald–Giemsa solution. The number of mitotic figures per 1000 cells was counted for mitotic index.

Measurement of DNA synthesis

For the examination of the effect of NCS on the synthesis of DNA, 0·5 μCi per ml of ^{3}H-thymidine (14·0 mCi/mM) was added to the cultures for a desired period of time. After incubation, the incorporation of ^{3}H-TdR was stopped by the addition of 10% cold perchloric acid (PCA), followed by successive treatments with 5% PCA and 1% PCA. PCA-insoluble

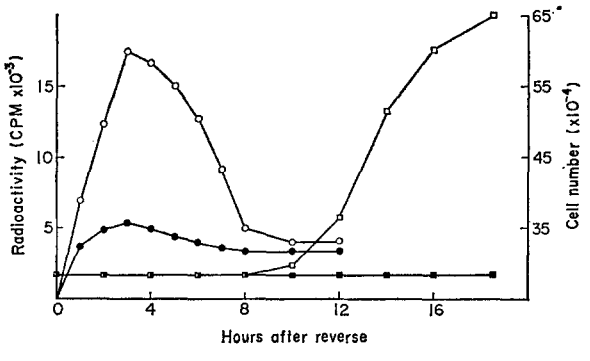


Fig. 1. Effects of NCS on DNA synthesis and cell division in synchronized HeLa-S3 cell. At various times after removal of anethopterin, cells were labeled with ^{3}H-thymidine for 30 min. Five micrograms per ml of NCS was added to cultures at 0 time. ○——○, DNA synthesis, control cells; ●——●, DNA synthesis, NCS treated cells; □——□, cell number, control cells; ■——■, cell number, NCS treated cells.

fraction was dissolved in 2 ml of 1N NH_4OH and 0·5 ml of aliquotes were dried on planchettes. The radioactivity was determined by Aloka Gas Flow counter.

RESULTS

Effects of NCS on DNA synthesis and cell division in synchronized HeLa-S3 cells

In order to confirm the previous result that NCS inhibits DNA synthesis at early S phase in double thymidine method [6], similar experiments were conducted in the system of amethopterin method. As shown in Fig. 1, the incorporation of ^{3}H-TdR into acid-insoluble fraction occurred immediately after the removal of amethopterin and the rate of DNA synthesis reached a maximum at 3 hr after the removal of amethopterin, while the increase of cell number was first observed at 10 hr. Preliminary autoradiographic study showed that about 75% of cells incorporated ^{3}H-TdR immediately after the removal of amethopterin, that is, 75% of cells treated with amethopterin were synchronized at S phase.

When 5 μg per ml of NCS was added to the synchronized cultures at 0 time, the incorporation of ^{3}H-TdR into acid-insoluble fraction was reduced by 70% compared with that of control cells and the division of cells was completely prevented (Fig. 1).

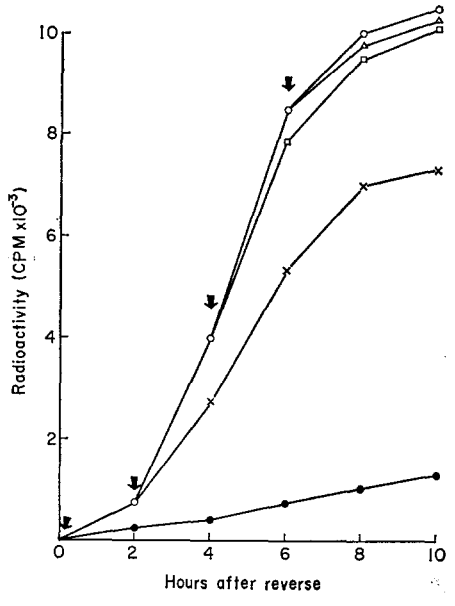


Fig. 2. Effect of time of addition of NCS on the synthesis of DNA in synchronized HeLa-S3 culture. To the synchronized culture was added 5 μg per ml of NCS at the time indicated by arrows. (●——●, 0 hr; ×——×, 2 hr; □——□, 4 hr; △——△, 6 hr; ○——○, control.) ^{3}H-thymidine was added at 0 time and cells were labeled continuously. At appropriate intervals after the addition of NCS, cells were taken out and the radioactivity in acid-insoluble fraction was measured.

As shown in Fig. 2, when 5μg per ml of NCS was added at 2 hr after the removal of amethopterin, the inhibition of ^{3}H-TdR incorporation was still observable. However, the addition of NCS at 4 and 6 hr was no more effective. These two results indicate that NCS inhibits the DNA synthesis at early S phase followed by the inhibition of cell division when NCS was left in the culture all the way through.

Effect of time of addition of NCS on cell division

To examine whether the inhibition of cell division induced by NCS is a consequence of the inhibition of DNA synthesis, the synchronized cells were exposed to NCS (5 μg/ml) at

different experimental times after the removal of amethopterin. The result shown in Fig. 3 indicates clearly that NCS inhibited cell division even at 9 hr after the amethopterin removal, when DNA synthesis in cells is completed as already shown in Fig. 1. This result suggests that the inhibition of cell division by NCS is not the consequence of the inhibition of DNA synthesis.

Location of NCS-induced G2 block

The result described above suggested that the location of NCS-induced block of cell progress in their life cycle is at G2 phase. In order to know the precise point of action of NCS at G2 phase, the following experiment

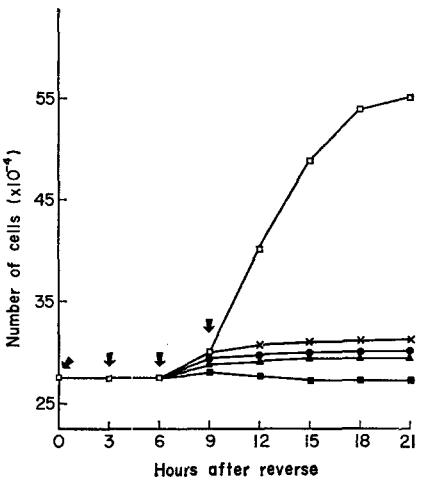


Fig. 3. Effect of time of addition of NCS on cell division in synchronized culture. Five micrograms per ml of NCS was added to cultures at the time indicated by arrows. ■——■, *0 hr;* ▲——▲, *3 hr;* ●——●, *6 hr;* ×——×, *9 hr;* □——□, *control.*

was conducted. When random cultures of HeLa-S3 cells were treated with 0·5 μg per ml of vinblastine sulfate, which is known as a metaphase arrest agent like colchicine, mitotic cells were accumulated linearly as shown in Fig. 4. When 5 μg per ml of NCS was added to the cultures one hr after the exposure of vinblastine sulfate, the accumulation of mitotic cells was prevented within one hr after the addition of NCS. This result suggested that the block point of NCS at G2 phase was almost one hr ahead of that of vinblastine sulfate.

Cytological study on NCS treated cells also confirmed that the antibiotic prevented cells at G2 phase from entering prophase in four stages of M phase (prophase, metaphase, anaphase and telophase).

DISCUSSION

It was revealed that the second site of action of NCS was at G2 phase, *i.e.*, one hr ahead of M phase (Fig. 4), resulting in the prevention of cells at G2 phase from entering prophase. This prevention at G2 phase by NCS was not dependent upon the inhibition of DNA synthesis by the antibiotic (Fig. 3).

Like NCS, actinomycin D and *p*-fluorophenylalanine also have a specific site of action at G2 phase [7], indicating that some specific RNA and protein are involved in the step for entering M phase. However, NCS is distinguished from these inhibitors, as the synthesis

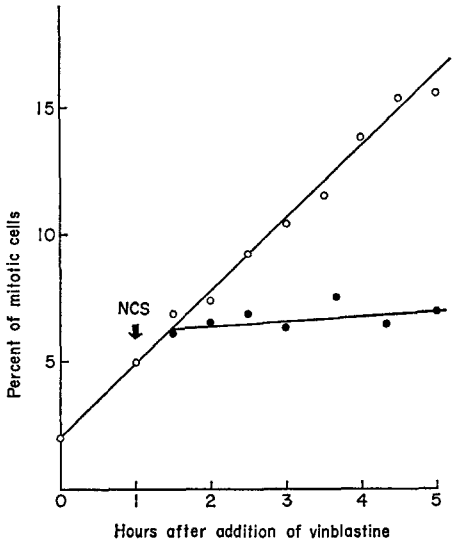


Fig. 4. Effect of NCS on accumulation of mitotic cells. To the random cultures of HeLa-S3 cells was added 5 μg per ml of NCS one hour after the treatment of 0·5 μg per ml of vinblastine sulfate. At the time indicated, the cultures were taken out and stained with May-Grünwald-Giemsa solution and the mitotic frequency was measured. ○——○, *control cells;* ●——●, *NCS treated cells.*

of RNA and protein are not inhibited by NCS at the concentration which completely blocks cell mitosis and DNA synthesis [6].

The mode of action of NCS is also very similar to those of phleomycin [8] and X-ray irradiation [9, 10], which inhibit DNA synthesis and block G2 phase cells. Phleomycin has been shown to inhibit the activity of DNA polymerase by direct interaction with primer DNA *in vitro* [11] and X-ray seems to effect directly DNA molecule, whereas NCS did not interact directly with DNA molecules in cells [12]. Accordingly, there must be another mechanism to be explained for the inhibition of DNA synthesis at early S and G2 block in synchronous culture by NCS.

Edelman *et al.* have proposed that the surface

receptors (glycoproteins) on the cell membrane interact with colchicine binding protein (microtubules) after the observation of the effect of colchicine and related drugs on the inhibition of receptor mobility by concanavalin A in lymphocytes [13]. Moreover, an electron microscopic observation showed that microtubules or microfilaments lie just beneath the plasma membrane [14], a fact which suggests that microtubular proteins are directly associated with membrane receptor glycoproteins.

From preliminary experiments using ^{3}H-labeled NCS, it was found that NCS adsorbed predominantly to the cell membrane fraction compared with cytoplasmic fraction and that the complete adsorption occurred within 30 min after the addition of ^{3}H-NCS, followed by immediate inhibitions of DNA synthesis and cell division. On the other hand, it has been reported that the replication of centrioles, which plays an important role for spindle microtubular formation, begins at late G1 and/or early S phase [15].

From the facts described above, it is possible to speculate that NCS of acidic nature bounds the specific receptor on cell membrane and NCS effects consequently microtubular proteins, *i.e.*, centriole tubular protein at early S phase and spindle fiber protein at G2 phase, followed by both early S phase block related to DNA synthesis and G2 block related to cell mitosis.

REFERENCES

1. N. Ishida, K. Miyazaki, K. Kumagai and Rikimaru, Neocarzinostatin, an antitumor antibiotic of high molecular weight; isolation, physicochemical properties and biological activities. *J. Antibiot. Ser. A (Tokyo)* **18,** 68 (1965).
2. J. Meienhofer, H. Maeda, C. B. Glaser, J. Czombos and K. Kuromizu, Primary structure of neocarzinostatin, an antitumor protein. *Science* **178,** 875 (1972).
3. T. Nishikawa, K. Kumagai, A. Kudo and N. Ishida, Cytological study on the effect of neocarzinostatin on sarcoma 180 *in vivo*. *J. Antibiot. Ser. A (Tokyo)* **18,** 223 (1965).
4. W. T. Bradner and D. J. Hutchison, Neocarzinostatin (NSC-69856): an antitumor antibiotic effective against ascitic leukemia L1210 in mice. *Cancer Chemother. Rep.* **50,** 79 (1966).
5. K. Hiraki, O. Kamimura, I. Takahashi, T. Nagao, K. Kitajima and S. Irino, Neocarzinostatin. A new chemotherapeutic approach to acute leukemia. *Nouv. Rev. fran. Hémat* **13,** 445 (1973).
6. M. Homma, T. Koide, T. Saito-Koide, I. Kamo, M. Seto, K. Kumagai and N. Ishida, Specific inhibition of the initiation of DNA synthesis in HeLa cells by neocarzinostatin. *Proc. int. Cong. Chemother.* **6,** 115 (1969).
7. G. C. Mueller and K. Kajiwara, Actinomycin D and *p*-fluorophynylalanine, inhibitors of nuclear replication in HeLa cells. *Biochem. Biophys. Acta.* **119,** 557 (1966).
8. K. Kajiwara, U. H. Kim and G. C. Mueller, Phleomycin, an inhibitor of replication of HeLa cells. *Cancer Res.* **26,** 233 (1966).
9. M. Yamada and T. T. Puck, Action of radiation on mammalian cells, IV. Reversible mitotic lag in the S3 HeLa cell produced by low doses of X-rays. *Proc. nat. Acad. Sci. (Wash.)* **47,** 1189 (1961).
10. Y. Doida and S. Okada, Radiation-induced mitotic delay in cultured mammalian cells (L5178 Y). *Radiat. Res.* **38,** 513 (1969).
11. A. Falaschi and A. Kornberg, Phleomycin, an inhibitor of DNA polymerase. *Fed. Proc.* **23,** 940 (1964).
12. Y. Ono, Y. Watanabe and N. Ishida, Mode of action of neocarzinostatin: inhibition of DNA synthesis and degradation of DNA in sarcina lutea. *Biochim. biophys. Acta* **119,** 46 (1966).
13. G. M. Edelman, I. Yahara and J. L. Wang, Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proc. nat. Acad. Sci. (Wash.)* **70,** 1442 (1973).
14. R. D. Goldman, The role of three cytoplasmic fibers in BHK-21 cell motility, I. Microtubules and the effects of colchicine. *J. Cell. Biol.* **51,** 752 (1971).
15. E. Robbins, G. Jentzsch and A. Micali, The centriole cycle in synchronized HeLa cells. *J. Cell Biol.* **36,** 329 (1968).

Europ. J. Cancer Vol. 11, pp. 159–167. Pergamon Press 1975. Printed in Great Britain

Proliferation Kinetics of a Virus-induced Sarcoma in the Rat Kidney*

H. ZOBL, W. LANG and A. GEORGII
Department of Pathology Medical School, Hanover, West Germany

Abstract—*In experiments with Polyoma-virus-induced autochthonous fibrosarcomas of the rat kidney, the cell cycle parameters, the proliferative fraction, and the rate of cell loss were determined as essential factors for tumor growth. The results demonstrate a mean cell cycle time of* 21·1 *hr* ± 12·8 *by the* 10*th day,* 26·3 *hr* ± 20·9 *by the* 20*th day, and* 29·8 *hr* ± 15·4 *by the* 30*th day of growth. The proliferative fraction was between* 39 *and* 50% *and remained constant during the course of tumor growth. The critical factor in the great decrease in tumor growth rate was the rate of cell loss which, in large tumors, was up to about* 100% *of the rate of cell production. These results differ only from those obtained in most solid transplanted or spontaneous neoplasms with regard to the constancy of the proliferative fraction.*

INTRODUCTION

FOR THE LAST ten years, research on the proliferation kinetics of tumors has been limited largely to transplantable, *i.e.*, secondary tumors [1]. Corresponding studies on primary induced or spontaneous neoplasms [1–10], and in virus-induced tumors have been performed rarely [11].

However, spontaneous or primary induced tumors are doubtlessly far more suitable for consideration of the factors which might be essential for growth of human neoplasms. The model of our SE-Polyomavirus (PV)—induced sarcoma in the rat kidney is especially sutiable not only for its high yield and short latency periods, but also for the exactly defined and easily reproducible experimental conditions [12, 13] to determine the critical parameters of proliferation kinetics.

MATERIAL AND METHODS

(*a*) *SE-Polyomavirus*

Cultivation and preparation of the virus have been discussed previously [12, 13]. The $TCID_{50}$ of the virus suspension used in these experiments was 10^6 or 10^7/0·3 ml.

(*b*) *Animals*

Pregnant Wistar rats were obtained from the conventional colony strain of the Breeding Institute for Experimental Animals, Hanover, West Germany.

Within the first 24 hr following birth, the newborn animals were inoculated s.c. in the back with 0·3 ml of a virus suspension. Each animal was separated and all weanlings were maintained under standard laboratory conditions (pellet diet and water *ad libitum*).

(*c*) *Autoradiographic techniques with* ^{3}*H-thymidine*

^{3}H-thymidine (^{3}H-TdR, specific activity 5 Ci/mmol, Amersham/England) was used and was stored in the refrigerator at 4°C.

The following experiments were performed in virus inoculated rats of 30, 40 and 50 days of age:

1. *Determination of the percentage of labelled mitoses following a single injection of* ^{3}*H-TdR* [14]

The rats were injected i.p. with a single dose of 1 μCi ^{3}H-TdR/g body weight. 1, 1·5, 2, 3, 3·5, 4·5, 5, 6, 8, 9, 10, 12, 14, 16, 20, 24, 28, 32 and 48 hr following the injection, 5 animals at a time were sacrificed by cervical fracture.

Accepted 16 October 1974.
*This work was supported by the DFG.

2. *Continuous labelling experiments with ^3H-TdR*

Continuous labelling experiments were performed according to the continuous infusion method of Schultze *et al.* [15], derived from Dr. Stöcker, Würzburg, West Germany, 1969. One μm Ci ^{3}H-TdR (Spec. activity 5 Ci/mmol) per g body weight was administered into virus-inoculated rats by a venous catheter in the tail, a micropump (Sage Pump) and a swivel [16] allowing for free movements of the animals. Four groups of 10 rats each were infused over a period of 40, 50, 60, and 70 hr.

(d) Histological and autoradiographic preparations

After sacrificing the animals, the kidneys were excised immediately, cooled for 5 min and fixed in 4% formalin for 24 hr with addition of non-radioactive thymidine to the solution. Paraplast sections of whole kidney were routinely cut at 5 μM. Following deparaffinization, the slides were mounted with stripping film (Kodak AR 10), the autoradiographs exposed 21 days at 4° C, and finally fixed and developed.

(e) Analysis of the autoradiographs

Usually, autoradiographs of 3 tumors were evaluated at a magnification of 1000 × by 2 to 3 observers using an ocular grid micrometer. The percentage of labelled mitoses (labelled mitoses/total number of mitoses × 100) was determined by counting on the average 200 mitoses/tumor/observer *i.e.*, 1200–1800 mitoses for one period. The labelling index (labelled cells/total number of cells) was evaluated at 3000 cells/tumor/observer, *i.e.*, about 12,000 to 15,000 cells for one period.

(f) Evaluation of cell cycle parameters by analysis of percent labelled mitoses curves (PLM-curves)

Cell cycle time and phase durations including the log normal distribution of cell cycle times were calculated by computer analysis. [17–19], (analysis performed by Dr. G. G. Steel, London).

(g) Evaluation of the increase in tumor volume as a function of time

The tumor volume was determined in animals of 25, 30, 40 or 50 days by the scanning point method [20]; in complete sections through the kidneys, the surface of the renal or the sarcomatous tissue was scanned totally using the ocular grid (Fa. Zeiss). The total volume of one kidney was calculated following the principle of Delesse quoted by Henning [20]. For details of this method see [21]:

$$V_N : V_T = F_N : F_T \qquad (1)$$

$$V_T = V_N \frac{F_T}{F_N}$$

V_N = volume of the kidney
V_T = volume of the tumor
F_N = surface of the kidney
F_T = surface of the tumor.

The mean value of tumor volume/day of observation was calculated for 14 tumors by 28 measurements. Tumor doubling time (T_D) of an experimental growing neoplasm was determined by

$$V_t = V_0 \times 2t\ T_D \qquad (2)$$

$$T_D = \frac{t \times \log 2}{\log. V_t - \log. V_0}$$

V_0 = tumor volume at time to
V_t = tumor volume at time t
t = time between to and t.

This equation is valid only for exponential growth.

RESULTS

(a) Morphology

About 20 days following infection of newborn rats, sarcomas developed predominantly in the outer zone of the renal medulla. They were consistent with a richly vascularized, scarcely fiber-producing fibrosarcoma [12]. Electron microscopy demonstrated these tumor cells to originate from connective tissue cells (interstitial cells) and not from pericytes [22]. In the beginning, the tumor cells infiltrated the interstitium between the still well preserved tubules and later, (between 25th–30th day) developed into a solid tumor nodule which did allow an optimal analysis of proliferation kinetics (Fig. 1). There were more than 95% neoplastic cells in smears taken from the tumor surface.

(b) Growth curve

For determination of the growth curve, 14 tumors were usually scanned per observation day. A semilogarithmic graph of the increase in the mean tumor volume shows a nearly straight line between the 25th and 40th day following virus inoculation which is still consistent with exponential growth. Until the 50th day, there was apparently a reduction of volume. In addition, the lower average volume

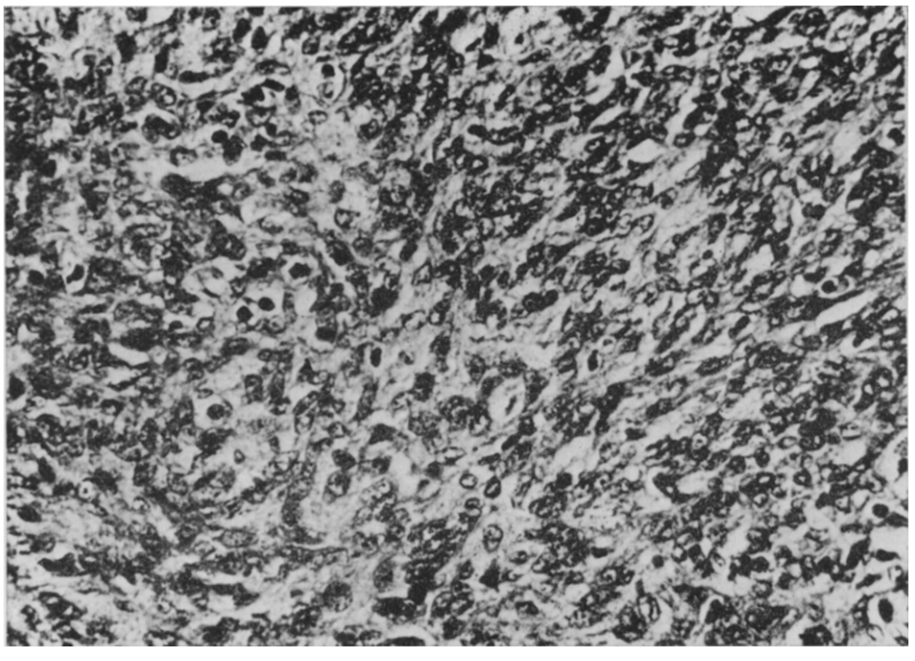


Fig. 1. *PV-induced sarcoma of the rat kidney,* 30 *days following neonatal virus inoculation. Haematoxilin and eosin* × 100.

(to face p. 160)

of the tumors at the 50th day of growth (Fig. 2) was caused by preferential of animals bearing large sarcomas.

As calculated from equation 1, the T_D is 47 hr at the 30th day and 72 hr at the 40th day of growth.

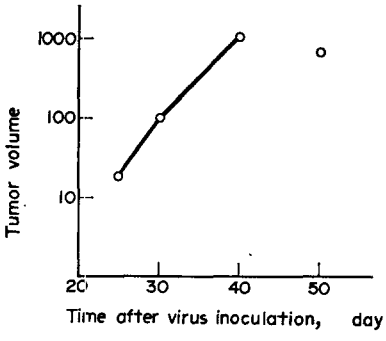


Fig. 2. Growth curve of PV-induced sarcomas in 25–40 day old rats with logarithmical drawn volumes of tumors in mm³. The value for the 50th day has been considered (see text).

(c) *Cell cycle time and phase duration*

Cell cycle time and the length of the single cell cycle phases were determined by PLM-curves which were drawn using the experimental data points. Because of the only weakly developed or even missing second peak of the PLM-curves of our kidney sarcoma, optimized PLM-curves were determined by computer analysis (method of Barrett [17, 18], modified by Steel and Hanes [19]. According to this, cell cycle time and the distributions of phase durations were calculated.

These computer analysed PLM-curves demonstrate an instant incline of the first peak to about 90% on the 30th, 92% on the 40th and 86% on the 50th day following virus inoculation, *i.e.*, the 10th, 20th and 30th days of tumor growth (Fig. 3a–c). The decline following the first peak was almost as steep in 30–40 day old animals, whereas the descending limb was flatter at the 50th day (Fig. 3c). A second peak was recognizable only in small tumors of 30 day old rats and was completely absent during the remaining observation days.

The calculated values for cell cycle time and phase durations obtained by computer analysis are shown in Table 1. The results demonstrate

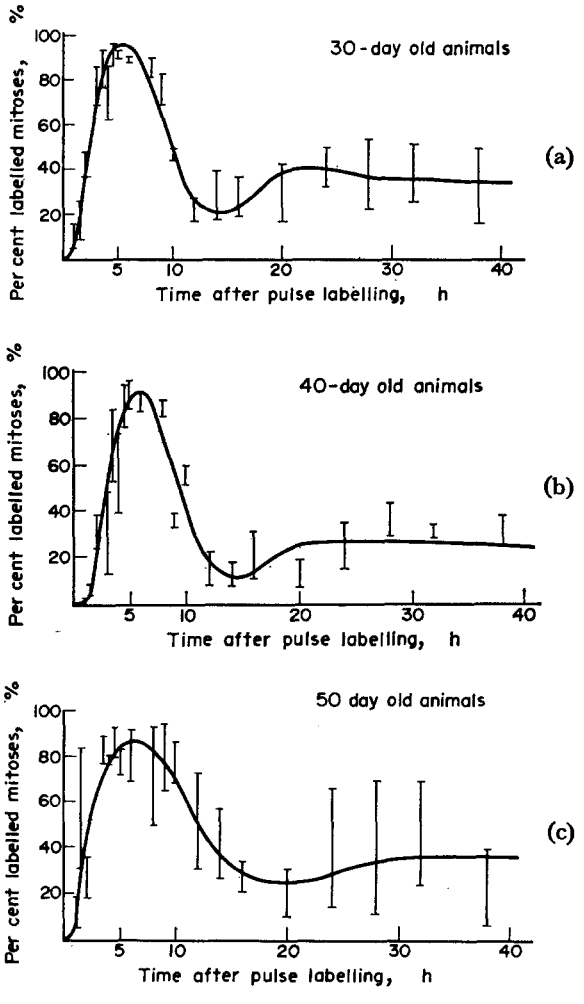


Fig. 3. PLM-curves as functions of time after a single injection of ^{3}H-thymidine, calculated by computer analysis following Barrett [17, 18], *and Steel and Hanes* [19].
(a) *in 30 day old animals, resp. 10th day of tumor growth*
(b) *in 40 day old animals, resp. 20th day of tumor growth*
(c) *in 50 day old animals, resp. 30th day of tumor growth*

Table 1. Cell cycle times and duration of cell cycle phases for virus-induced kidney sarcomas of 10–30 days growth. Determination by computer analysis following Barrett [17, 18] *modified by Steel and Hanes* [19]. *LI = percentage labelled cells measured at t = 60 after injection of ^{3}H-thymidine*

Age of tumors (days)	LI (%)	Computed PLM-curves: Mean durations (hours) and standard deviations T_{G2}	T_s	T_{G1}	T_C
10	13·3	2·5 ± 1·1	7·8 ± 2·6	10·8 ± 12·5	21·1 ± 12·8
20	14·7	3·3 ± 1·4	6·5 ± 2·1	16·5 ± 20·7	26·3 ± 20·9
30	14·8	3·0 ± 2·5	10·5 ± 5·1	16·3 ± 14·3	29·8 ± 15·4

a mean cell cycle time of 21·1 hr ± 12·8 by the 10th day, 26·3 hr ± 20·9 by the 20th day, and 29·8 hr ± 15·4 by the 30th day of growth. With increasing tumor size the cycle time distributions exhibit a wider spread, especially an increase in cells with a cycle time longer than 40 hr (Fig. 4).

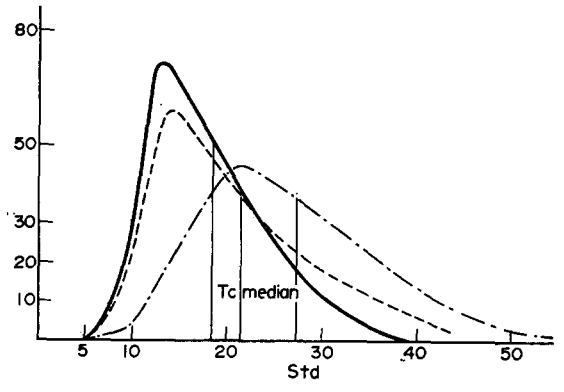


Fig. 4. Frequency distribution curves of cell cycle times (Tc) of PV-induced sarcomas following a computer analysis of Barrett [17, 18] *and Steel and Hanes* [19].
——— *in* 10 *day old tumors*
— — — *in* 20 *day old tumors*
— . — *in* 30 *day old tumors*

At all times of observation, even as soon as one hour following the injection of ^{3}H-TdR, a few labelled mitoses were already seen. This means that in these tumors, the minimal G_2-length was shorter than one hour.

(d) Proliferative fraction (PF)

1. By knowing the cell cycle time, the S-phase, G_2-phase and ^{3}H-TdR labelling index can be calculated for an exponential growing cell population in which all cells are cycling by the equation of Cleaver [23]. The labelling index is indicated as $LI_{theor.}$ or $LI_{calc.}$

$$LI_{theoretical} = \exp.\left(\frac{T_{G_2}}{T_C} \times \ln 2\right) \exp.\left[\left(\frac{T_S}{T_C} \times \ln 2\right) - 1\right] \quad (3)$$

where: $LI_{theor.}$ = ^{3}H-thymidine-labelling index in case all cells proliferate; $T_{G2} = G_2$-phase duration; T_C = cell cycle time; and T_S = S-phase duration. The experimentally measured ^{3}H-TdR-labelling index is equal to the theoretical ^{3}H-TdR-labelling index if all the cells of the population proliferate. But if only a part of the tumor cell population is proliferating, the experimentally found ^{3}H-TdR-labelling index is smaller than the theoretical one. The quotient of $LI_{exp.}$ and $LI_{theor.}$ results in the proliferative fraction [24].

$$PF = \frac{LI_{exp.}}{LI_{theor.}} \quad (4)$$

The determination of the *PF* following this method implies exponential growth which, however, cannot be assumed at the 30th day of tumor growth. Here the theoretical ^{3}H-TdR-labelling index was calculated assuming a rectangular "steady state" age distribution according to the formula:

$$LI = \frac{T_S}{T_C} \text{ [25]} \quad (5)$$

In addition, *PF* was determined following a growth model by Lang and colleagues [26] in which after every division of cells there originates b proliferative (P) cells and $(1-b)$ non proliferative, *i.e.*, quiescent (Q) cells. The formula developed from the growth model is

$$LI_t = \frac{1}{1 + 2\,\dfrac{(1-b)\,(1-2b)}{(2b-1)\,(2-2b-(2b)\,t/T_C}} \quad (6)$$

LI_t: labelling index during continuous infusion at a time t

b = distribution factor

t = duration of infusion

T_C = median cell cycle time

Index curves are drawn for different theoretical values of *PF* (*PF* = 10, 20, 30% etc.) and estimated as to which curve is in best accordance with the experimentally found labelling indices. *PF* ranges between 39% and 50% and did not markedly decrease during the 20 days of growth (Table 2).

(d) Rate of cell loss

To estimate the rate of cell loss as the third most important parameter of tumor growth, one must know the so-called potential doubling time ($T_{D\,pot.}$). Potential tumor doubling time is defined as the period needed to double the total number of cells provided a T_C and *PF* are given and assuming that there is no cell loss. This potential doubling time is to be calculated following Steel [27] when that T_C and *PF* are known.

$$T_{D\,pot.} = \frac{\ln 2}{\ln (2b)} \times T_C \quad (7)$$

$PF = 2b - 1$ [see equation (6)]

Table 2. Proliferative fraction (PF), potential doubling time and rate of cell loss for PV-induced sarcomas of the kidney at the 10th to 30th day of tumor growth. (+) PF following the growth model by Lang et al. *(see text). (*) calculation following Lala* [19]

Age of tumors (days)	Proliferative fraction (%)		Potential doubling (hr)	Cell loss rate (φ) (%)
	(Cleaver, 1965; Lala a. Patt, 1966)	(Lang *et al.*)	(+)	
10	39	45	39·3	15·8
20	45	50	37·2	48·0
30	42 (*)	40	42·3	

2*b* (Lang *et al.*) corresponds to α of Steel (1968). $T_{D\ pot.}$ is calculated to be 39·3 hr in 10 day old tumors and is 42·3 hr in 30 day old tumors (Table 2).

Cell loss rate may now be calculated by comparing the potential with the actual doubling time ($T_{D\ eff}$) of the tumor. Cell loss is best indicated by calculating the so-called cell loss factor after Steel [27].

$$\varphi = 1 - \frac{T_{D\ pot.}}{T_{D\ eff.}} \qquad (8)$$

This cell loss factor (φ) shows the cell loss rate as a fraction of cell-production rate [27]. φ amounts in small 10 day old sarcomas is 15·8%, in 20 ± day old tumors is 48% (Table 2)

DISCUSSION

The characterization of the proliferation kinetics of this primary tumor is established by the calculation of cell cycle time, proliferative fraction, and the rate of cell loss.

The PV-induced sarcoma of the kidney are practically growing exponentially over a period of 15 days. Later, a reduction in volume is observed. The decrease in the average tumor volume between the 20th and 30th days might be explained by the preferential death of those animals bearing especially large neoplasms.

The growth curves of this primary sarcoma are comparable to those of other solid primary and secondary neoplasms [28–30].

The reduction in growth with increasing volume of the tumor generally might be caused by either prolongation of cell cycle time, reduction of *PF*, or rate of cell loss or all three of these factors.

Therefore, it was quite important to determine these single parameters. The *PF* of 39–50% calculated by two different methods is highly comparable to those of other solid primary tumors [31–34]. It is remarkable that *PF* did not decline markedly with progressive tumor growth. In contrast, other authors demonstrated a decrease in *PF* with increasing age of the tumors [31, 35–37], while a constancy of *PF* was observed only occasionally [10, 38].

The decrease in *PF* generally has been explained by a reduced supply of the neoplastic cells with O_2 [37, 39, 40]. Tannock [39] demonstrated this fact particularly in neoplasms of the mammary gland in which cells are closely located around vessels and show a decline in *PF* with increasing distance from the vessel walls, even though there was no influence of hypoxemia on cell cycle time of the proliferating cells [40, 41].

Whether this conception is realistic cannot be answered by our experiments, because of the nearly identical values of *PF* found in the small tumors of the 10th and in the large tumors of the 30th days. Apparently, the large sarcomas either exhibit overt necroses or areas consisting of cell populations preserving a proliferative fraction comparable to that of cells in younger tumors. In general, it can be stated that these fast-growing primary sarcomas exhibit a relatively small *PF* and a constant cell fraction responsible for growth. The relatively low *PF* of primary tumors as compared to the transplanted neoplasms has been noted previously [28, 42, 43].

PF following ^{3}H-TdR-continuous labelling was neither estimated by the incline nor the plateau forming of the ^{3}H-TdR-labelling indices [44]. This method is not accurate in our opinion since undoubtedly there exist labelled cells after every mitoses in the non-proliferative compartment and therefore the *PF* is smaller than seen in the ^{3}H-TdR-labelling index following continuous labelling. Therefore, the relation between *PF* and the LI is expressed by the mathematical equation (6) derived by Lang *et al.* [26].

While generally there is a distinct second peak of the PLM-curves of transplanted tumors, this was missing in the autochthonuous

tumors which we examined. Steel [45] emphasized that in human tumors and spontaneous neoplasms in animals, this second peak is usually missing or at most very weakly developed. The duration of the measured cell cycle time and the cycle phases are in strong accordance with the values found in other solid tumors [4, 8, 11, 46–49]. There is an increase in the mean cell cycle time during the course of the 20 days of growth amounting to 8·7 hr which is probably not significant. From Fig. 3(a), 3(b) and 3(c) it is apparent that the form of the curves is not significantly different, corresponding at periods later than 15 hr following the labelling at the days of experiments. This lack of significance is further supported by the high s.d. of T_C mean at all 3 days of experiments.

In the pertinent literature, a constancy of cell cycle time was usually reported [10, 35, 37, 50–52], whereas the only reference for an increase of cell cycle time in a solid tumor was given for a transplantable tumor in the mouse [38]. The potential doubling time is increased only slightly throughout the 20 days of observation and the cell production rate may not have been restricted substantially. Therefore, the essential factor for the reduced growth rate must be due to the increase in the cell loss rate, already associated with experimental [25, 27, 29, 53] and human tumors [54–56]. Especially in spontaneous neoplasms, cell loss rate might reach the value of cell production rate causing stagnation of growth [10].

In virus-induced renal sarcomas, cell loss rate amounts to only 16% at the 30th day following viral inoculation (10th day of tumor growth) but, 10 days later it is already 48%. For this reason there must be an additional and remarkable increase in cell loss rate as demonstrated by the stagnation of growth and by the morphological findings of large necrotic areas. In the PV-induced kidney sarcomas, the cell loss is explained substantially by cell death. A shedding of cells as in neoplasms growing on surfaces or metastazising [27] can be excluded.

Two explanations might be given for the necroses: First, an increasing hypoxemia and decrease in supply of metabolites, [31, 53, 57, 58] secondly, influences of the immune system of the host [10, 53, 59–61]; thymectomized or congenital thymusless animals with depletion of cellular immunity show a lesser degree of necrosis [62, 63].

The decrease in the growth rate of the viral-induced renal sarcoma is consequently substantial depending on an increase of the cell loss rate. In summary, the proliferation kinetics of this neoplasm correspond with already-known findings in other tumors, but they also exhibit some peculiarity. This applies especially to the constancy of the *PF* during the observed period of tumor growth.

Acknowledgements—The authors are indebted to Dr. M. F. Rajewski, Tübingen, West Germany for his valuable suggestions and critical discussion of our paper and to Dr. G. G. Steel, London, England, for the computer analysis of the PLM-curve experiments.

REFERENCES

1. G. G. Steel The cell cycle in tumours: An examination of data gained by the technique of labelled mitoses. *Cell Tiss. Kinet.* **5,** 87 (1972).
2. M. L. Mendelsohn, Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. II Growth and survival of cells labelled with tritiated thymidine. *J. nat. Cancer Inst.* **25,** 485 (1960).
3. M. L. Mendelsohn, The kinetics of tumor cell proliferation. In *Cellular Radiation Biology. 18th Annual Symposium on Fundamental Cancer Research,* Houston 1964. p. 498. Williams & Wilkins, Baltimore (1965).
4. F. Bresciani, A comparison of the cell generative cycle in normal hyperplastic and neoplastic mammary gland of the C3H mouse. In *Cellular Radiation Biology. 18th Annual Symposium on Fundamental Cancer Research,* Houston 1964. p. 547. Williams & Wilkins, Baltimore (1965).
5. L. N. Owen and G. G. Steel, The growth and cell population kinetics of spontaneous tumours in domestic animals. *Brit. J. Cancer* **23,** 493 (1969).
6. I. L. Hansteen and S. B. Refsum, Cell proliferation and time of appearance of sarcomas induced with 20-methylcholanthrene in mice. *Virch. Arch. Abt. B,* **4,** 8 (1969).
7. L. Simpson-Herren and D. P. Griswold, Jr., Studies of the kinetics of growth and regression of 7, 12-Dimethylbenz(α)anthracene-induced mammary adenocarcinoma in Sprague–Dawley rats. *Cancer Res.* **30,** 813 (1970).
8. A. Schauer, E. Kunze und K. Boxler, Generationszeitzyklus von 1, 2-Dimethylhydrazininduzierten Adenocarcinomen des Rattencolon. *Naturwissenschaften* **58,** 221 (1971).

9. M. S. Aksyutina, L. P. Lipchina, N. A. Spryshkova, L. Ya. Yablovskaya, Radioautographic analysis of cell proliferation in primary gliomas of rats induced by 9, 10-dimethyl-1, 2-benzanthracene. *Tsitologiia* **13,** 850 (1971).
10. D. Cohen and G. G. Steel, Thymidine labelling studies in a transmissible venereal tumor of the dog. *Brit. J. Cancer* **26,** 413 (1972).
11. A. L. Rashad and C. A. Evans, Radioautographic study of epidermal cell proliferation and migration in normal and neoplastic tissues of rabbits. *J. nat. Cancer Inst.* **41,** 845 (1968).
12. A. Georgii, K. Prechtel und H. Zobl, Sarkombildung durch Polyoma-Virus in der Niere von Ratten. *Z. Krebsforsch.* **72,** 1 (1969).
13. U. Desselberger, A. Georgii, H. Zobl und H. Ostertag, Zur onkogenen Aktivität des Polyomavirus bei der Ratte. *Zbl. Bakt. I. Abt. Orig. A* **219,** 28 (1972).
14. H. Quastler and F. G. Sherman, Cell population kinetics in the intestinal epithelium of the mouse. *Exp. Cell Res.* **17,** 420 (1959).
15. B. Schultze, E. A. Löbbecke und W. Maurer, Autoradiographische Untersuchungen zur Variabilität der Generationszeit von fetalen Zellarten der Ratte mit Hilfe von Dauerinfusionen mit 3 H-Thymidin. *Acta histochem. (Jena)* Suppl. VIII, 351 (1968).
16. Ch. Eve and S. H. Robinson, Apparatus for continuous longterm intravenous infusions in small animals. *J. Lab. clin. Med.* **62,** 169 (1963).
17. J. C. Barrett, A mathematical model of the mitotic cycle and its application to the interpretation of percentage labelled mitoses data. *J. nat. Cancer Inst.* **37,** 443 (1966).
18. J. C. Barrett, Optimized parameters for the mitotic cycle. *Cell Tiss. Kinet.* **3,** 349 (1970).
19. G. G. Steel and S. Hanes, The technique of labelled mitoses: Analysis by automatic curve-fitting. *Cell Tiss. Kinet.* **4,** 93 (1971).
20. A. Henning, Kritische Betrachtungen zur Volumen- und Oberflächenmessung in der Mikroskopie. *Zeiss-Werkschr.* **30,** 78 (1958).
21. K. Bergius, Über das Wachstumsverhalten von Polyomavirus-induzierten Nierensarkomen bei Ratten. *Dissertation,* München, 1972.
22. A. Georgii, J. Thiele und E. Reale, Die Feinstrukutur eines Polyomavirus-induzierten Sarkoms der Rattenniere während seines Wachstums. *Z. Krebsforsch.* **80,** 255 (1973).
23. J. E. Cleaver, The relationship between the duration of the S-phase and the fraction of cells which incorporate 3 H-thymidine during exponential growth. *Exp. Cell Res.* **39,** 697 (1965).
24. P. K. Lala and H. M. Patt, Cytokinetic analysis of tumor growth. *Proc. nat. Acad. Sci. (Wash.)* **56,** 1735 (1966).
25. P. K. Lala, Evaluation of the mode of cell death in Ehrlich ascited tumor. *Cancer (Philad.)* **29,** 261 (1972),
26. W. Lang, F. Wingert, H. Zobl und A. Georgii, Modell zur Berechnung der Wachstumsfraktion. Bestimmung am PV-induzierten Nierensarkom der Ratte. In Vorbereitung (1974).
27. G. G. Steel, Cell loss from experimental tumors. *Cell Tiss. Kinet.* **1,** 193 (1968).
28. G. G. Steel, K. Adams and J. C. Barrett, Analysis of the cell population kinetics of transplanted tumors of widely-differing growth rate. *Brit. J. Cancer* **20,** 784 (1966).
29. K. H. Clifton and M. B. Yatvin, Cell population growth and cell loss in the MTG-B mouse mammary carcinoma. *Cancer Res.* **30,** 658 (1970).
30. L. Simpson-Herren and H. H. Lloyd, Kinetic parameters and growth curves for experimental tumor systems. *Cancer Chemother. Rep.* **1,** 143 (1970).
31. I. F. Tannock, Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. *Cancer Res.* **30,** 2470 (1970).
32. M. L. Mendelsohn, Autoradiographic analysis of cell proliferation in spontaneous breast of C3H mouse. III. The growth fraction. *J. nat. Cancer Inst.* **28,** 1015 (1962).
33. J. M. Brown and R. J. Berry, Effects of X-irradiation on cell proliferation in normal epithelium and in tumours of the hamster cheek pouch. In *Effects of Radiation on Cellular Proliferation and Differentiation,* p. 475. International Atomic Energy Proceedings Series, Vienna (1968).

34. J. Denekamp, The cellular proliferation kinetics of animal tumors. *Cancer Res.* **30,** 393 (1970).
35. E. Frindel, E. P. Malaise, E. Alpen and M. Tubiana, Kinetics of cell proliferation of an experimental tumor. *Cancer Res.* **27,** 1122 (1967).
36. M. Tubiana, E. Frindel and E. Malaise, The application of radiobiologic knowledge and cellular kinetics to radiation therapy. *Amer. J. Roentgenol.* **102,** 822 (1968).
37. P. K. Lala, Age-specific changes in the proliferation of Ehrlich ascites tumor cells grown as solid tumors. *Cancer Res.* **32,** 628 (1972).
38. M. H. L. Gibson and F. D. Bertalanffy, The *in vivo* cell kinetics of transplantable B16 mouse melanoma growth. *Growth* **36,** 263 (1972).
39. I. F. Tannock, The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Brit. J. Cancer* **22,** 258 (1968).
40. I. F. Tannock and G. G. Steel, Tumor growth and cell kinetics in chronically hypoxic animals. *J. nat. Cancer Inst.* **45,** 123 (1970).
41. J. W. Harris, F. Meyskens and H. M. Patt, Biochemical studies of cytokinetic changes during tumor growth. *Cancer Res.* **30,** 1937 (1970).
42. F. Bresciani, Cell proliferation in cancer. *Europ. J. Cancer* **4,** 343 (1968).
43. M. Tubiana, The kinetics of tumor cell proliferation and radiotherapy. *Brit. J. Radiol.* **44,** 325 (1971).
44. L. M. Schiffer, J. S. R. Nelson, B. Dilettuso, D. Migliorato and W. Randolph, Cytokinetics of the S180 ascites tumor system. *Cell Tiss. Kinet.* **6,** 165 (1973).
45. G. G. Steel, Quoted. In The growth kinetics of solid tumors. Summary report of meeting, by Mendelshon, M. F. and S. E. Shackney. *Cell Tiss. Kinet.* **3,** 410 (1970).
46. K. Hempel, Analyse des Zellcyclus der Melanocyten des Harding-Passey-Melanoms der Maus unter besonderer Berücksichtigung der "Growth Fraction". *Virch. Arch. Abt. B* 1, 15 (1968).
47. W. B. Looney, A. A. Mayo, M. Y. Janners, J. G. Mellon, P. Allen, D. Salak and H. P. Morris, Cell proliferation and tumor growth in Hepatomas 3924A. *Cancer Res.* **31,** 821 (1971).
48. P. Janik and G. G. Steel, Cell proliferation during immunological perturbation in three transplanted tumours. *Brit. J. Cancer* **26,** 108 (1972).
49. J. Denekamp, The relationship between the "Cell Loss Factor" and the immediate response to radiation in animal tumors. *Europ. J. Cancer* **8,** 335 (1972).
50. A. F. Hermens and C. W. Barendsen, Cellular proliferation patterns in an experimental rhabdomyosarcoma in the rat. *Europ. J. Cancer* **3,** 361 (1967).
51. L. A. Dethlefsen, J. M. S. Prewitt and M. L. Mendelsohn, Analysis of tumor growth curves. *J. nat. Cancer Inst.* **40,** 389 (1968).
52. P. K. Lala, Cytokinetic control mechanism in Ehrlich ascites tumor growth In *Effects of Radiation on Cellular Proliferation and Differentiation.* p. 463. International Atomic Energy Proceedings Series. Vienna (1968).
53. E. H. Cooper, The biology of cell death in tumors. *Cell Tiss. Kinet.* **6,** 87 (1973).
54. O. H. Iversen, Kinetics of cellular proliferation and cell loss in 13 human carcinomas. *Europ. J. Cancer* **3,** 389 (1967).
55. G. G. Steel, Cell loss as a factor in the growth rate of human tumors. *Europ. J. Cancer* **3,** 381 (1967).
56. J. J. Terz, H. P. Curutchet, W. Lawrence, Jr., Analysis of the cell kinetics of human solid tumors. *Cancer* **28,** 1100 (1971).
57. N. V. Krylova, Characteristics of microcirculation in experimental tumors. In *5th European Conference on Microcirculation,* Gothenburg 1968 (Edited by H. Harders) In *Bibl. anat.* (Edited by G. Wolf-Heidegger,) p. 301. S. Karger, Basel (1969).
58. C. W. Song, Th. Payne and S. H. Lewitt, Vascularity and blood flow in x-irradiated Walker carcinoma 256 of rats. *Radiology* **104,** 693 (1972).
59. G. Klein, Tumor specific transplantation antigenes. GHA Clowes Memorial Lecture. *Cancer Res.* **28,** 1625 (1968).
60. M. F. Rajewsky and A. A. Grüneisen, Cell proliferation in transplanted rat tumors: Influence of the host immune system. *Europ. J. Immunol.* **2,** 445 (1972).
61. S. Rockwell and R. F. Kallman, Growth and cell population kinetics of single and multiple KHT sarcomas. *Cell Tiss. Kinet.* **5,** 449 (1972).

62. W. LANG, H. ZOBL, G. SIEGISMUND und A. GEORGII, Proliferationskinetik von Polyomavirus-induzierten Nierensarkomen. II. wissenschaftliche Tagung der Deutschen Krebsgesellschaft, Hanover 1971.
63. A. GEORGII and H. OSTERTAG, Multiple primary renal sarcomas in rats: A dose response study with polyoma-virus. V. Perugia Quadrennial International Conferences on Cancer (1973).

Enrop. J. Cancer Vol. 11, pp. 169–173. Pergamon Press 1975. Printed in Great Britain

The Effect of Cytostatic Hexitol Derivatives on Haematopoietic Stem Cells, the L_{1210} Leukaemia and C22LR Osteosarcoma*

M. FUZY†, P. LELIEVELD and L. M. VAN PUTTEN
Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands;
†*National Oncological Institute, Rath Gyorgy u.5 Budapest XII, Hungary*

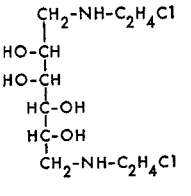
Abstract—*In a comparative study the effect of six hexitol derivatives, mannitol mustard, dibromomannitol, dianhydromannitol, dibromodulcitol, dianhydrodulcitol and dimesyldianhydroiditol (DMDAI) on haematopoietic stem cells was studied by the spleen colony technique. All drugs investigated, except DMDAI, have a low ratio of effectiveness on resting compared to rapidly-proliferating stem cells. In the L_{1210} system, the drugs have less effect on the leukaemic cells than on the rapidly-proliferating haematopoietic stem cells. In the C22LR osteosarcoma solid tumour system, the hexitol derivatives present a moderate or strong antitumour activity. Among the drugs studied, DMDAI deserves special attention, because of its sparing effect on resting haematopoietic stem cells.*

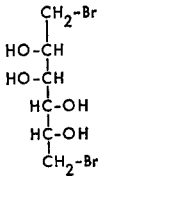
INTRODUCTION

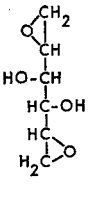
THE CYTOSTATIC hexitol derivatives represent a distinct type of cytostatic agents. The first compound of this group, mannitol mustard, was synthetized in 1955, and mannitol was used as a carrier for terminal beta-chloroaethylamine groups [1] (see Table 1).

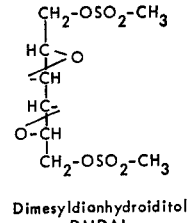
It was soon realized that halogen- and mesyl-substituted hexitols were also active [2, 3]. Dimesylmannitol and dibromomannitol (DBM) are myelotoxic agents, have a moderate effect on transplantable tumours, and have proved to be useful drugs for the treatment of chronic myelogenous leukaemia [4]. The diastereo-isomer of DBM, dibromodulcitol (DBD) shows a strong inhibition of several transplantable tumours and is also active against L_{1210} leukaemia [5, 6]. DBD gives remissions in chronic myelogenous leukaemia and in some patients with squamous cell carcinomas, but the latter data need further support [7, 8].

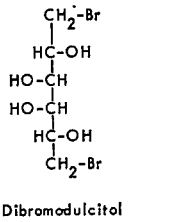
Table 1

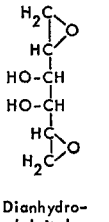












In vitro, at slightly alkaline pH, alpha–omega substituted hexitols are converted into dianhydrohexitols, which are far more active

Accepted 5 November 1974.
*This study was made possible by a fellowship (to M.F.) under the Hungarian-Netherlands Cultural Exchange Agreement.

cytostatic drugs than the parent compounds [9]. *In vivo*, DBM and DBD are converted into a series of alkylating agents, such as bromo-epoxides, diepoxides (dianhydrodulcitol DAHD, dianhydromannitol DAHM) and other compounds [10]. All the hexitol derivatives exert characteristic but undesirable side effects on blood-elements, thus limiting their clinical usefulness for the treatment of solid tumours. A number of publications was related to this problem describing the type of this action on the blood and bone marrow [9, 11, 12]. Mesyl- and halogen-substituted hexitols affect mainly the myelopoesis; epoxy- and mustard-derivatives predominantly depress lymphopoiesis but after higher doses, all blood elements are depressed. Tetrafunctional agents with mixed groups show a much less characteristic effect on haematopoiesis [13]. Among the tetrafunctional agents with mixed groups, dimesyl-dianhydroiditol (DMDAI) deserves special attention because of its strong antitumour and relatively mild blood depressing effect [14].

It was thought worthwhile to study the effects on both an experimental tumour and on the haematopoietic system with cell survival assay techniques. Use was made of the spleen colony assay for the normal haematopoesis and of L_{1210} leukaemia as a representative malignancy and six hexitol derivatives were tested on these systems. In addition, anti-tumour activity was measured on a solid sarcoma.

MATERIAL AND METHODS

All drugs were administered s.c. in a single dose either suspended in 2% carboxymethylcellulose or dissolved in saline in a volume of 0.01 ml/g of body weight.

The $LD_{50/30}$ was determined by the Probit-method. The method of determining stem cell survival by the spleen colony assay was described earlier by van Putten *et al.* [15]. $(DBA_2 \times C57BL)F_1$ hybrid mice (further D_2BF_1) were used in all experiments except for part of the stem cell survival studies after administration of DAHM which were performed in $(CBA \times C57BL)F_1$ mice. Donors were male mice; recipients were both male and female. The mice were sacrificed 16 hr after administration of the drugs. Rapidly proliferating spleen colony forming cells were also exposed to chemotherapeutic agents [16]. Such CFU (colony-forming units) were obtained by injection of a standard amount of bone marrow (about 1/4 femur content) into lethally irradiated mice. In the spleens of such mice, rapidly proliferating CFU may be found and the survival of spleen CFU seven days after bone marrow administration was used for comparison with the survival of femur bone marrow CFU in normal mice.

The L_{1210} leukaemia cell survival was determined by the method of Skipper *et al.* [17]. L_{1210} cell suspensions were diluted to contain the following cell numbers per 0·5 ml; 10^5, 10^4, 10^3, 10^2, 10 and 1. One half ml quantities of each of these dilutions were injected i.v. into 10 D_2BF_1 hybrid mice. A number of similar groups of mice received 10^5 cells i.v. and different doses of the drugs were injected s.c. 24 hr later. The mice were observed daily and the median survival time (MST) was noted for each group. Reduction in survival of leukaemia cells was estimated by comparison of the MST of treated with that of the reference groups.

The effect on a solid tumour was studied by use of a transplantable osteosarcoma described earlier by van Putten *et al.* [16]. For the osteosarcoma studies, drug effectiveness was expressed in the form of a tumour volume reduction ratio which expresses the relative volume reduction at day 4 after s.c. injection of the drugs.

RESULTS

Acute toxicity values for hexitols in D_2BF_1 mice are listed in Table 2. Dose–effect curves for resting and rapidly-proliferating colony-forming units and L_{1210} leukaemia cells after exposure to mannitol mustard, DBM, DAHM, DBD, DAHD and DMDAI are presented in Fig. 1. For each curve of resting and rapidly proliferating CFU, as well as for L_{1210} cells, the slope and its reciprocal, D_0 were calculated. The results for the normal cells are presented in Table 3 and for L_{1210} cells in Table 4. The tumour volume reduction ratios were calculated as described above and the results for the

Table 2

$LD_{50/30}$ s.c.

Mannitol mustard	85	mg/kg
DBM	2000	"
DAHM	22,5	"
DBD	1500	"
DAHD	16	"
DMDAI	1850	"

Table 3

Drugs	Resting CFU* D_o, mg/kg A	Rapidly proliferating CFU D_0, mg/kg B	Ratio A/B	CFU Survival at $LD_{10/30}$
Mannitol mustard	13.57	8.38	1.62	0.2%
DBM	317.8	237.1	1.34	1.0%
DAHM	4.12	2.65	1.55	0.8%
DBD	257.4	171.9	1.60	0.5%
DAHD	2.64	2.04	1.29	1.3%
DMDAI	1308.0	374.1	3.49	10 %

*CFU (colony forming unit)

Table 4

Drugs	L1210 Leukaemia cells D_o, mg/kg	L1210 extrapolated cell survival at LD_{10}
Mannitol Mustard	24.0	10^{-1}
DBM	713.7	2.10^{-1}
DAHM	4.34	10^{-2}
DBD	186.2	10^{-3}
DAHD	1.85	5.10^{-3}
DMDAI	385.0	10^{-2}

Table 5

Drugs	Dose LD_{10} mg/kg	Osteosarcoma Tumor Volume Reduction Ratio
Mannitol mustard	75	0.48 +
DBM	1800	0.30 +
DAHM	20	0.27* +
DBD	1300	0.17 ++
DAHD	14	0.14 ++
DMDAI	1700	0.37 +

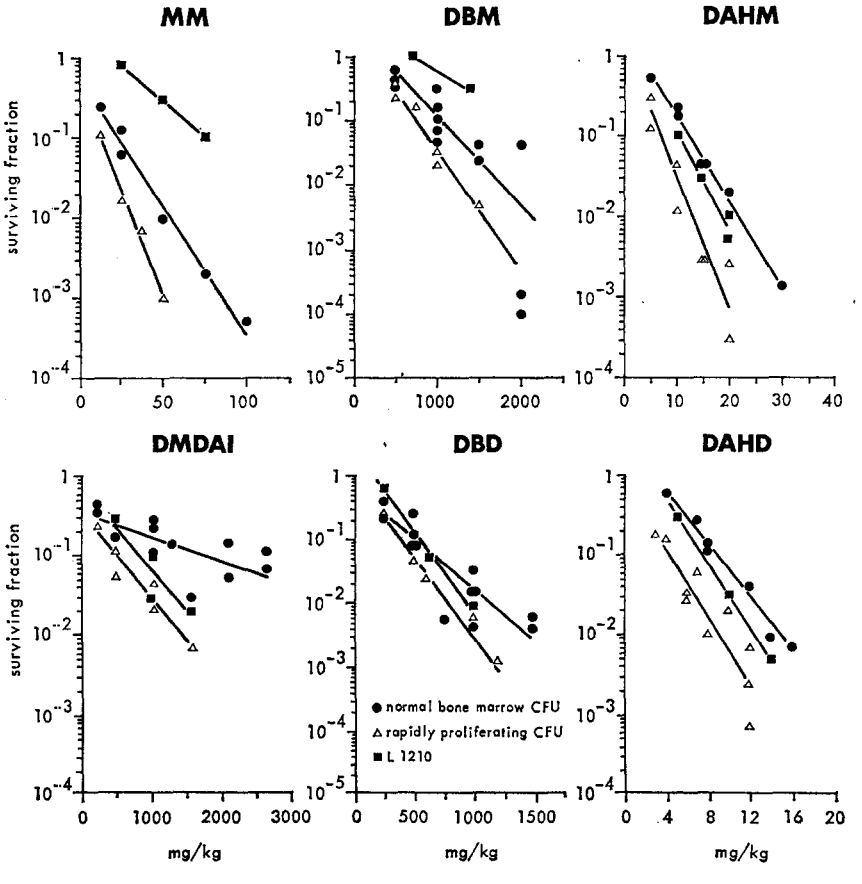
*A volume reduction of 0.14 was derived from an earlier observation (15) Recent repeat studies performed simultaneously with other drugs gave the higher value of 0.27

osteosarcoma are presented in Table 5. These osteosarcoma studies were carried out after exposure to an LD_{10} dose of the drug; these doses are also presented in Table 5.

DISCUSSION

The cytostatic hexitol derivatives investigated

Table 6



give rise to stem cell survival curves which decrease exponentially with increasing dose. The slope ratios of survival curves for resting and rapidly proliferating haematopoietic colony forming cells cluster for the different drugs around 1·5 except for DMDAI, which shows a much higher ratio: 3·5. In comparison with other drugs studied in a similar way the group of hexitols is similar to most other alkylating agents, but DMDAI has an exceptionally high slope ratio, indicating that the sensitivity of cells to this drug seems highly dependent upon the rate of proliferation. In addition, DMDAI has certainly a markedly lower toxicity for resting hematopoietic stem cells as is evident from the last column of Table 3. The similarity of slope ratios for the other agents supports the theory, that the halogenated hexitols are converted into dianhydrohexitols and act as such. It must be emphasized, however, that the ratios calculated have no absolute validity for the investigated drugs. Variations in dose response curves depend on the time of assay, route of administration, and other experimental conditions; *e.g.*, the low toxicity of DMDAI for resting stem cells may be partly related to the route of subcutaneous injection. However, the effects of the hexitol derivatives on the L_{1210} leukaemic cells are more variable and this may not fit with a simple explanation of transformation as was postulated [9]. L_{1210} killing by mannitol mustard and DBM was ineffective; the slope of the survival curves was not steeper than that of resting CFU. For DAHM, L_{1210} survival was rather similar to that of normal resting CFU but even for the more active drugs DMDAI, DBD and DAHD, the killing of L_{1210} cells was not more effective than the killing of rapidly proliferating mouse CFU.

This is an unusual finding, indicating that the inherent cellular sensitivity of L_{1210} leukaemia is relatively low, compared to that of normal stem cells when proliferating at a similar rate. In other studies, [18] the sensitivity of L_{1210} was found to be higher and of the same order as that of another mouse lymphoma, NK/Ly, indicating that the results in our L_{1210} studies may not be representative of mouse lymphomas studied by others. Partly the difference may be a consequence of the higher local drug concentration for leukaèmic cells in the ascites when drugs are given i.p., partly it may be due to the difference in cell lines used [6, 11].

All the hexitol derivatives investigated show strong or moderate anti-tumour effect in the mouse osteosarcoma system. DBD and DAHD have the strongest antitumour activity. This is in conformity with the conclusion that the antitumour activity of the dibromo compounds is due to transformation into diepoxy derivatives. Mannitol mustard shows a very moderate effect on the osteosarcoma; in this respect it is similar to many classical alkylating agents. The mannitol derivatives are, in the simultaneously performed recent studies, moderately effective, and the dulcitol derivatives are markedly effective in this system. DMDAI deserves special attention. Although in our single dose test it has shown only moderate antitumour effectiveness, its low hematopoietic toxicity may be promising nevertheless for studies on protracted treatment. The special sparing effect of DMDAI for resting stem cells could make it especially effective in the treatment of slowly growing tumours.

REFERENCES

1. L. Varga, L. Toldy, O. Fehér and S. Lendvai, Synthesis of new sugar derivatives of potential antitumour activity—I. Ethyleneimino-and 2-chloroethylaminoderivatives. *J. chem. Soc.* **151,** 805 (1957).
2. L. Institorisz, I. P. Horváth and E. Csanyi, Cytostatic activity of certain halogenated polyalcohols. *Proc. 2nd Int. Symp. Chemother. Naples, 1961*, p. 250 Karger, New York (1963).
3. A. Haddow, G. M. Timmis and S. S. Brown, Tumour inhibiting action of 1,6-Dimethanesulphonyl-D-mannitol. *Nature (Lond.)* **182,** 1164 (1958).
4. S. Eckhardt, C. Sellei I. P. Horváth and L. Institorisz, Effect of 1,6-dibromo-1,6-dideoxy-D-mannitol on chronic granulocytic leukaeia. *Cancer Chemother. Rep.* **33,** 57 (1963).
5. B. Kellner, L. Németh, I. P. Horváth and L. Institorisz, 1,6-Dibromo-1,6-dideoxy-dulcitol: a new antitumor agent. *Nature (Lond.)* **213,** 402 (1967).
6. S. K. Carter, Dibromodulcitol (NSC-104800)-Clinical brochure. *Cancer Chemother. Rep.* III. **1,** 165 (1968).

7. C. Sellei, S. Eckhardt, I. P. Horváth, J. Kralovánszky and L. Institorisz, Clinical and pharmacologic experience with dibromodulcitol. (NSC-104800) a new antitumor agent. *Cancer Chemother. Rep.* **53,** 377 (1969).
8. J. W. Keyes, Jr., O. S. Selawry and H. H. Hansen, Initial clinical trial of dibromodulcitol (NSC-104800) in patients with advanced cancer. *Cancer Chemother. Rep.* **55,** 583 (1971).
9. L. A. Elson, M. Jarman and W. C. J. Ross, Toxicity, haematological effects and antitumour activity of epoxides derived from disubstituted hexitols. Mode of action of mannitol myleran and dibromomannitol. *Europ. J. Cancer* **4,** 617 (1968).
10. I. P. Horváth, J. Csetényi and S. Eckhardt, Clinico-pharmacological studies on the metabolism of cytostatic hexitol derivatives. *8th Int. Congress Chemotherapy*, September 8–14, 1973, Athens–Greece. In press (1974).
11. L. Döbrösy and L. Németh, Jr., The haematological effect of a new cytostatic: 1,6-dibromo-1,6-dideoxy-dulcitol (DBD) in animal experiments. *Neoplasma* **16,** 33 (1969).
12. C. Sellei, S. Eckhardt and L. Németh, *Chemotherapy of Neoplastic Diseases.* Publishing house of the Hungarian Acad. Sciences, Budapest (1970).
13. G. Kendrey, J. Balo, J. Juhász, G. Gyenes and M. Sellyei, Experimental investigation into the antitumoral activity of 1,2,5,6-tetra-o-methanesulfonyl-D-mannitol. *Acta Un. int. Cancer* **20,** 141 (1964).
14. E. Csanyi and M. Halász, 1,6-di-o-methanesulphonyl-2,3–4,5-dianhydro-L-Iditol. A new potential antitumour agent. *Arzneimittel-Forsch.* **23,** 961 (1973).
15 L. M. Van Putten and P. Lelieveld, Factors determining cell killing by chemotherapeutic agents *in vivo*—I. Cyclophosphamide. *Europ. J. Cancer* **6,** 313 (1970).
16. L. M. Van Putten, P. Lelieveld and L. K. J. Kram-Idsenga, Cell-Cycle Specificity and therapeutic effectiveness of cytostatic Agents. *Cancer Chemother. Rep.* **56,** 691 (1972).
17. H. E. Skipper, F. M. Schabel, Jr., and W. S. Wilcox, Experimental evaluation of potential anticancer agents—XIII. On the criteria and kinetics associated with curability of experimental leukaemia. *Cancer Chemother. Rep.* **35,** 1 (1964).
18. L. Németh, L. Institorisz, S. Somfai, F. Gál, I. Pályi, J. Sugár, O. Csuka, Z. Szentirmay and B. Kellner, Pharmacologic and antitumor effects of 1,2:5,6-dianhydrogalactitol (NSC-132313). *Cancer Chemother. Rep.* **56,** 593 (1972).

Europ. J. Cancer Vol. 11, pp. 175–179. Pergamon Press 1975. Printed in Great Britain

Cytokinetic Considerations of Murine Neuroblastoma (C1300) as a Screening Model for the Childhood Disease*

JERRY Z. FINKLESTEIN† and JOHN WEINER‡

†*Department of Pediatrics, University of California, Los Angeles, and Harbor General Hospital, Torrance, California, and* ‡*the University of Southern California, U.S.A.*

Abstract—*Kinetic studies are presented for the Murine Neuroblastoma C1300 system. Animals receiving subcutaneous inoculations of* 10^6 *cells develop a palpable tumor with a mean diameter of* 1·3 *cm in* 10 *days. The growth characteristics of palpable disease are consistent with a Gompertzian curve distribution; the doubling time of palpable disease is* 10 *days. Autoradiographic studies demonstrated a DNA synthesis time of* 4·52 *hr and a total generation time of* 14·1 *hr. These considerations and the sensitivity of the model to cell cycle nonspecific agents suggest that the C1300 system deserves further study as a potential model of the human disease.*

INTRODUCTION

THE C1300 murine neuroblastoma system has been proposed as a model for the human disease [1]. In this model cyclophosphamide, BCNU and cytosine arabinoside prolong significantly the median duration of survival of animals treated with measurable tumor, whereas vincristine, actinomycin D, methotrexate, imidazole carboxamide and cyanocoblamin are ineffective in the dosage and regimes used [1]. All the above agents, with the exception of cyanocoblamin, are active in one or more of the classical animal tumor systems; murine L1210 leukemia, murine P388 leukemia, murine B16 melanoma, murine Lewis lung carcinoma, Rat Walker 256 tumor and murine carcinoma 755 [2, 3]. However, only cyclophosphamide is generally accepted as the primary agent to which a significant number of children with metastatic neuroblastoma will respond [4]. BCNU and cytosine arabinoside have not received an adequate trial in children with this entity. Although vincristine has been used alone with limited success, most clinicians prefer to use it with cyclophosphamide [5]. These results indicate that further study of the C1300 model is warranted, to determine whether it is a valid model for the screening of chemotherapeutic regimens proposed for use in children with metastatic disease.

The murine neuroblastoma model has a number of factors which make it unique among animal tumor systems. The tumor cells produce acetylcholine esterase and the enzymes necessary for the synthesis of neurotransmitters *in vitro*, and under certain culture conditions axon formation takes place [6]. Morphological maturation can be accelerated *in vitro* and differentiating cells can be selected with BUDR [7, 8]. Histopathologic and electron microscopic chemical studies of the tumor *in vivo* reveal the presence of catecholamines [9]. These biological properties suggest that the murine neuroblastoma model has many features of the human disease.

The purpose of this communication is to report results of growth kinetic studies of the C1300 model and to compare its characteristics

Accepted 18 October 1974.

*Supported in part by Public Health Service Grants CA-14658, CA-14560 and CA-14089 from the National Cancer Institute.

Reprint address: Jerry Z. Finklestein, M.D., Department of Pediatrics, Harbor General Hospital, 1000 West Carson Street, Torrance, California 90509, U.S.A.

with the published data of various common animal and human tumors.

MATERIAL AND METHODS

Tumor induction

Details of the C1300 neuroblastoma tumor have been described previously. Briefly, tumor induction was performed by injecting adult A/J mice (more than eight weeks of age) with 10^6 tumor cells in the interscapular area. Animals were observed for the appearance of palpable disease and the subcutaneous tumors were measured with a vernier calipers at least thrice weekly until the animals expired. Tumor volume was calculated from the mean diameter taking into account the double thickness of the skin overlying the tumor (2 × 0·5 mm) [10].

Generation time

The generation cycle of the tumor *in vivo* was calculated by determining the percent labeled mitosis in animals with a palpable tumor. Ninety animals with s.c. tumors of 1 cm in diameter were given single i.p. injections of 2 μCi/gm weight of tritiated thymidine (Sp Act 5 c/mm-Amersham/Searle); two mice were sacrificed at various times after injection of the DNA precursor. The tumors were dissected and fixed in Bouin's fixative for two hours, then processed for routine histology. Sections 4 μm thick were prepared and autoradiographs were made by the dipping method, using Kodak Nuclear Track Emulsion NTB2. After one to three weeks of exposure at 4°C in a plastic light-tight box, slides were developed in an anhydrous sodium sulfite and potassium bromide developer, with amidol 2,4-diaminophenol dihydrochloride as the developing agent, according to the method of Kopriwa and Leblond [11]. The developed slides were then stained with Harris hematoxylin and 1% alcoholic eosin. Cells with five or more grains were considered labeled. The percent of labeled mitoses (PLM) was determined from the available number of mitoses per slide, which was always over 100. The PLM after the pulse label were then plotted against time, and the form of the curve was obtained. The cell cycle time and the components of the cell cycle were determined using the analysis of Mendelsohn and Takashi [12].

RESULTS

Animals receiving s.c. inoculations of 10^6 cells develop a palpable tumor with a mean diameter of 1·3 cm in 10 days. The frequency distribution of the measured mean diameters of 119 different mice observed 10 days following injection are given in Table 1. The relationship between time from injection to maximum tumor size is shown in Fig. 1. The least square line was calculated by the formula: estimated size = 2·14 + 0·08 (Time—16·66). The measured doubling time is 10 days.

Table 1. Frequency table for the mean diameters of subcutaneous tumors in 119 mice at 10 days following the injection of 10^6 viable neuroblastoma cells. The average diameter was 1·35 cm with a S.D. of 0·25351 and a range of 1 to 1·9 cm

Mean dia.	Frequency	Percentage	Cumulative frequency	Cumulative percentage
1·00	15	12·60	15	12·60
1·10	10	8·40	25	21·00
1·20	15	12·60	40	33·61
1·30	17	14·28	57	47·89
1·40	24	20·16	81	68·06
1·50	15	12·60	96	80·67
1·60	8	6·72	104	87·39
1·70	13	10·92	117	98·31
1·80	1	0·84	118	99·15
1·90	1	0·84	119	100·00

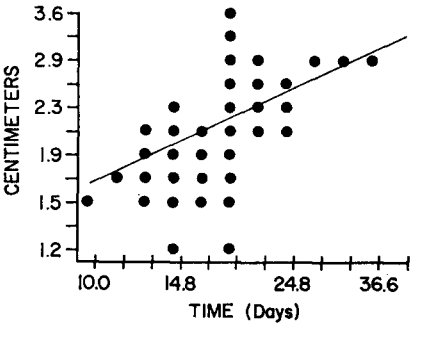


Fig. 1. Maximum tumor size with time in 119 control animals. The scales in the figure are the arithmetic equivalent of the log of the measurements. Each dot reflects one or more observations at the same time and maximum tumor size intervals. The correlation coefficient between time and tumor size is 0·70 ($P < 5\%$). The least square line based on the original arithmetic scales was equivalent with a correlation of 0·6. The measured doubling time is 10 days.

The percentage of labeled mitosis was determined in tumors from animals sacrificed at various times, from 1 to 40 hr following the injection of the pulse label of tritiated thymidine. Variation of transit time was observed and an asymmetrical curve was obtained (Fig. 2). The

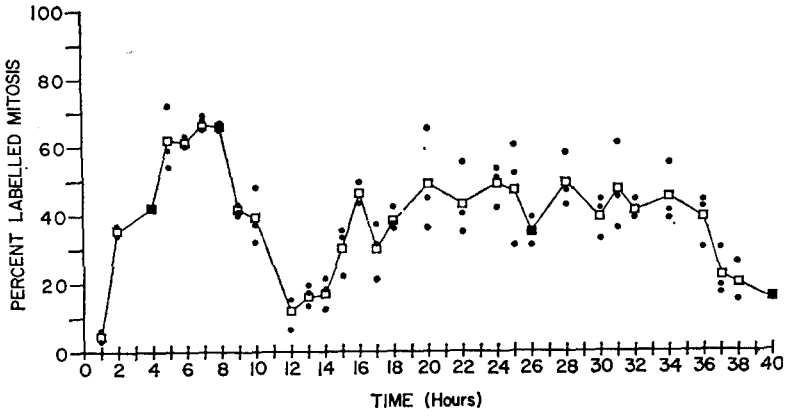


Fig. 2. Cell cycle in vivo *of C1300 neuroblastoma. Each point represents the percent labeled mitosis after a developing time of* 6, 9 *or* 21 *days.*

cell cycle parameters were calculated by the Mendelsohn–Takahashi method [12], using the following estimates:

1. The height of the first peak, H peak = 0·66.
2. The height of the first trough, H trough = 0·12.
3. Time to last peak on the plateau Tn 34 hr.
4. The area to Tn, An = 10·825 hr.

Two waves of labeled mitosis were obtained. The peak was reached by 7·5 hr and was equal to 66%. The cell cycle of the measurable tumor revealed a DNA synthesis time of 4·52 hr and a total generation time of 14·1 hr. The results are compared to other widely used tumor models in Table 2. Also illustrated in the Table is the result of an autoradiographic study of the generation cycle of the embryonic neural tube in a mouse of 10 days' gestation.

DISCUSSION

While any number of solid tumors in animals can serve as a useful model for human neoplasia, the lack of correlation between anticancer drug activity in animal screens and in childhood neuroblastoma is striking. The C1300 murine system characteristics which make it attractive to the chemotherapist are: transplantability, uniform lethality, responsiveness to cyclophosphamide [1] and to radiotherapy [13]. Furthermore, the tumor has been found to have catecholamine-like substances on electron microscopic chemical studies [9].

The growth of a tumor depends on the length of the cell cycle, the fraction of proliferating cells and the rate of cell loss. In a uniformly distributed, uniformly cycling population, pulse-labeling with tritiated thymidine results in a "trapezoid" percent labeled mitosis (PLM) curve with 100% of the cells labeled. However, if cells are distributed uniformly in cycle, they have transit times which obey Gaussian distributions with variation in the transit times. Methods used to analyze these cell cycle curves have included the "boundary method" including the "half-height method" [12]. Since curves with various transit times demonstrate isocyclic waves, it is difficult to analyze them without arbitrarily extrapolating the tails of the first wave to zero PLM or inserting an arbitrary boundary between the first and second troughs. However, PLM curves consist not only of variable transit times but also exponential age distributions. The error with the boundary method increases as the height of the first peak lowers; it is for this reason that Mendelsohn and Takahashi proposed the peak-height estimator level based on a synthetic PLM curve; their methodology was utilized in the calculation reported for the C1300 neuroblastoma model.

The PLM peak of 66% is another reflection of a commonly observed finding; namely, damping-out of labeled mitosis. It emphasizes the point that the growth of a tumor is the result of cell production and various types of cell loss. Deviations in the median cell cycle time and the population doubling time of solid tumors may result from a decreasing growth fraction, increasing cell loss, increasing cell cycle time, or any combination thereof [14].

The murine system has a short DNA synthesis (Ts) time, which is similar to that seen in the ten-day murine embryonic neural tube [15]. The 4·0 hr Ts in the embryo suggests the intriguing speculation that the murine C1300's kinetic characteristics are consistent with that of undifferentiated neural tissue [16]. The short generation time (Tc) in the embryonic neural tube is in keeping with the observation that during the course of differentiation, the need for specific proteins

Table 2. Kinetic parameters of the C1300 murine neuroblastoma system and selected animal and human tumors

Tumor	Cell mass (days after) implant)	Approximate doubling time (days)	(*a*) Ts (hr)	(*b*) Tc (hr)	Ts/Tc	References
Murine C1300	(10 days)	10	4·52	14·1	0·32	
L1210 Leukemia	(0 days)		9	12·8	0·7	[19]
L1210 Leukemia	(6 days)	0·6 or >	8·9	11·8	0·74	[20]
Mouse Embryo Neural Tube	n/a	n/a	4·0	8·4	0·48	[15]
Lewis Lung	340 mg	2·9	8·5	18·8	0·45	[20]
Sa 180	600 mg	1·2	7·5	13·0	0·57	[21]
B16 Melanoma	560 mg	1·5	7·0	20·0	0·35	[3]
Leukemia Man	10^{12} ca	4 or >	ca 20	ca 40–80	ca ·25–·50	[22]
Neuroblastoma Children		4·2		40		[23, 24]

(*a*) Ts = DNA synthesis time.
(*b*) Tc = Total generation time.

results in a lengthening of the duration of the cell cycle.

The cell cycle characteristics of the C1300 system resemble other rodent tumors in that the generation time is between 11 and 20 hr (Table 2). The ratio of Ts/Tc closely parallels those observed in the Lewis lung and B16 melanoma. The doubling time of 10 days was obtained using time of maximum tumor size; this is consistent with a Gompertzian curve distribution. Kinetic data can be of help in the design of chemotherapy. In that respect, the C1300 resembles these rodent tumors since it appears to be preferentially sensitive to cell cycle phase nonspecific agents. This is not surprising since most animal tumor systems are sensitive to relatively high doses of alkylating agents [17]. Nevertheless, the kinetic characteristics and behavior of the C1300 system does provide the advantage of gaining basic knowledge regarding the relationship of kinetic and therapeutic data in a "neural" neoplasm with its short DNA synthesis time. Its relevance to human disease requires further study.

The cellular kinetics of the murine model with its short DNA synthesis time may explain the lack of activity of cell cycle specific agents. Furthermore, a review of the active agents in children with metastatic disease reveals that cyclophosphamide and other cell cycle nonspecific agents should be attempted in humans with this disease. In fact, an increased response rate in children with metastatic disease is found in patients receiving combination therapy with cyclophosphamide and imidazole carboxamide, two cell cycle nonspecific agents, plus vincristine [18]. The sensitivity of the murine neuroblastoma to cytosine arabinoside [1] may be due to its unique biologic characteristics and emphasizes the need to further evaluate the model as a chemotherapy model.

Acknowledgement—The authors wish to acknowledge the excellent technical assistance of Mss. Rowie Meshnik, Joan Scher and Karen Tittle.

REFERENCES

1. J. Z. Finklestein, E. Arima, P. E. Byfield, J. E. Byfield and E. W. Fonkalsrud, Murine neuroblastoma: A model of human disease. *Cancer Chemotherp. Rep.* **57,** 405 (1973).
2. S. A. Schepartz, Screening. *Cancer Chemother. Rep.* Part 3, **2,** 3 (1971).
3. D. P. Griswold, Jr., Consideration of the subcutaneously implanted B16 melanoma as a screening model for potential anticancer agents. *Cancer Chemother. Rep.* Part 2, **1,** 315 (1972).
4. W. G. Thurman and M. H. Donaldson, Cyclophosphamide (NSC-26271) therapy for children with neuroblastoma. *Cancer Chemother. Rep.* **51,** 399 (1967).
5. A. E. Evans, R. M. Heyn, W. A. Newton, Jr., and S. Leikin, Vincristine sulfate and cyclophosphamide for children with metastatic neuroblastoma. *J. Amer. med. Ass.* **207,** 1325 (1969).

6. G. Augusti-Tocco and G. Sato, Establishment of functional clonal lines of neurons from mouse neuroblastoma. *Proc. nat. Acad. Sci.* (*Wash.*) **64,** 311 (1969).
7. D. Schubert, S. Humphreys, C. Baroni and M. Cohn, *In vitro* differentiation of a mouse neuroblastoma. *Proc. nat. Acad. Sci.* (*Wash.*) **64,** 316 (1969).
8. N. W. Seeds, A. G. Gilman, T. Amano and M. W. Nirenberg, Regulation of axon formation by clonal lines of a neural tumor. *Proc. nat. Acad. Sci.* (*Wash.*) **66,** 160 (1970).
9. J. Z. Finklestein, K. L. Tittle, F. Hirose, H. Itabashi, A. Mitchell and J. Weiner, A new approach to the design of effective treatment for neuroblastoma. *Pediat. Res.* **8,** 400 (1974).
10. E. Frindel, E. P. Malaise, E. Alpen and M. Tubiana, Kinetics of cell proliferation of an experimental tumor. *Cancer Res.* **27,** 1122 (1967).
11. B. M. Kopriwa and C. P. Leblond, Improvements in the coating technique of radioautography. *J. Histochem. Cytochem.* **10,** 269 (1962).
12. M. L. Mendelsohn and M. Takahashi, A critical evaluation of the fraction of labeled mitosis method as applied to the analysis of tumor and other cell cycles. In *The Cell Cycle and Cancer.* (Edited by R. Baserga) p. 58. Marcel Dekker, New York (1971).
13. E. Arima, J. E. Byfield, J. Z. Finklestein and E. W. Fonkalsrud, An experimental model for the therapy of mouse neuroblastoma. *J. Pediat. Surg.* **8,** 757 (1973).
14. R. Baserga, The relationship of the cell cycle to tumor growth and control of cell division: A review. *Cancer Res.* **25,** 581 (1965).
15. S. L. Kauffman, An autoradiographic study of the generation cycle in the ten day mouse embryo neural tube. *Exp. Cell Res.* **42,** 67 (1966).
16. D. Malamud, Differentiation and the cell cycle, In *The Cell Cycle and Cancer.* (Edited by R. Baserga) p. 132. Marcel Dekker, New York (1971).
17. H. E. Skipper, The cell cycle and chemotherapy of cancer, In *The Cell Cycle and Cancer.* (Edited by R. Baserga) p. 358. Marcel Dekker, New York (1971).
18. J. Z. Finklestein, S. Leikin, A. Evans, M. Klemperer, I. Bernstein, R. Hittle and G. D. Hammond, Chemotherapy for metastatic neuroblastoma. *Proc. Amer. Ass. Cancer Res.* **15,** 44 (1974).
19. G. P. Wheeler, B. J. Bowdon, L. J. Wilkoff and E. A. Dulmadge, The cell cycle of leukemia L1210 cells *in vivo* and *in vitro. Proc. Soc. exp. Biol.* **126,** 903 (1967).
20. R. A. Yankee, V. T. Devita and S. Perry, The cell cycle of leukemia L1210 cells *in vivo. Cancer Res.* **27,** 2381 (1967).
21. D. P. Griswold, F. M. Schabel, W. S. Wilcox, L. Simpson-Herren and H. E. Skipper, Success and failure in the treatment of solid tumors. I. Effects of cyclophosphamide (NSC-26271) on primary and metastatic plasmacytoma in the hamster. *Cancer Chemother. Rep.* **52,** 345 (1968).
22. S. Killmann, Acute leukemia: The kinetics of leukemic blast cells in man. *Ser. Haematol.* **1,** 38 (1968).
23. H. P. Wagner and H. Käser, Cell proliferation in neuroblastoma. *Europ. J. Cancer* **6,** 369 (1968).
24. W. Aherne and P. Buck, The potential cell population doubling time in neuroblastoma and nephroblastoma. *Brit. J. Cancer* **25,** 691 (1971).

Europ. J. Cancer Vol. 11, pp. 181–185. Pergamon Press 1975. Printed in Great Britain

Mechanism of the Immunosuppressive Effect of Ehrlich Ascitic Tumour

IVO HRŠAK and TANJA MAROTTI

Laboratory for Experimental Therapy, Institute "Ruder Bošković", Zagreb, Yugoslavia

Abstract—*Mice were given one i.p. injection of 0·3 ml of the cell-free Ehrlich ascitic fluid (EAF). Seven to 14 days later, the generation of antibody-forming cells (PFC's) in their spleens was suppressed. However, when spleen cells from EAF treated mice were transfererd into normal lethally irradiated recipients, the cells were able to generate normal numbers of PFC's. On the other hand, spleen cells or bone marrow and thymus cell mixtures from normal donors were also completely inefficient in producing PFC's when assayed in EAF-treated and irradiated recipients. Incubation of spleen cells, bone marrow cells or thymus cells with EAF prior to transplantation, as well as simultaneous injection of spleen cells together with EAF into irradiated animals, did not inhibit the normal immune performances of these cells. These data suggest that EAF may induce immunosuppression by changing the microenvironment in the spleen, rather than by acting directly on the cells involved in the immune response.*

INTRODUCTION

THE APPEARANCE of the growth of malignant tumours is often closely related to the deficient immunologic functions of the organism [1, 2]. Although it is not always clear as to whether the immunosuppression is the cause or only the consequence of the tumour growth the data show that humoral and cellular immunologic reactivities of tumour-bearing animals are very often depressed. This seems to be especially valid for non-specific tumours, like Ehrlich carcinoma, which grow in a variety of mouse strains [3, 4].

We have shown that the cell-free ascitic fluid of Ehrlich tumour contains an agent which, after transfer into normal mice, can inhibit strongly the immune reactivity of their spleen cells against sheep erythrocytes [5]. Normal immune responses to particulate antigens require the function of three different cell types, *i.e.*, macrophages, T and B lymphocytes [6, 7]. It thus seemed a reasonable assumption that the immunosuppressive agent in Ehrlich ascitic fluid (EAF) could act by directly inhibiting these cells. Our data indicate, however, that this agent does not affect the function of either of these cells selectively, but rather, may initiate changes in the microenvironment of the spleen resulting in an impaired immune response [8]. This paper presents additional data which corroborates this hypothesis.

Accepted 18 October 1974.

MATERIAL AND METHODS

Mice

Male and female LAF_1 mice (hybrids between C57BL ♀ and A ♂ strains) were used when 3–5 months old. They were kept in plastic cages on a standard diet with food and water given *ad libitum.*

Ehrlich ascitic fluid (EAF)

Near-tetraploid line of Ehrlich ascites carcinoma was maintained in LAF_1 mice by serial i.p. transplantations. Cell-free ascitic fluid of this tumour—*i.e.*, supernatant of the whole ascites that had been spun down for 10 min at 2·100 rev/min, was collected from 10

to 30 mice with terminal ascites, and pooled. The ascitic fluid of one pool was distributed into glass vials (2 ml each), frozen at −18°C, and the quantity for each experiment was thaw off just before use. To obtain maximal immunosuppression, the mice received 0·3 ml of EAF i.p. 7 to 14 days prior to antigen.

PFC assay

The immunologic response to sheep erythrocytes (SRBC) was determined by counting the cells forming haemolytic antibodies (PFC's) in the spleen. Mice were immunized with 5×10^8 SRBC and 4 days later the number of PFC's was counted according to the method of Jerne *et al.* [9] modified for use on microscopic slides [5].

In transfer experiments, where the reactivity of spleen cells or mixtures of bone marrow and thymus cells were assayed in lethally irradiated (850 r of X-rays) recipients, the procedure described by Playfair was followed [10]. Four hours after irradiation (day 0) mice received 25×10^6 viable syngeneic spleen cells, or mixtures of thymocytes (50×10^6 cells) and bone marrow cells ($2{\cdot}5 \times 10^6$), along with 2×10^7 sheep erythrocytes. Four days later they were boosted with 2×10^8 SRBC and on day 8 were sacrificed for PFC assay.

In some experiments, transplanted cells were incubated with EAF just before transfer. Cell suspensions were incubated in Hank's solution at 37°C for 30 min with 0·3 ml of EAF per 50×10^6 thymocytes or splenocytes, and with 0·1 ml of EAF per 10×10^6 bone marrow cells. After incubation the cells were washed once, and then injected into the recipients.

Assay for in vitro *phagocytic activity*

Spleen cells were mixed with fresh suspensions of Escherichia coli K-12 bacteria in the ratio of 20 bacteria to 1 cell. Five millilitres of splenocytes ($5–7 \times 10^6$ cells per ml) in Hank's solution were incubated with adequate numbers of E. coli for 1 hr at 37°C. To some suspensions 0·1 ml of EAF was added before incubation. Thereafter, the suspensions were centrifugated to sediment the cells, the supernatant was discarded, and the pellet resuspended to the original volume with Hank's solution. Several smears were made from each suspension, and were stained by the standard May–Grünwald–Giemsa method. One thousand cells were examined on each smear, and only the cells which had engulfed 5 or more bacteria were counted as phagocytes.

RESULTS

The number of antibody-forming cells (PFC's) in the spleens of mice injected with EAF or Hank's solution 11 days before sheep erythrocytes is shown in Fig. 1. Animals injected with Hank's were used as controls because earlier results showed that injection of reticulosarcoma ascitic fluid [5], as well as the injection of heat-inactivated EAF [8], did not alter the PFC forming capacity of the recipients. At the time of maximal response in control animals, *i.e.*, 4 days after antigen injection, the formation of PFC's was markedly suppressed in mice treated with EAF (14·5% of control value). Significant suppression persisted through day 7 of the immune response. This clearly shows that one injection of EAF inhibits, rather than postpones the normal response to sheep erythrocytes.

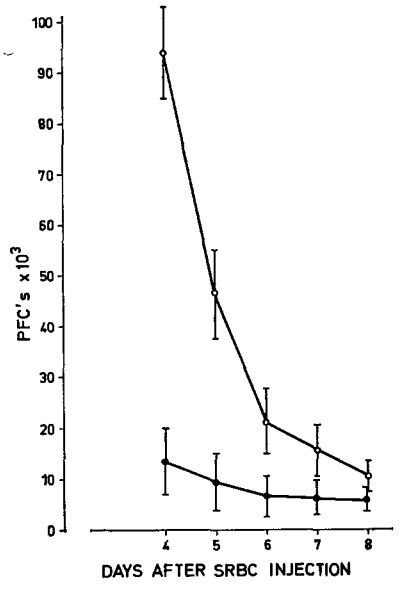


Fig. 1. *Total number of PFC's per spleen in mice injected with EAF (black circles) or Hank's solution (white circles) 11 days before SRBC. Each point represents mean value ± SE of 4–8 individual results.*

In the next experiment the ability of spleen cells as well as of thymocytes plus bone marrow cell mixtures (T plus B cells) to form PFC's was assayed in lethally irradiated recipients. The cells from EAF treated animals were able to initiate the same numbers of PFC's as could the corresponding cell suspensions from normal donors (Table 1, groups 2 and 5). On the other hand, splenocytes and T plus B cell mixtures from normal donors could not induce a PFC response in recipients injected with EAF 11 days earlier (Table 1, groups 3 and 6).

Lethally irradiated mice were used to assay the immune reactivity of cells incubated *in vitro* with EAF before transplantation. Thymocytes,

Table 1. *Total number of PFC's (mean ± S.E.) in the spleens of lethally irradiated mice* 8 *days after transplantation of splenocytes or thymocytes plus bone marrow cells. Donors were injected with EAF or Hank's* 7 *days before adoptive transfer, and recipients* 7 *days before irradiation*

Group	Donor	Recipient	Cells transplanted	No. of mice	PFC No.	PFC %
1.	Normal	Normal	25×10^6 splenocytes	4	33·065 ± 10·980	100
2.	EAF-treated	Normal		6	28·434 ± 6·434	89
3.	Normal	EAF-treated		6	365 ± 122	1·1
4.	Normal	Normal	50×10^6 thymocytes plus $2{\cdot}5 \times 10^6$ bone marrow cells	9	7·230 ± 2·318	100
5.	EAF-treated	Normal		6	8·058 ± 1·997	111
6.	Normal	EAF-treated		8	30 ± 26	0·4

bone marrow cells and splenocytes from normal donors were incubated separately with EAF for 30 min and were then transplanted into irradiated recipients. The results presented in Table 2 show that irrespective of cell type used EAF did not alter their immune performance in irradiated recipients.

Table 2. *Total number of PFC's (mean ± S.E.) in the spleens of lethally irradiated recipients of thymocytes plus bone marrow cells or splenocytes. Transplanted cells were incubated with EAF or media alone for* 30 *min prior to adoptive transfer*

Thymocytes + Bone marrow cells incubated with		No. of mice	PFC No.	PFC %
Media alone	Media alone	6	5·292 ± 3·340	100
EAF	Media alone	8	7·132 ± 2·578	134
Media alone	EAF	8	6·153 ± 3·215	116
EAF	EAF	8	8·229 ± 3·057	155
Splenocytes incubated with				
EAF		6	20·882 ± 4·416	132
Media alone		6	15·715 ± 2·266	100

Moreover, when spleen cells and SRBC were injected into irradiated recipients simultaneously with EAF, normal numbers of PFC's were generated. On the other hand, if irradiated recipients were given spleen cells and EAF but no antigen on day 0 and SRBC on day 4, the production of PFC's was again blocked (Table 3). Evidently, EAF may induce immunosuppression only if injected into the recipients several days before antigen.

In the last experiment, the influence of EAF on the phagocytic activity of spleen cells was tested *in vitro*. If the number of cells phagocyting

Table 3. *Total number of PFC's (mean ± S.E.) in the spleens of lethally irradiated mice* 8 *days after injection of spleen cells and EAF. SRBC were given either together with the spleen cells (day* 0), 4 *days later, or at both times*

Treatment of the recipients in addition to splenocytes day 0	day 4	No. of mice	PFC No.	PFC %
SRBC	SRBC	6	14·880 ± 4·935	100
SRBC + EAF	SRBC	8	15·486 ± 2·098	104
EAF	SRBC	6	218 ± 92	1·4
Hank's	SRBC	6	13·670 ± 5·304	91

E. coli bacteria in the spleens of mice treated with EAF is compared with similar cells in the spleens of control mice, no differences could be observed. However, normal splenocytes assayed in the medium containing EAF, showed significantly reduced phagocytic activity (Table 4).

Table 4. In vitro *phagocytic activity of spleen cells from mice treated* 11 *days previously with EAF or Hank's; and the phagocytic activity of splenocytes in the presence or absence of EAF. The results represent the number of cells phagocytizing E. coli bacteria per* 1000 *splenocytes*

Mice treated with	No. of mice	% of phagocytes range	% of phagocytes mean	p*
EAF	8	38–84	52·8	N.S.
Hank's	6	39–90	58·2	
Normal splenocytes incubated with				
EAF	7	31–56	44.4	0·014
Media alone	8	47–94	62·0	

*Mann-Whitney U test [11].

DISCUSSION

The principal aim of the experiments described was to investigate the cellular mechanism of the immunosuppressive effect of Ehrlich ascitic fluid. Two main questions were raised: Firstly, is EAF cytotoxic for lymphoid cells or does it simply block their function? Secondly, what type of cells are affected: T cells, B cells or macrophages?

The fact that cells from immunosuppressed mice, as well as the cells incubated *in vitro* with EAF prior to transplantation, are entirely immunocompetent in the irradiated normal recipients, strongly suggests that the active factor in EAF does not destroy the cells of the immune system, but only temporarily impairs their function.

Studies of the others have indicated that EAF possibly affects T and B cells. Thus, Loring and Schlesinger observed that theta positive (T) lymphocytes disappeared in thymuses and lymph nodes of RIII mice bearing Ehrlich tumour [12]. Futhermore, a virus similar to Friend virus was isolated from the Ehrlich ascitic cells [13], and Friend virus has been shown to selectively inhibit the function of B lymphocytes [14]. Therefore, the immunosuppressive effect of EAF could be attributed to deficient T cell function, deficient B cell function, or both, but transfer experiments described herein disagree with this explanation.

Alternative candidates as the target for the action of EAF would be the macrophages. In our experiments the phagocytic activity of spleen cells *in vitro* was depressed if EAF was added to the incubating medium. EAF has been shown to contain a dialysable factor, which inhibits the formation of pseudopodia and adhesion of *L cells* in tissue culture to the glass [15]. It might be speculated, therefore, that this factor could affect interactions of macrophages with antigens or lymphoid cells. In the transfer experiments described, either recipient's or donor's macrophages could have been affected in this way. These two possibilities will be discussed.

Cells from normal donors (representing mixtures of T cells, B cells and macrophages) were blocked completely in their reactivity when transferred into lethally irradiated recipients treated with EAF 7 days before. One explanation could be that in this experimental system macrophages of the *recipient* are needed for processing the antigen [7], and that PFC-formation failed because these cells, under the influence of EAF, did not function. The weak point of this idea is that the ability of irradiated macrophages to participate in the immune response against SRBC has been denied [15]. In addition, it has been shown that irradiated macrophages can only engulf particulate antigens, but not destroy and process them [16].

Therefore, if the role of recipient's macrophages is doubtful, another explanation of the observed data would be that the immunosuppressive agent has remained active in the recipient for some time after EAF injection, and incapacitated macrophages of the *donor*. This idea can be questioned on the basis of the experimental data presented. Firstly, the phagocytic activity of cells obtained from the immunosuppressed animals (as well as their PFC-producing ability) recoveres promptly and completely if the cells are in the environment without EAF. Secondly, there is no immunosuppression if spleen cells are incubated with EAF before transfer into irradiated test animals, or if spleen cells and EAF are inoculated simultaneously.

Because of these uncertainties we prefer the following explanation for the mechanism by which the ascitic fluid of the Ehrlich tumour inhibits immune responses. We suppose that EAF exerts its effect by affecting the environment in which the immune response is initiated. Indeed, Frost and Lance [17] have reported that the "trapping" of recirculating lymphocytes in the spleen (which is probably a necessary event in the immune response to antigens like sheep erythrocytes), was greatly reduced in mice bearing an ascitic tumour. Moreover, normal animals injected with syngeneic cell-free ascitic fluid also demonstrated defective trapping. Thus, we think that after injection of EAF, the splenic environment is very probably altered in that the accumulation of immunocompetent cells is inhibited and the necessary cooperation processes between various cell types in the immune response are prevented.

Acknowledgements—We acknowledge the discussions with Dr. M. Boranić, and greatly appreciate the technical assistance of Mrs. Lj. Bošković and Mrs. J. Miholjević.

REFERENCES

1. N. Trainin, M. Linker-Israeli, M. Small and L. Boiato-Chen, Enhancement of lung adenoma formation by neonatal thymectomy in mice treated with dimethylbenzantracene or urethane. *Int. J. Cancer* **2,** 326 (1967).
2. R. Gershon and R. L. Carter, Facilitation of metastatic growth by antilymphocyte serum. *Nature (Lond.)* **226,** 368 (1970).
3. R. E. McCarthy, Modification of the immune response of mice to skin homografts and heterografts by Ehrlich carcinoma cells. *Cancer Res.* **24,** 915 (1964).
4. A. Nowotny, J. Grohsman, A. Abdelnoor, N. Rote, C. Yang and R. Waltersdorff, Escape of TA3 tumors from allogeneic immune rejection: theory and experiments. *Europ. J. Immunol.* **4,** 73 (1974).
5. I. Hršak and T. Marotti, Immunosuppression mediated by Ehrlich ascites fluid. *Europ. J. Cancer* **9,** 717 (1973).
6. G. F. Mitchell and J. F. A. P. Miller, Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. exp. Med.* **128,** 821 (1968).
7. K. Shortman, E. Diener, P. Russel and W. D. Armstrong, The role of nonlymphoid accessory cells in the immune response to different antigens. *J. exp. Med.* **131,** 461 (1970).
8. I. Hršak and T. Marotti, Mode of immunosuppressive action of Ehrlich ascitic fluid. *J. nat. Cancer Inst.* In press (1974).
9. N. K. Jerne, A. A. Nordin and O. Henry, The agar plaque technique for recognizing antibody forming cells. In *Cell Bound Antibodies.* Edited by B. Amos and H. Koprowski, p. 109. Wistar Institute Press, Philadelphia (1963).
10. J. L. Playfair and E. C. Purves, Antibody formation by bone marrow cells in irradiated mice. I. Thymus-dependent and thymus-independent responses to sheep erythrocytes. *Immunology* **21,** 113 (1971).
11. S. Siegel, Mann-Whitney U test. In *Nonparametric Statistics for the Behavioural Sciences,* p. 116, McGraw-Hill, New York (1956).
12. M. Loring and M. Schlesinger, The θ antigenicity of lymphoid organs of mice bearing the Ehrlich ascites tumor. *Cancer Res.* **30,** 2204 (1970).
13. C. Friend, Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. *J. exp. Med.* **105,** 307 (1957).
14. M. Bennett and R. A. Steeves, Immunocompetent cell functions in mice infected with Friend leukemia virus. *J. nat. Cancer Inst.* **44,** 1107 (1970).
15. B. Holmberg, Inhibition of cellular adhesion and pseudopodia formation by dialysable factor from tumour fluids. *Nature (Lond.)* **195,** 45 (1962).
16. L. E. Gordon, D. B. Cooper and C. P. Miller, Clearance of bacteria from the blood of irradiated rabbits. *Proc. Soc. exp. Biol. (N.Y.)* **89,** 577 (1955).
17. P. Frost and E. M. Lance, Abrogation of lymphocyte trapping by ascitic tumours. *Nature (Lond.)* **246,** 101 (1973).

Europ. J Cancer Vol. 11, pp. 187–191. Pergamon Press 1975. Printed in Great Britain

Cell-mediated and Humoral Immunity in Dogs with Spontaneous Lymphosarcoma*

L. N. OWEN, D. E. BOSTOCK and R. E. W. HALLIWELL†‡
School of Veterinary Medicine, University of Cambridge, England

Abstract—*Full thickness skin grafts were made in* 10 *dogs with spontaneous lymphosarcoma, in normal dogs and in new born dogs previously injected with donor spleen cells* in utero. *The rejection of skin grafts appeared histologically as a graft rejection and was essentially similar in both lymphosarcoma and normal dogs. In some of the injected new born dogs skin graft survival was greatly prolonged.*

For measurement of humoral immunity, a modification of the red cell linked antigen antiglobulin test was used. In normal adult dogs and dogs with lymphosarcoma there was no significant difference in the primary response of the two groups. There was, however, a difference in the secondary response, the lymphosarcoma group being significantly lower than the normals.

INTRODUCTION

LYMPHOSARCOMA or lymphatic leukosis in the dog is a common disease with malignant features which make it a suitable model for the study of lymphosarcoma in man. Jarrett, Crighton and Dalton [1] found 22% of 630 fatal neoplasms in the dog to be of this type. The condition occurs as either an alimentary/mesenteric complex or as a multicentric type. All cases reported in the present study were of multicentric type and were all confirmed by histological examination of a lymph node biopsy. In this manifestation of the disease there is bilateral enlargement of superficial lymph nodes, those most frequently involved being the submandibular, prescapular, superficial inguinal, and popliteal. Hepato-splenomegaly and tumour involvement of many organs including tonsils, heart, lungs, kidneys, bone marrow and pancreas are common. Some cases have skin involvement and in others the anterior chamber of the eye and the spinal meninges are invaded by neoplastic cells.

As the clinical signs and pathological appearance of the condition in the dog so closely resemble lymphosarcoma in man, the dog could be a useful model for immunological studies. In all domestic animal models for cancer in man, basic information on the immunological status of the animal is required. The present studies were designed to increase knowledge in this field so that studies in immunotherapy can develop further in this relatively common malignant disease in the dog.

Full thickness skin grafts have been made and the detection of anti-DNP antibodies performed.

Accepted 15 October 1974.

*Supported by grants from the Medical Research Council and the Cancer Research Campaign.

†Present address: University of Pennsylvania, The School of Veterinary Medicine, 3800 Spruce Street, Philadelphia, Pennsylvania, 19104, USA.

‡Recipient of a Wellcome Research Fellowship.

MATERIAL AND METHODS

Lymphosarcoma cases

Diagnosis was made by lymph node biopsy. Of the 10 lymphosarcoma dogs, 4 were males

and 6 females. The ages varied from 2–8 yr. Eight different breeds and one mongrel were represented.

Skin grafting

Full thickness skin grafts were made from healthy donor outbred Collies, Collie crosses and mongrels. Grafts were circular, 1·5 cm in diameter and were sutured with 5/0 silk. Some grafts were made on a hind limb and others on one flank. Grafts were covered with Vaseline gauze and bandaged firmly to avoid any self-inflicted trauma. After 2 days the bandage was changed and dry dressings only were used. Grafts were subsequently examined daily [2].

The main criterion used to determine rejection was a progressive change in skin colour. Skin colour in grafts undergoing rejection varied from deep red to bluish–brown and within 2–3 days of the appearance of these colour changes, epithelial necrosis was usually apparent followed either by scab formation or ulceration.

Ten control dogs, (5 adult and 5 new born) and 10 dogs with lymphosarcoma were skin grafted. Five of the latter (Nos. 1–5) were injected also with dinitrophenylated human serum albumin as later described.

Antigens employed

Human serum albumin (Cohn fraction V) was obtained from Koch–Light Laboratories and dinitrophenylated according to the method of Eisen, Bellman and Carsten [3]. Protein was separated from the reactant mixture by filtration through a column of Sephadex G25 (Pharmacia, Uppsala, Sweden). Protein determination was made by the Folin–Ciocalteau reaction using unconjugated HSA as the standard. The extent of substitution was estimated by measuring the absorbance at 360 mμM and assuming a molar extinction coefficient of $1{\cdot}75 \times 10^4$ for the dinitrophenyl group. The value for moles of DNP per mg of protein was $2{\cdot}4 \times 10^{-4}$.

Preparation of antisera

Antisera specific for the canine immunoglobulin classes and sub-classes were prepared as previously described [4].

Rabbit anti-sheep red cell serum was prepared by injecting rabbits with 3 bi-weekly injections of 0·5 ml of packed washed SRBCs intravenously.

Detection of anti-DNP antibodies

A modification of the red cell linked antigen antiglobulin test (RCLAAT) of Coombs, Howard and Mynors [5] was employed for the detection of the class-specific anti-DNP response. Sheep red blood cells (SRBCs) coated with a dinitrophenylated monovalent Fab preparation of rabbit-anti-SRBCs formed the indicator cells. A 40% ammonium sulphate precipitate of rabbit-anti-SRBC was digested with 1% crystalline papain (Sigma) in 0·1M phosphate buffer, pH 7, in the presence of 0·01M L-cysteine and 0·002M EDTA at 37°C for 18 hr. The digested IgG was separated from any undigested antibody by filtration through a column of Sephadex G200, and every third tube of the eluate tested for SRBC agglutinating activity. The pooled and concentrated Fab'-anti-SRBC was heavily dinitrophenylated as described above.

Optimum ratios of DNP/Fab' anti-SRBC, SRBCs, and of each antiglobulin serum were determined in preliminary experiments using serum from a dog hyperimmunised against DNP. Indicator SRBCs were prepared by incubating washed SRBCs with DNP/Fab'-anti-SRBC at room temperature for 1 hr. After washing, equal quantities of indicator cells and dilutions of the test sera were incubated at room temperature for 30 min. The final wash utilized 0·01% bovine serum albumin in PBS, and the agglutination reaction was carried out in microtitre trays.

Immunisation schedules

Ten dogs received two injections of 10 mg of DNP/HSA in PBS s.c. at two-week intervals. Five of these dogs were also skin grafted (No. 1–5).

Six normal dogs served as controls.

RESULTS

Skin grafting

The survival times of full thickness skin from normal dogs grafted on to lymphosarcoma dogs and on to control normal new born (5 dogs) and adult (5 dogs) animals is shown in Table 1. The survival times varied from 4–11 days in lymphosarcoma dogs and 4–10 days in control dogs.

The technique of transplantation would appear to be satisfactory as experiments on transplantation tolerance in the dog have shown previously [2]. Approximately 10^8

Table 1. *Survival times in days of normal full thickness skin grafts in dogs with lymphosarcoma, in normal dogs and in dogs injected with donor spleen cells as foetuses* in utero *and skin grafted after birth*

Lymphosarcoma dogs		Control dogs		Dogs injected *in utero*	
1. Alsatian	5	A	6	4	54 days gestation
2. Mongrel	4	NB	4	8	
3. Gt. Dane	10	A	8	10	50 days gestation
4. Schnauzer	11	NB	9	60	
5. Scottish Terrier	6	A	6	10	49 days gestation
6. Scottish Terrier	6	NB	5	10	
7. Geman Schnauger	6	A	7	27	
8. Cocker Spaniel	6	A	7	20	41 days gestation
9. Boxer	6	NB	8	240	
10. Springer Spaniel	11	NB	10	—	

A = Adult
NB = Newborn (36 hr approx.)
Lymphosacoma dogs 1–5 also injected with DNP/HSA.

allogeneic spleen cells from totally unrelated dogs were injected i.p. into canine foetuses and skin grafts from the donors made 36 hr after the puppies were born. Results are shown in Table 1. Three of 9 animals injected between 41–54 days of gestation sustained grafts for 27, 60 and 240 days—considerably longer than control animals. In 5 lymphosarcoma dogs and in all control dogs where grafts were rejected between 4–11 days, biopsies were made from these animals 2–3 days after the grafts had been assessed as rejected. Histological examination of the rejected grafts revealed extreme infiltration of the deeper layers of the dermis by mononuclear cells among which macrophages, plasma cells and lymphocytes were conspicuous. Grafts from control and lymphosarcoma dogs had a similar appearance which was typical of a cell-mediated graft rejection reaction.

Humoral immunity

All animals were sampled 14 days after initial antigenic stimulation (primary response) and again 7 days after the second injection (secondary response). This timing was dictated by the frequency with which the lymphosarcoma cases, most of which were seen as outpatients, could be seen. The control animals were sampled twice weekly, and their antibody response was maximal around 7 days after each injection. Thus, the primary response in Table 2 does not represent a peak response, but the secondary response does. In some instances, the lymphosarcoma dogs could not be sampled for both primary and secondary responses and the tests in 2 dogs were technically unsatisfactory and have not been included.

The titres of both control and lymphosarcoma dogs in the primary response at the time this was sampled were low. Direct agglutination was usually either undetectable or detectable with undiluted sera only. Considerable enhancement, however, was produced by the antiglobulin sera. This was most marked using antisera to IgG_{2ab}, IgG, and IgM. One of the lymphosarcoma dogs, however, (Sealyham) had an excellent response, agglutinating with anti IgM to a titre of 128. Three lymphosarcoma dogs and one control animal had a low level of pre-existing anti-DNP activity. In calculating the titres, therefore, the pre-existing value was deducted from that following antigenic challenge prior to recording. One animal, an Airedale with lymphosarcoma, gave no detectable antibody response.

Following secondary antigenic stimulation, higher titres were obtained. Two of the normal animals gave titres of 64, one with IgG_{2ab}, and one with IgG . However, two of the lymphosarcoma dogs lacked a demonstrable antibody response. One was the Airedale which had no primary response and another was a Scottish Terrier in which no detectable increase in titre was recorded in the three samples taken at intervals after secondary immunisations.

Table 2. The primary and secondary antibody response to DNP/HSA in normal dogs and in dogs with lymphosarcoma

Normal dogs	Primary						Secondary					
No.	Direct Agg.	IgG_{2ab}	IgG_{2c}	IgG_1	IgA	IgM	Direct Agg.	IgG_{2ab}	IgG_{2c}	IgG_1	IgA	IgM
1.	0	1	0	1	0	1	2	16	2	4	2	4
2.*	1	2	1	1	1	1	2	4	2	2	2	2
3.	0	0	0	1	0	1	0	64	1	1	0	1
4.	1	4	1	1	1	4	1	8	1	2	1	16
5.	0	1	0	8	1	2	1	2	1	8	1	2
6.	1	4	1	8	1	8	0	8	1	64	2	8
Lymphosarcoma dog												
1. Alsatian	NT	NT	NT	NT	NT	NT	0	1	2	1	1	0
2. Mongrel	1	4	1	2	2	4	NT	NT	NT	NT	NT	NT
3. Gt. Dane*	2	4	2	2	4	4	4	4	2	4	4	4
4. Schnauzer*	4	4	4	4	8	2	8	16	16	16	16	16
5. Scottish Terrier*	NT	NT	NT	NT	NT	NT	0	0	0	0	0	0
6. Sealyham	4	64	32	64	16	128	4	8	4	16	4	32
7. Mongrel	1	0	1	2	2	2	1	2	2	1	1	1
8. Airedale	0	0	0	0	0	0	0	0	0	0	0	0

N.T. Test not performed.

* Animals having a pre-existing anti DNP titre which was deducted from the post-injection levels before recording the titre.

DISCUSSION

With the recognition that many tumours have antigens distinct from those of the host, a study of the immunologic status of tumour-bearing animals assumes great importance. This is of particular interest when the tumours involve organs concerned with the immune response, as is the case with lymphosarcomas.

It is established that there are two major lymphocyte populations, *viz.* T lymphocytes, whose prime concern is cell-mediated immunity, and B lymphocytes which are precursors of antibody forming cells. It is not clear whether lymphosarcomas can involve T or B lymphocytes or both, and so theoretically the tumour can interfere with either function. Furthermore there is the possibility that the tumour may involve B lymphocytes late in their differentiation and produce monoclonal gammopathies but a preliminary study of 15 sera from canine LS has failed to reveal any such cases in the dog (Halliwell and Owen, unpublished data).

The cell mediated immune response as determined by skin grafting in 10/10 dogs with spontaneous lymphosarcoma was similar to that found in normal dogs. The reports of Dennis *et al.* [6] on split thickness skin grafting in normal Beagles state that adult dogs usually rejected skin grafts 9–11 days after application. These times are longer than those of some of our dogs with full thickness grafts but the criteria of rejection also vary. The rejection of our full thickness skin grafts appeared histologically as a graft rejection and was essentially similar in both lymphosarcoma and normal dogs.

It is difficult to quantitate the true antibody response, despite apparently excellent information as to its degree and class. The sum of the titres recorded for all classes may be misleading for comparative purposes and is possibly more meaningful to compare the maximum titres recorded in each case, irrespective of class. If this is done, there is no statistically significant difference in the primary response of the two groups. There is, however, a difference in the secondary response, the lymphosarcoma group being significantly lower than the normals ($P = 0{\cdot}042$).

Howard [7] found that the early agglutinating antibody titre in the serum of 4 mastocytoma bearing dogs following injection of *Brucella abortus* (strain 19) vaccine indicated a significant deficiency of antibody production when compared with normal dogs.

Perryman, Hoover and Yohn [8] found there was immunosuppression in experimental feline lymphosarcoma in the pre-neoplastic period. Cats inoculated with Fe LV at birth had significantly longer cutaneous allograft survival times if tested after 5 weeks of the age than control cats. Humoral antibody responsiveness, measured by haemagglutination titres to sheep red blood cells, was not altered significantly during leukaemogenesis. Based on our

results reported in the dog, the cell-mediated immune response in lymphosarcoma in this species resembles lymphosarcoma in man rather than Hodgkin's disease as in patients with the latter condition skin grafts often have greatly prolonged survival times.

Acknowledgements—The investigation was supported by grants from the Medical Research Council and the Cancer Research Campaign. R.E.W.H. was in receipt of a Wellcome Research Fellowship.

REFERENCES

1. W. F. H. Jarrett, G. W. Crighton and R. G. Dalton, Leukaemia and lymphosarcoma in animals and man. I. Lymphosarcoma or leukaemia in the domestic animals. *Vet. Rec.* **79,** 693 (1966).
2. L. N. Owen, Transplantation of canine lymphocytic leukaemia. In *Unifying Concepts of Leukaemia.* (Edited by R. M. Dutcher and L. Chieio-Bianchi), p. 139. Karger, Basel (1973).
3. H. N. Eisen, S. Belman and M. E. Carsten, The reaction of 2,4-dinitrobenzenesulphonic acid with free amino group of proteins. *J. Amer. chem. Soc.* **75,** 4583 (1953).
4. R. E. W. Halliwell, R. M. Schwartzman and J. H. Rockey, Antigenic relationship between human IgE and canine IgE. *Clin. exp. Immunol.* **10,** 399 (1972).
5. R. R. A. Coombs, A. W. Howard and L. S. Mynors, A serological procedure theoretically capable of detecting incomplete or non-precipitating antibodies to soluble protein antigens. *Brit. J. exp. Path.* **34,** 525 (1953).
6. R. A. Dennis, R. O. Jacoby and R. A. Griesemer, Development of immunity in fetal dogs: Skin allograft rejection. *Amer. J. vet. Res.* **30,** 1511 (1969).
7. E. B. Howard, Immunologic defect in mastocytoma-bearing dogs. *J. Amer. vet. med. Ass.* **151,** 1308 (1967).
8. L. E. Perryman, E. A. Hoover and D. S. Yohn, Immunologic reactivity of the cat: Immunosuppression in experimental feline leukaemia. *J. nat. Cancer Inst.* **49,** 1357 (1972).

Europ. J. Cancer Vol. 11, pp. 193–197. Pergamon Press 1975. Printed in Great Britain

Antibody-induced Redistribution of Surface Immunoglobulins in Chronic Lymphatic Leukaemia

C. PALENCIA, G. BAUMGARTNER and W. KNAPP

*Institut für Immunologie der Universität Wien and 1. Medizinische Abteilung and Ludwig Boltzmann-Institut für Hämatologie und Leukämieforschung im Hanusch-Krankenhaus, Wien, Austria**

Abstract—*Membrane dynamics of chronic lymphatic leukaemia (CLL) cells treated with anti IgG $(Fab)_2$ antibodies were studied by means of the membrane immunofluorescence technique. It was found that cap formation of CLL lymphocytes was in 19 of 24 cases within the range found in healthy subjects. Only 5 patients showed a decrease in the percentage of capping cells (mean 34%). None of the 24 patients showed no capping at all and the fluorescence pattern also of the non-capping cells was never homogeneous but patchy. No correlation between cytostatic treatment of the clinical picture and cap formation could be found. These findings show that CLL is a heterogeneous disease not only in respect to the intracytoplasmic localization of Ig but also in respect to the membrane motility. Therefore to explain the functional impairment of leukaemic lymphocytes in general with a defect of the membrane motility is not, in our opinion, justified.*

INTRODUCTION

IT IS KNOWN that a subpopulation of lymphocytes, considered to be of B-cell nature, bears easily detectable immunoglobulins on the surface [1–4]. Chronic lymphatic leukaemia (CLL) is regarded as a malignant proliferation of B Lymphocytes, i.e. of precursors of antibody secreting cells [5, 6]. The further differentiation of CLL cells to immunoglobulin secreting cells, however, seems to be blocked, since cells with cytoplasmic Ig are rarely found in CLL [7]. Also, the finding of crystalline Ig inclusions in some cases of CLL may be interpreted as a side step from the normal differentiation to Ig secreting cells [8–10]. In addition to that, there are several reports in literature about membrane alterations in CLL [5, 11–13].

The information about the dynamic state of membrane Ig on CLL lymphocytes, however, is still limited. We observed in preliminary investigations with the immunofluorescent technique, impaired, or even the absence of capping of membrane Ig in some cases of CLL, while membrane Ig in other cases behaved completely normally in that respect. A similar observation has been made by Pernis (personal communication). Huber *et al.*, found with the autoradiographic technique, impaired or even absent capping in all 8 cases of CLL studied [14]. Since capping, endocytosis and shedding of membrane proteins after reaction with antibodies are possibly not only of interest in respect to the trigerring of cells but also in respect to antigenic modulation of cell membranes, we found it worthwhile to study the cap formation of lymphocytes more systematically in a series of patients with CLL.

Accepted 12 August 1974.

*This investigation was supported by "Fonds zur Förderung der Wissenschaftlichen Forschung in Osterreich".

MATERIAL AND METHODS

EDTA blood was obtained from 24 patients with chronic lymphatic leukaemia (CLL). A chronic lymphatic proliferation consisting mainly of mature small lymphocytes with compatible morphology in bone marrow and blood smears was the main criterion for diagnosis of CLL.

The separation of lymphocytes and the fluorescence staining technique was performed essentially as described previously [10, 15]. From one to three million viable lymphocytes suspended in 50 μl phosphate buffered saline with 1 % bovine serum albumine were stained for 30 min at 0°C with a fluorescein isothiocyanate labelled anti human IgG [$F(ab)_2$], washed and further incubated for 10 min at 37°C. The reactivity of the anti IgG [$F(ab_2$] conjugate with the three main Ig classes IgG, IgM and IgA was checked with the monoclonal bone marrow system [16]. For microscopical examination, one drop of the cell suspension was placed on a slide with a cover slip. Epi-illumination [17] was used as a standard procedure. Each microscope field was examined for fluorescence and also in phase contrast for cell morphology. Cap formation was considered if staining was confined to less than half of the cell surface.

RESULTS

In Table 1 (a), the results in patients under treatment with different drugs and radiotherapy are listed in order of increasing percentage of fluorescent capping cells in the peripheral blood. The number of lymphocytes ranged between 20,750 and 140,000 with a mean of 66,075. In the differential white blood cell count, the lymphocytes made up 80–97 % (mean of 91·5 %). The percentage of fluorescent lymphocytes ranged between 16 and 96 % (mean 56·1 %), and in 25–97 % of those capping formation was observed (mean 73·7 %).

Table 1 (b) shows the observation in CLL patients without treatment. The number of lymphocytes per mm^3 ranged between 10,900 and 220,000 with a mean of 57,490. In the

Table 1. *Percentage of membrane Ig positive cells with polar distribution of the membrane Ig after reaction with anti-Ig and incubation at* 37°C

Patient's name	Treatment	No. Leucocytes	% Lymphocytes	% Membrane Ig-positive	% Capping
(a) Treated patients					
V.H.	cyt.	72·000	93	16	25
R.H.	cyt.	31·000	93	55	38
K.M.	cyt.	65·000	92	96	70
W.L.	cyt.	25·000	90	28	75
B.A.	cyt., cort.	20·750	93	48	79
H.A.	cyt.	83·000	80	71	83
N.H.	cyt.	71·000	97	87	86
M.M.	cyt., cort.	140·000	97	58	91
P.F.	cyt.	126·000	90	43	93
A.A.	irr.	27·000	90	59	97
(b) Untreated patients					
W.M.	without	37·000	85	57	18
H.E.	without	38·000	88	69	39
K.A.	without	21·000	76	75	49
M.F.	without	53·000	93	80	74
M.M.	without	57·000	97	70	79
S.J.	without	44·000	87	61	82
P.M.	without	51·000	97	84	82
P. Mar.	without	19·700	83	78	83
K.H.	without	22·000	*	73	90
N.K.	without	65·000	99	61	90
S.E.	without	151·000	*	73	92
Z.H.	without	10·900	90	53	96
D.F.	without	31·000	83	69	97
F.A.	without	220·000	87	41	98

cyt = cytostatic drug *No test performed at date of investigation.

differential white blood cell count, the lymphocytes made up 76–99 % (mean 89 %). The percentage of fluorescent cells ranged between 41–84 % (mean 66 %) and those with a cap-like fluorescent pattern made up 18–98 % (mean 76 %).

DISCUSSION

The mobility of surface immunoglobulins as well as of other membrane bound antigens has been the object of extensive studies [3, 18], which have demonstrated that the formation of polar "caps" is the consequence of cross-linking by antibodies of surface components that can move freely in the plane of the lymphocytic membrane. These findings were in agreement with the hypothesis that the membrane proteins are partly embedded as particles in a double layer of phospholipids and free to move in the plane of the double layer itself [19]. The process of capping seems to be sequential, beginning with a microprecipitation of Ig–anti-Ig complexes after a simple diffusion which is followed by an active transport of these microprecipitates to one pole of the cell [18, 20]. In immunofluorescence, the microprecipitates can be seen as fluorescent patches and after transport to one pole of the cell as cap.

The capping phenomenon is not restricted to Ig–anti-Ig complexes but also includes several other membrane proteins. Concanavalin A, for example, induces capping of lymphocyte membrane glycans [21–23], anti-HL-A antibodies induce capping of transplantation antigens [24] and also tumor specific antigens are capped by the appropriate antibodies [25].

The formation of caps is followed by the disappearance of the capped membrane constituents from the surface. This is due mainly to pinocytosis, but also to some extent to shedding of the complexes [20, 26, 27]. The biological importance of the capping phenomenon is not as yet completely clear. It may play a role in lymphocyte stimulation or tolerance induction and it definitely should have some sort of cleaning function [18, 20]. The last point may be of special interest in regard to the expression of tumor specific antigens on the surface of the respective cells.

Therefore, it was of special interest to see if malignant B cells are also capable of forming Ig–anti-Ig caps. Reports about cap formation of human lymphocytes are controversial to some extent. Some authors report an impairment or complete absence of human lymphocytes [14, 27, 28], while others had no problems with it and used this phenomenon for the separation and characterization of independently moving structures on individual cells [15, 29, 30]. It seems that the induction of cap formation is not only dependent on the capability of cells to transport the membrane constituents, but also to some extent on the cap inducing reagents (Raff, personal communication). Besides that, the use of different techniques might also at least partially explain the controversial reports. For our investigations we used a FITC labelled rabbit anti-human IgG–$F(ab)_2$ conjugate which induced in normal peripheral human lymphocytes after incubation at 37°C for 10 min cap formation in 70–90 % of membrane Ig positive lymphocytes. Our results with this conjugate show that cap formation of CLL lymphocytes was in 19 of 24 cases within the range found in healthy subjects. Only five patients showed a decrease in the percentage of capping cells. The mean percentage of caps in these five patients was 34 % with a range of 18–48 %. None of the 24 patients showed no capping at all and the fluorescence pattern also of the non capping cells was never homogeneous but patchy. This indicates that there was in none of the cases a defect in the first step of the cap formation—the microprecipitation. The second step, however, the active transport of the aggregates to one pole of the cell, was obviously impaired in some cases. We do not know as yet the reason for that. Our results show that there is no correlation between cytostatic treatment and cap formation and as far as the limited number of patients allows any conclusion, there is also no correlation between the cap formation and the clinical picture.

Thus, it can be concluded that only in a proportion of CLL patients is capping of Ig–anti-Ig complexes impaired. It is therefore not justified to consider an impairment of membrane dynamics of CLL lymphocytes as a general phenomenon.

REFERENCES

1. E. Klein, T. Eskeland, M. Inove, R. Strom and B. Johannson, Surface immunoglobulin moieties on lymphoid cells. *Exp. cell. Res.* **62,** 133 (1970).
2. B. Pernis, L. Forni and L. Amante, Immunoglobulin spots on the surface of rabbit lymphocytes. *J. exp. Med.* **132,** 1001 (1970).

3. S. Petris and M. Raff, Normal distribution, patching and capping of lymphocyte surface immunoglobulin studied by electron microscopy. *Nature New Biology* **241,** 257 (1973).
4. M. C. Raff, Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology* **19,** 637 (1970).
5. A. C. Aisenberg and K. J. Bloch, Immunoglobulins on the surface of neoplastic lymphocytes. *New Engl. J. Med.* **287,** 272 (1972).
6. J. L. Preud'homme, M. Klein, P. Verroust and M. Seligmann, Immunoglobulins monoclonales de membrane dans les leucémies lymphoïdes chroniques. *Europ. J. clin. biol. Res.* **16,** 1025 (1971).
7. W. Hijmans, H. R. E. Schuit, Immunofluorescence studies on immunoglobulins in the lymphoid cells of human peripheral blood. *Clin. exp. Immunol.* **11,** 483 (1972).
8. D. Hurez, G. Flandrin, J. L. Preud'homme and M. Seligmann, Unreleased intracellular monoclonal macroglobulin in chronic lymphatic leukaemia. *Clin. exp. Immunol.* **10,** 223 (1972).
9. I. C. Cawley, C. R. Barker, R. D. Britchford and J. L. Smith, Intracellular IgA immunoglobulin crystals in chronic lymphocytic leukaemia. *Clin. exp. Immunol.* **13,** 407 (1973).
10. W. Knapp, H. R. E. Schuit, R. L. H. Bolhuis and W. Hijmans, Surface immunoglobulins in chronic lymphatic leukaemia, macroglobulinaemia and myelomatosis. *Clin. exp. Immunol.* **16,** 541 (1974).
11. M. Schweitzer, C. J. M. Melief, V. P. Eijsvogel, The nature of the transforming lymphocyte in chronic lymphocytic leukaemia. *Europ. J. Immunol.* **3,** 121 (1973).
12. H. -D. Menne and H. -D. Flad, Membrane dynamics of HL-A–anti-HL-A complexes of human normal and leukaemic lymphocytes. *Clin. exp. Immunol.* **14,** 57 (1973).
13. G. Michlmayr and H. Huber, Receptor sites for complement on certain human peripheral blood lymphocytes. *J. Immunol.* **105,** 670 (1970).
14. C. Huber, G. Micalmayr, H. Braunsteiner and H. Huber, Redistribution of immunoglobulin determinants on human lymphocytes in lymphoproliferative disorders. *Europ. J. Cancer* **10,** 517 (1974).
15. W. Knapp, R. L. H. Bolhuis, J. Rádl and W. Hijmans, Independent movement of IgD and IgM molecules on the surface of individual lymphocytes. *J. Immunol* **111,** 1295 (1973).
16. W. Hijmans, H. R. E. Schuit and F. Klein, An immunofluorescent procedure for the detection of intracellular immunoglobulins, *Clin. exp. Immunol.* **4,** 457 (1969).
17. J. S. Ploem, The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. *Z. wiss. Mikr.* **68,** 129 (1967).
18. R. B. Taylor, W. H. Duffus, M. C. Raff and S. Petris, Restribution and pinocytosis of lymphocytes surface immunoglobulin molecules induced by anti-immunoglobulin antibodies. *Nature New Biology*, **233,** 225 (1971).
19. S. J. Singer and G. L. Nicholson, The fluid mosaic model of the structure of cell membranes. *Science (N.Y.)* **175,** 720 (1972).
20. F. Loor, L. Forni and B. Pernis, The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins. *Europ. J. Immunol.* **2,** 203 (1972).
21. M. F. Greaves, S. Bauminger and G. Janossay, Lymphocyte activation. *Clin. exp. Immunol.* **3,** 537 (1972).
22. M. Inbar, B. Hannah and L. Sachs, Exp.-temperature sensitive activity on the surface membrane in the activation of lymphocytes by lectins. *Exp. Cell Res.* **76,** 143 (1973).
23. E. Karsenti and S. Avramras, The use of Concanavalin A in the study of the dynamics of lymphocyte membrane glycans. *Febs Letters* **32,** 238 (1973)
24. F. M. Kourilsky, D. Silvestre, C. Neauport-Sautes, Y. Loosfelt and J. Dausset, Antibody-induced redistribution of HL-A antigens at the cell surface. *Europ. J. Immunol.* **2,** 249 (1972).
25. J. E. Leonard, Cell surface antigen movement induction in hepatoma cells by antitumor antibody. *J. Immunol.* **111,** 4 (1973).

26. H. D. ENGERS and E. R. UNANUE, The fate of anti-Ig—surface Ig complexes on B lymphocytes. *J. Immunol.* **110,** 465 (1973).
27. E. R. UNANUE and M. J. KARNOSKY, Redistribution and fate of immunoglobulin complexes on surface of B lymphocytes: functional implications and mechanisms. *Transplant Rev.* **14,** 184 (1973).
28. K. A. AULT, M. J. KARNOVSKY and E. R. UNANUE, Studies on the distribution of surface immunoglobulins on human B lymphocytes. *J. clin. Invest.* **52,** 2507 (1973).
29. J. L. PREUD'HOMME, C. NEAUPORT-SAUTES, S. PIAT, D. SILVESTRE and F. M. KOURILSKY, Independence of HL-A antigens and immunolobulin determinants on the surface of human lymphoid cells. *Europ. J. Immunol.* **2,** 297 (1972).
30. S. D. ROWE, K. HUG, L. FORNI and B. PERNIS, Immunoglobulin D as a lymphocyte receptor. *J. exp. Med.* **138,** 965 (1973).

Europ. J. Cancer Vol. 11, pp. 199–201. Pergamon Press 1975. Printed in Great Britain.

Experimental Bilharzial Squamous Metaplasia of the Urinary Bladder in Hamsters

B. EL-MORSI*, M. SHERIF* and E. S. EL-RAZIKI†

*Cancer Institute and †Department of Tropical Medicine, Cairo University, Cairo, Egypt

Abstract—*The aim of this study was to induce experimental schistosomiasis of the urinary bladder through a natural route of infection in hamsters using* Schistosoma haematobium. *Schistosomal cystitis was produced in* 11/18 *animals. In* 8 *animals there were epithelial changes, hyperplasia only in* 4, *and both hyperplasia and squamous metaplasia in* 4 *animals. Two forms of squamous metaplasia were seen, one simulating that which develops in the urinary bladder of man and looks like epidermis, the other consisting of many layers of active basal cells with* 2–3 *layers of flattened cells on the surface.*

Development of squamous metaplasia is particularly interesting since it is considered a precancerous lesion.

INTRODUCTION

BILHARZIASIS of the urinary bladder is significantly associated with bladder carcinoma in Egypt [1]. The pathological characteristics are quite different from carcinoma of the non-bilharzial bladder; the tumour is commonly of the squamous cell type with a low grade malignancy [1, 2].

The nature of this association is not yet clarified and a solid epidemiological or experimental proof of a causal relationship is still lacking. This is due partly to the difficulty of establishing an experimental model since, unlike with *S. mansoni* and *S. japonicum*, it is very difficult to infect laboratory animals with *Schistosoma haematobium* [13].

This report presents an account of the experimental induction of bilharzial squamous metaplasia, a known precancerous lesion, in the urinary bladder of hamsters.

MATERIAL AND METHODS

Eighteen male hamsters, 8 weeks old, average weight 150 g, were used. Cercariae of *S. haematobium* were obtained from naturally infected snails in the local canals near Cairo and kept in the laboratory.

The total body immersion technique was used. Each hamster was put in a special jar containing 250 cercariae for one hr. The immersion fluid was then tested to be sure that it became cercariae free. The infection was repeated after 3 months.

The animals were killed after 7–11 months from the first exposure. The viscera were examined for gross pathological lesions and samples taken for detailed histological examination. The urinary bladder was filled with 0·5–1 ml of 10% neutral buffered formalin for 12 hr. The bladder was cut anteroposteriorly and examined for any gross lesions. Serial sections were then cut and stained with haematoxylin and eosin.

Accepted 1 July 1974.

*Mailing Adress: M. Sherif (M.Ch.), Department of Surgery, Cancer Institute, Kasr El-Aini Str. Fom El-Khaleeg, Cairo, Egypt.

RESULTS

Two animals died, one after 2 months with no pathological lesions in the urinary bladder, the second after 6 months and the bladder showed only congestion of the submucosa but no epithelial change.

Sixteen animals survived and were killed at 7–11 months. Five of them did not show any evidence of vesical schistosomiasis (killed at 7, 8, 8, 9 and 9 months). Eleven animals developed manifestations of schistosomal cystitis.

In these 11 animals, the bladder was rubbery, its mucosa was haemorrhagic and irregular, and the submucosa appeared thickened and oedematous with dilatation of the serosal venules. On cut section, polypoid patches were seen to occupy the lumen (Fig. 1).

Histologically, bilharzia ova were aggregated in the submucosa with surrounding granulomatous reaction (Fig. 2). This consisted mainly of lymphocytes, eosinophils and plasma cells. Fibrous tissue was scanty and surrounded clumps of eggs. Most of the ova were viable, but a few eggs were degenerated and showed partial or complete calcification.

In 4 animals (killed at 9, 9, 10 and 10 months) the submucosal lesions were associated with epithelial hyperplasia (Fig. 3). The basal cells were elongated and prominent, with varying degrees of proliferation to form many layers. In some areas, this proliferation formed epithelial pegs invaginating the lamina propria. The active basal cells were surmounted by 2–3 layers of cuboidal cells, sometimes with a distinct line of separation (Fig. 3).

In 4 animals, killed at 8, 9, 9 and 11 months, the epithelial changes consisted of both hyperplasia and squamous metaplasia. The squamous metaplasia occurred in 2 forms. In one form, it consisted of about 12 layers or even more of elongated basal cells with sharp margins and evident mitotic figures (Fig. 4). The superficial cells showed pyknosis and shrinkage of their nuclei. The basal cells were surmounted with 2–3 layers of flattened cells. In the other form, the epithelium consisted of 1–2 layers of active basal cells, then 3–4 layers of prickle cells followed by 1–2 layers of flattened cells covered by a thin layer of keratin (Fig. 5). The latter form resembles the structure of the epidermis. These lesions were patchy in distribution and showed variations in the number of cell layers.

DISCUSSION

In Egypt, the association of bilharziasis of the urinary bladder and squamous carcinoma is so striking that a causal relationship is strongly suspected. There is, however, considerable diversity of opinion on the possible role played by schistosomiasis in the pathogenesis of bladder cancer; mechanical irritation by the ova [4], miracidial toxins, [5] and associated long standing pyogenic infection [6], have all been cited as possible pathogenic factors. The design of an experimental model in mammals will certainly help in the study of this complex problem.

Monkeys and rodents can be infected with *S. haematobium*, but the infection usually affects the digestive tract [3]. De Paoli [7] reported a natural infection of the urinary bladder, and other organs, of a male chimpanzee with *S. haematobium*. The infection took the form of granulomata containing eggs. Obuyn [8] infected vervet monkeys with *S. haematobium*, and the urinary bladder showed sandy and haemorrhagic patches but no histological details were given. Sadun *et al.* [3] infected chimpanzees with varying numbers of cercariae of *S. haematobium* and numerous inflammatory patches and granulomata were seen. Microcytic Brunn's nests and patchy squamous metaplasia with intercellular bridges were described. Wright and Knowles [9] infected hamsters with *S. haematobium* and killed them at 10-day intervals. Eggs were recovered from the bladder in older infections but no macroscopic lesions were described.

In the present work, hamsters were infected with *S. haematobium* in a manner simulating natural infection. The animals were killed after a relatively long period, considering the life span of the hamster which is 12–24 months. The high rate of infection was very promising, 11 animals contracted the infection and showed evidence of vesical schistosomiasis. The development of squamous metaplasia is of special interest since this lesion is known to be precancerous and is noted in 81·8% cases of squamous cell carcinoma in the urinary bladder in man [10]. One form of the squamous metaplasia occurring in these animals took the form of squamous metaplasia occurring in man (Fig. 5) and exhibited all the structural layers of the epidermis.

The development of these lesions is encouraging. Probably with longer periods and more repetitions of infection, more will be learned about the complexity of schistosomiasis of the urinary bladder and its possible relation to malignancy.

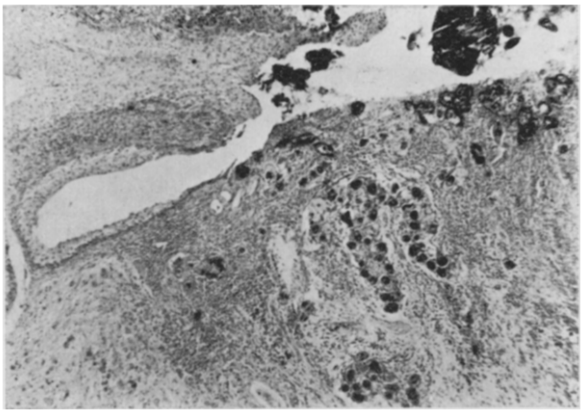


Fig. 1. *Hamster urinary bladder infected with* S. haematobium *showing thickened mucosa with polypoid patches in the lumen and submucosal granuloma* (× 185). *H & E.*

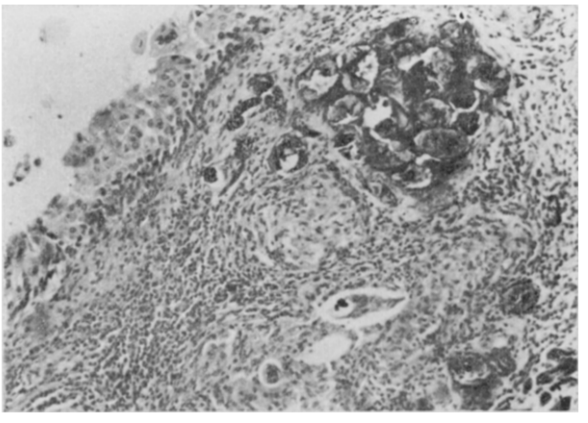
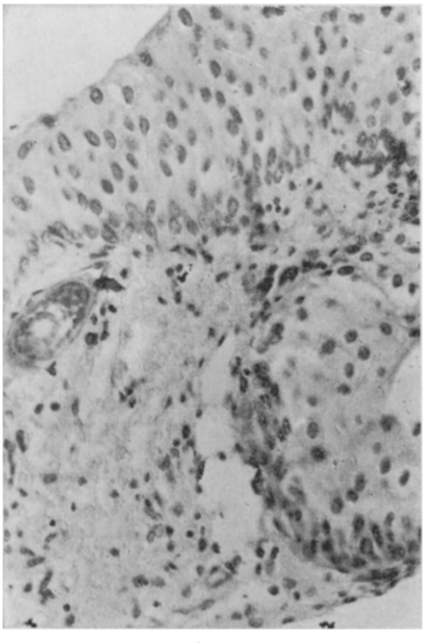


Fig. 2. *Hamster urinary bladder infected with* S. haematobium *showing submucosal cellular infiltration surrounding clumps of eggs* (× 250). *H & E.*



Fig, 3. *Patches of hyperplastic transitional epithelium of the urinary bladder with calcified ova in the submucosa.* (× 350). *H & E.*

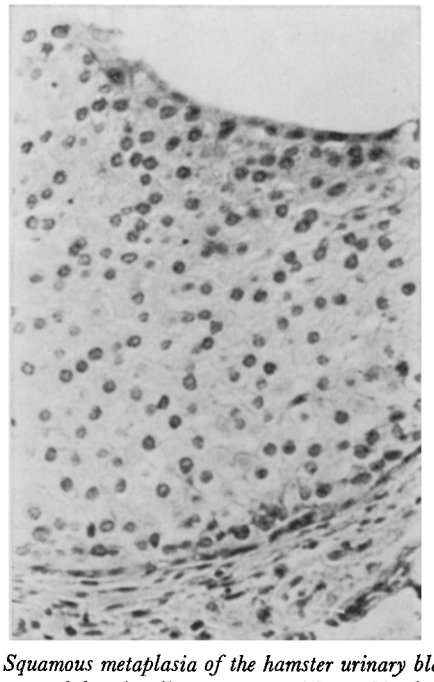


Fig. 4. *Squamous metaplasia of the hamster urinary bladder. Many layers of basal cells are seen with a thin layer of flattened cells on top* (× 350). *H & E.*

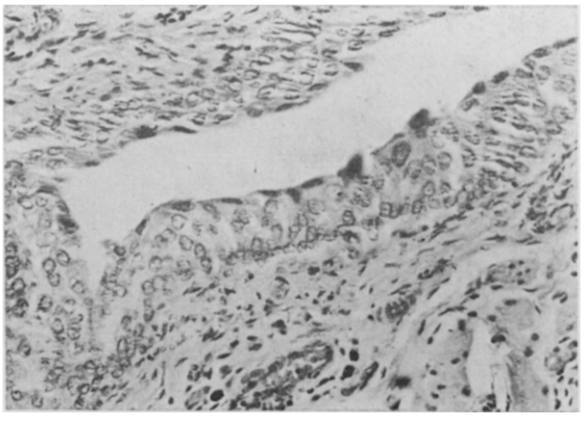


Fig. 5. *Squamous metaplasia of the urinary bladder in hamsters with superficial parakeratotic cells and submucosal cellular infiltration* (× 250). *H & E.*

(to face p. 200)

REFERENCES

1. EL-SEBAI, Cancer of the bladder in Egypt. *Kasr-El-Aini-J. Surg.* **2,** 183 (1961).
2. A. L. ABOUL NASR, M. E. GAZAYERLI, R. M. FAWZI and I. EL-SEBAI, Epidemiology and pathology of cancer of the bladder in Egypt. *Acta Un. int. Cancer* **18,** 528 (1962).
3. E. H. SADUN, F. VON LICHTENBERG, A. W. CHEEVER, D. G. ERICKSON and R. L. HICKMAN, Experimental infection with *Schistosoma haematobium* in chimpanzees. *Amer. J. trop. Med. Hyg.* **19,** 427 (1970).
4. A. R. FERGUSSON, Associated bilharziasis and primary malignant disease of the urinary bladder with observations on a series of forty cases. *J. Path. Bact.* **16,** 76 (1911).
5. N. MAKAR, A note on the pathogenesis of cancer of the bilharzial bladder. *Acta Un. int. Cancer* **8,** 323 (1952).
6. A. S. MOHAMED, The association of bilharziasis and malignant disease in the urinary bladder. Pathogenesis of bilharzial cancer in the urinary bladder. *Ann. trop. Med. Parasit.* **64,** 395 (1970).
7. A. DE PAOLI, *Schistosoma haematobium* in the chimpanzee, A nautral infection. *Amer. J. trop. Med. Hyg.* **14,** 561 (1965).
8. C. K. A. OBUY, Preliminary communication on gross pathological lesions in the genito-urinary systems of vervet monkeys (*Cercopithecus ethiops*) experimentally infected with *S. haematobium. Ann. trop. Med. Parasit.* **64,** 395 (1970).
9. C. A. WRIGHT and R. J. KNOWLES, Studies on *Schistosoma haematobium* in the laboratory—III. Strains from Iran, Mauritius and Ghana. *Trans. roy. Soc. trop. Med. Hyg.* **66,** 10 (1972).
10. M. M. KHAFAGY, M. N. EL-BOLKAINY and M. A. MANSOUR, Carcinoma of the bilharzial urinary bladder. A study of the associated mucosal lesions in 86 cases. *Cancer* **30,** 150 (1972).

Europ. J. Cancer Vol. 11, pp. 203–204. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Cellular Cyclic AMP Content Independent of Ascites Tumour Growth Rate *In Vivo*

A. JAKOBSEN
The Institute of Cancer Research, Radiumstationen, DK-8000 Aarhus C, Denmark*

RECENT reports from several laboratories indicate that cyclic AMP may be involved in the regulation of the cellular proliferation rate *in vitro*. The proliferation of various malignant cells is inhibited when cyclic AMP is present in the nutrient media [1], and cyclic AMP restores "contact-inhibited growth" to transformed fibroblasts [2]. A decrease in cyclic AMP may be essential for the malignant transformation of normal cells by oncogenic viruses [3]. Furthermore, rapidly growing cells have lower cyclic AMP concentrations than more slowly growing cells [4]. In contact-inhibited fibroblasts, the cyclic AMP concentration rises at confluency. These observations suggest that cellular levels of cyclic AMP are correlated inversely to the proliferation rate. However, all these experiments were carried out *in vitro*. The present study represents an *in vivo* investigation of the relationship between the cellular cyclic AMP concentration and growth rate of two ascites tumour lines.

It is well known that ascites tumours grow rapidly for the first few days after inoculation, but with increasing age the growth rate decreases continuously leading to a plateau phase with a constant number of tumour cells. This is probably due to some feedback regulation [5], whereas an increased rate of cell loss and unfavourable growth conditions are only of minor or no importance.

In the present study, two transplantable ascites tumours were used, the JBI ascites tumour [6], a hypotetraploid plasmacytoma maintained syngenically in the AKR/Aa strain of mice, and a hypotetraploid line of the Ehrlich ascites tumour grown in the C_3H strain of mice. The growth pattern of these tumours has been investigated recently in this laboratory. After inoculation of $2{\cdot}5 \times 10^6$ cells i.p. both tumours reach a plateau phase of about 10^9 cells per mouse 10–14 days later [7]. The doubling time of the JB-1 tumour increased from day 4 to day 10 from 22·8 to 240 hr. During the same period, the generation time increased from 14 to 41 hr, whereas no increased cellular death rate was observed [8].

The cellular concentration of cyclic AMP was determined at two points of the growth curve, *viz* on day 4 (rapid growth) and on day 10 (plateau phase).

After aspiration of the ascites tumour, the cells were washed once with ice-cold phosphate-buffered saline. Cyclic AMP was extracted in 5% T.C.A. Preliminary experiments demonstrated that 5% T.C.A. disrupted the cells sufficiently for extraction of cyclic AMP, so that homogenization was not required. The T.C.A. precipitate was removed by centrifugation (10,000 × *g* for 10 min). The supernatant was extracted 5 times with acidified water-saturated ether. An aliquot was lyophilized and redissolved in 0·2 ml of assay buffer. A sample of 0·1 ml was used in each cyclic AMP

Accepted 13 November 1974.
*Sponsored by the Danish Cancer Society.
Correspondence to: A. Jakobsen, The Institute of Cancer Research, Radiumstationen, Nörrebrogade 44, DK-8000 Aarhus C, Denmark.

assay. Cyclic AMP was determined using Gilman's method [9]. A standard curve was run with each assay to ensure comparable results. The specificity of the cyclic AMP determination was controlled by phosphodiesterase treatment. In five experiments it was found that cyclic AMP disappeared completely after digestion with phosphodiesterase from beef heart (Boehringer catalogue No. 15153). The recovery of cyclic AMP varied from 84–94%. Protein was measured by the method of Lowry *et al.* [10].

The results of the present study are shown in Table 1. It appears that no difference was found in cyclic AMP levels between tumours with different growth rates. The data presented here are apparently inconsistent with *in vitro* observations indicating that cyclic AMP is involved in the growth regulation of both normal and malignant cells; but this apparent discrepancy may be due to the more complex *in vivo* situation. However, stimulation of cell proliferation by cyclic AMP has also been reported [11].

Thus, the role of cyclic AMP in growth regulation appears to be a rather complicated problem and needs further elucidation.

Table 1. *Cyclic AMP levels (±standard deviations) in Ascites tumours of different ages*

Tumour	Age (days)	Picomol cyclic AMP / mg protein
JB–1	4	11·1 ± 1·6
JB–1	10	11·2 ± 2·0
Ehrlich	4	12·6 ± 1·6
Ehrlich	10	13·3 ± 1·8

The values shown are the means of 4 separate experiments. Each experiment included 5 specimens from each mouse.

REFERENCES

1. M. L. Heidrick and W. L. Ryan, Cyclic nucleotides on cell growth *in vitro*. *Cancer Res.* **30,** 376 (1970).
2. J. R. Sheppard, Restoration of contact-inhibited growth to transformed cells by dibutyryl adenosine 3′,5′-cyclic monophosphate. *Proc. nat. Acad. Sci. (Wash.)* **68,** 1316 (1971).
3. A. Rein, R. A. Carchman, G. S. Johnson and I. Pastan, Simian virus 40 rapidly lowers cAMP levels in mouse cells. *Biochem. biophys. Res. Commun.* **52,** 899 (1973).
4. J. Otten, G. S. Johnson and I. Pastan, Cyclic AMP levels in fibroblasts: Relationship to growth rate and contact inhibition of growth. *Biochem. biophys. Res. Commun.* **44,** 1192 (1971).
5. P. Bichel, Editor, *Autoregulation of growth in a murine ascites tumour.* S. Møller Christensen A/S, Aarhus, 1973.
6. J. Bichel, A transplantable plasma cell leukemia in mice. *Acta path. Microbiol. Scand.* **29,** 464 (1951).
7. P. Bichel, Specific growth regulation in three ascitic tumours. *Europ. J. Cancer* **8,** 167 (1972).
8. P. Dombernowsky, P. Bichel and N. R. Hartmann, Cytokinetic analysis of the JB-1 ascites tumour at different stages of growth. *Cell Tiss. Kinet.* **6,** 347 (1973).
9. A. G. Gilman, A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. nat. Acad. Sci. (Wash.)* **67,** 305 (1970).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
11. J. F. Whitfield, R. H. Rixon, J. P. Macmanus and S. D. Balk, Calcium, cyclic adenosine 3′,5′-monophosphate, and the control of cell proliferation: A review. *In Vitro* **8,** 257 (1973).

Europ. J. Cancer Vol. 11 pp. 205–206 Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Tamoxifen (ICI 46,474) and the Human Carcinoma 8S Oestrogen Receptor

V. C. JORDAN* and S. KOERNER

Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 01545, *U.S.A.*

TAMOXIFEN has been used for the treatment of breast cancer in postmenopausal women [1–3]. Reports concerning its mechanism of action though have been conflicting since some workers [4] claim that tamoxifen inhibits the binding of tritiated oestradiol-17β to oestrogen receptors derived from breast carcinomas, whereas others claim that tamoxifen is inactive [5]. Since the previous workers both used charcoal assay methods, we have used sucrose density gradient analysis to identify human tumour 8S oestrogen binding components and investigated the *in vitro* effects of tamoxifen upon the binding of tritiated oestradiol-17β.

Samples of breast and uterine adenocarcinomas were frozen in liquid nitrogen after pathological examination. Tissues were stored at the temperature of liquid nitrogen until transportation in dry ice. The procedure for sucrose density gradient analysis (10–30%) was that described by Jensen *et al.* [6] with some minor modifications. The precise method has been reported elsewhere [7] and is described briefly as a legend to Fig. 1. [6,7^{3}H] oestradiol-17β (49·3 Ci/mM purity > 97%) was obtained from New England Nuclear Corp., Boston, U.S.A. Tamoxifen (ICI 46,474. *trans* 1-(p-β-dimethyl aminoethoxy phenyl)-1,2-diphenyl but-1-ene) was a gift from ICI United States Inc.

The proportion of 8S oestrogen receptor positive breast tumours was found to be consistent with the previous report by Jensen *et al.* [6]. All uterine adenocarcinomas analysed were found to be positive (Table 1). Of interest was the presence of large quantities of 4S binding components in uterine adenocarcinoma cytosols (Fig. 1). Large quantities of 4S binding components are also seen in other non-cancerous conditions of the human endometrium and it seems to be a characteristic of human uterine cytosols [8].

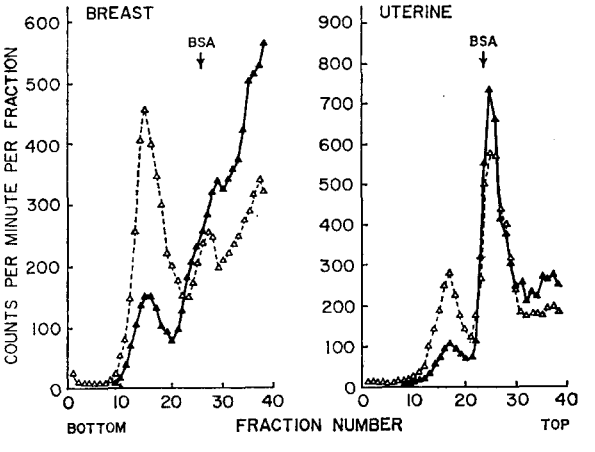


Fig. 1. Sucrose density gradient analysis (10–30%) of cytosol derived from human breast or uterine carcinomas. Cytosol (150 µl) was incubated (0°C) for 15 min with 50 µl Tris buffer (pH 7·4) containing 5×10^{-6}M tamoxifen (▲———▲) or with 50 µl buffer alone (△———△) prior to the incubation of both samples with 50 µl 5×10^{-9}M [6, 7-^{3}H] oestradiol-17β for 30 min. Samples (200 µl) were layered onto gradients and centrifuged for 15 hr (4°) at 250,000 × g using an SW56 swinging bucket rotor in a Beckman L5–75 centrifuge. Bovine serum albumin (BSA) was run on separate gradients as a sedimentation standard (4·6S).

Accepted 27 September 1974.

*Present address: Department of Pharmacology, School of Medicine, Leeds LS2 9NL, Yorkshire, England.

Table 1. *Table of the presence* (+) *or absence* (−) *of* 8*S oestrogen receptors determined by* 10–30% *sucrose density gradient analysis in cytosol from human breast and uterine carcinomas.* (±) *indicates the presence of border line quantities of receptor*

Origin of carcinoma	No. carcinomas analyzed	8S Oestrogen receptor
Breast	4	+
	3	±
	5	—
Uterus	4	+

In all the cases which were positive for 8S oestrogen receptors, incubation with tamoxifen prior to incubation with tritiated oestradiol-17β resulted in a lowered binding in the 8S region of the gradient with an increase in the binding in the 4S region. Alternatively in tumours with low levels of 4S binding components, excess unbound oestradiol remained near the top of the gradient.

Since the results suggest that tamoxifen inhibits the actions of oestradiol by occupying the 8S oestrogen receptors then the present findings are consistent with the report that tamoxifen inhibits the *in vivo* binding of tritiated oestradiol in human uterine endometrium [9].

In the treatment of breast cancer, if oestrogen responsiveness is indicated by the presence of oestrogen receptors [6] then clearly, patient preselection on the basis of oestrogen receptor positive tumours may provide a more logical approach to antioestrogen therapy. Furthermore, the presence of oestrogen receptors in uterine adenocarcinomas suggests that the metastases, which usually develop several years after the removal of the primary tumour at hysterectomy, may also be oestrogen sensitive. Antioestrogen therapy may be beneficial in the treatment of these women.

Acknowledgement—We wish to thank ICI United States Inc. for funds to support one of us (SK) and ICI Ltd for providing a grant for the study. VCJ was supported by NCI contract No. 1–CB–43967.

REFERENCES

1. M. P. Cole, C. T. A. Jones and I. D. H. Todd, A new antioestrogen agent in late breast cancer. An early clinical appraisal of ICI 46,474. *Brit. J. Cancer* **25,** 270 (1971).
2. H. W. C. Ward, Anti-oestrogen therapy for breast cancer: a trial of Tamoxifen at two dose levels. *Brit. med. J.* **1,** 13 (1973).
3. M. J. O'Hallaran and P. G. Maddock, ICI 46,474 in breast cancer. *J. Irish med. Assoc.* **67,** 38 (1974).
4. R. Hahnel, E. Twaddle and T. Ratajczak, The influence of synthetic antiestrogens on the binding of tritiated estradiol-17β by cytosols of human uterus and human breast carcinoma. *J. steroid Biochem.* **4,** 687 (1973).
5. H. Braunsberg, Studies on the effect of antioestrogens and dithiothreitol on oestrogen binding by preparations from human breast cancer tissue. *Acta endocr. (Kbh.) Suppl.* **177,** 27 (1973).
6. E. V. Jensen, G. E. Block, S. Smith, Kay Kyser and E. R. Desombre, Estrogen Receptors and Breast Cancer Response to Adrenalectomy. *NCI Monograph* **34,** 55 (1971).
7. V. C. Jordan and S. Koerner, Inhibition of oestradiol to mouse uterine and vaginal oestrogen receptors by triphenylethylenes. *J. Endocr.* (in press).
8. R. E. Hunter and V. C. Jordan, Detection of the 8S oestrogen binding component in human endometrium during the menstrual cycle. Submitted for publication. *J. Endocr.*
9. C. B. Lunan and B. Green, Effect of compound ICI 46,474 (Nolvadex) on the uptake of [^{3}H] oestradiol by human uterine endometrium. *Biochem. Soc. Trans* **1,** 500 (1973).

Europ. J. Cancer Vol. 11, p. 207. Pergamon Press 1975. Printed in Great Britain.

Erratum

R. J. BERRY, and D. K. BEWLEY. The importance of change in biological response with depth in tissue for collimated neutron beams—further experimental verification. *Europ. J. Cancer* **10,** 323 (1974).

Page 324, Fig. 4 is incorrect; the correct Fig. 4 with legend, appears below.

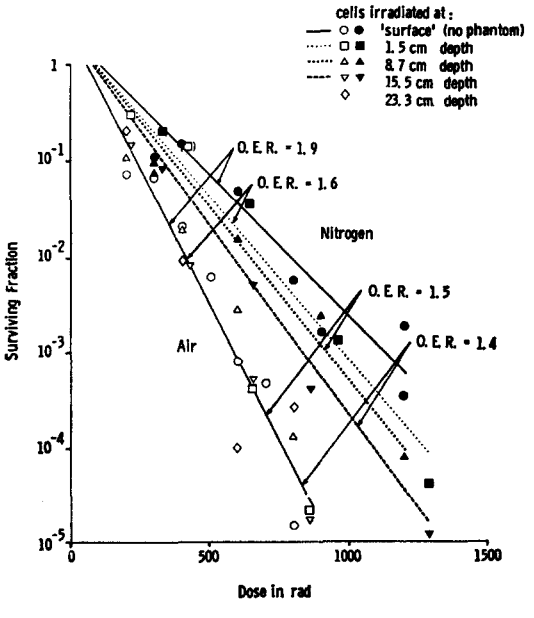


Fig. 4. Survival in vivo *of P–388 leukaemia cells irradiated* in vitro *with fast neutrons from the MRC cyclotron, Hammersmith Hospital (Berry and Bewley).*

Europ. J. Cancer Vol. 11, pp. 209–212. Pergamon Press 1975. Printed in Great Britain.

Announcements

Symposium on the Vascular Physiology of Tumors

A one-day meeting will be held on tumor microcirculation and closely related topics, on Saturday, June 14, 1975 at Roswell Park Memorial Institute, Buffalo, New York. This meeting will be immediately before the Toronto First World Congress for Microcirculation. The topics to be discussed will be focussed around the problems concerning tumor vascular supply, the physiology of blood flow, the tumor microcirculation and the influence of therapy on the microvasculature.

Preliminary Program

Morning Session Chairmen—Dr. Pietro Gullino (NCI); Dr. M. Intaglietta (LaJolla); Dr. Rafael Rubio (University of Virginia).

Topics—Tumor Blood Flow and Physiology; Tumor Microcirculation; Regulatory Mechanisms.

Afternoon Session Chairmen—Dr. R. F. Kallman (Stanford); Dr. Roger Inch (London, Ontario); Dr. Raymond Bush (Toronto).

Topics—Oxygenation and Drug Diffusion; The Effect of Treatment on the Microvasculature; The Response of the Microcirculation to Treatment.

Scientific papers dealing with any of the above subjects, relevant to tumor microcirculation and blood supply are invited. The abstracts should reach the organizers before March 1, 1975.

Organizing Committee—Dr. Richard Johnson—Department of Radiation Medicine, Roswell Park Memorial Institute; Dr. Hubert Reinhold—Department of Radiation Medicine, Roswell Park Memorial Institute; Dr. Hubert Eddy—Division of Radiation Oncology, University of Rochester Cancer Center.

All correspondence should be directed to: Dr. Hubert Reinhold, c/o Roswell Park Memorial Institute, 666 Elm Street, Buffalo, New York 14203.

The Johananoff International Fellowship for Advanced Biomedical Studies

The "Mario Negri" Institute for Pharmacological Research of Milan, Italy, and the Mario Negri Institute Foundation of New York City, U.S.A., announce the establishment of "*the Johananoff International Fellowship for Advanced Biomedical Studies*", through the generosity of Mr. S. Johananoff, a distinguished international industrialist.

The *purpose* of the Johananoff Fellowship is to offer an internationally known scientist the opportunity of spending his sabbatical year at the "Mario Negri" Institute for Pharmacological Research in Milan.

His *assignment* will be to evaluate and integrate the knowledge available on a specific subject, within the research areas of the Institute, to produce a comprehensive review, representing a constructive synthesis of the chosen subject.

The *grantee*, to be named "*the Johananoff Fellow*" will present his work at the end of the year through a public conference, called "*the Johananoff Lecture*". The lecture and the review will be published.

The *candidates* should be non-Italian citizens, known for outstanding contributions in cancer chemotherapy, cardiovascular pharmacology, neuropsychopharmacology, drug metabolism. Only candidates from academic or non-profit organizations will be considered, who are willing to spend their sabbatical year in Milano.

Applications for the academic year 1975–76 should be submitted by April 1st, 1975 and should include curriculum vitae, publications and outline of the proposed studies.

The *amount* of the fellowship is: $15,000. =

Submit applications to: The Johananoff Fellowship Committee, Istituto di Ricerche Farmacologiche "Mario Negri", 20157 Milano, Via Eritrea, 62, Italy—Tel. 35.54.546–35.70.546, Telex 37268 Negrinst.

Symposium on Human and Clinical Tumor Immunity

The Annual EORTC Symposium will be held in Brussels at Institut Jules Bordet, 1, rue Héger-Bordet, 1000 Brussels, Belgium, from May 26 to May 29, 1975. Fee: 1500 Belgian francs.

The topic is *Human and clinical tumor immunity*. The various aspects covered by European and American speakers will include: *General tumor immunity, Immunological tests in cancer patients, Tumor immunotherapy,* and *New aspects in leukemias and lymphomas.*

There will be some free communications related to the above topics which will be selected on the basis of abstracts to be submitted *before April* 19, 1975.

For all information regarding the Symposium and the abstracts, please write to: Dr. Joseph Wybran, Service d'Immunologie, Hôpital Universitaire Saint Pierre, rue Haute, 322, 1000 Bruxelles, Belgique.

Symposium on Microtubules and Microtubular Inhibitors

September 3–5, 1975

Organized by the Janssen Research Foundation under the auspices of the Belgian Society for Cell Biology.
Chaired by Professor P. Dustin (Brussels)
Place: 2340 Beerse, Belgium (near Antwerp)

Topics include: *Fundamental aspects of structure and biochemistry of microtubules; Functional aspects of microtubules in mitotic and interphase cells; Applied aspects of microtubular function.*

Most of the leading people in this field have already agreed to participate in this meeting. However, short communications concerning new data, in the form of exhibits, are welcome.

The total attendance at the meeting will be limited to 100 persons.

Information and application forms can be obtained from: M. Borgers or M. De Brabander, Janssen Pharmaceutica, Research Laboratories, 2340 Beerse, Belgium.

Europ. J. Cancer Vol. 11, pp. 213–216. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer *February 1975*

J. S. Rhim, H. S. Ro, E. B. Kim, R. V. Gilden and R. J. Huebner: "Transformation of horse skin cells by type C sarcoma viruses".

G. Steele, Jr., J. Ankerst, H. O. Sjögren, J. Vang and O. Lannerstad: "Absorption of blocking activity from human tumor-bearer sera by *Staphylococcus aureus*, Cowan I".

T. Katsuki and Y. Hinuma: "Characteristics of cell lines derived from human leukocytes transformed by different strains of Epstein–Barr virus".

J-L. H. Li, M. Jerkofsky and F. Rapp: "Demonstration of oncogenic potential of mammalian cells transformed by DNA containing viruses following photodynamic inactivation".

M. Lieber, C. Sherr, M. Potter and G. Todaro: "Isolation of type C viruses from the Asian feral mouse *Mus musculus molossinus*".

M. R. Proffitt, M. S. Hirsch, B. Gheridian, I. F. C. McKenzie and P. H. Black: "Immunological mechanisms in the pathogenesis of virus-induced murine leukemia. I. Autoreactivity".

M. R. Proffitt, M. S. Hirsch, I. F. C. McKenzie, B. Gheridian and P. H. Black: "Immunological mechanisms in the pathogenesis of virus-induced murine leukemia. II. Characterization of autoreactive thymocytes".

R. Auerbach, R. Arensman, L. Kubai and J. Folkman: "Tumor-induced angiogenesis: Lack of inhibition by irradiation".

J. M. MacSween: "The antigenicity of carcinoembryonic antigen in man".

C. H. Evans, S. H. Ohanian and A. M. Cooney: "Tumor specific and Forssman antigens of guinea pig hepatoma cells: Comparison of tumor cells grown *in vivo* and *in vitro*".

U. Mintz and L. Sachs: "Changes in the surface membrane of lymphocytes from patients with chronic lymphocytic leukemia and Hodgkin's disease".

M. V. Pimm and R. W. Baldwin: "BCG therapy of pleural and peritoneal growth of transplanted rat tumours".

A. A. Verstraeten, R. van Nie, H. G. Kwa and Ph. C. Hageman: "Quantitative estimation of mouse mammary tumour virus (MTV) antigens by radio-immunoassay".

D. M. Goldenberg and R. A. Pavia: "Oncogenesis by interspecific interaction of malignant murine and non-malignant hamster cells *in vitro*".

H. W. Taylor and P. Nettesheim: "Influence of administration route and dosage schedule on tumor response to nitrosoheptamethyleneimine in rats".

B. M. Vose, M. Moore and G. D. Jack: "Cell mediated cytotoxicity to human pulmonary neoplasms".

K. Nilsson and J. Pontén: "Classification and biological nature of established human hematopoietic cell lines".

M-R. Martin-Chandon, F. Vanky, C. Carnaud and E. Klein: "*In vitro* 'education' on autologous human sarcoma generates non-specific killer cells".

J. Cerny, M. Essex, M. A. Rich and W. D. Hardy, Jr.: "Expression of virus-associated antigens and immune cell functions during spontaneous regression of the Friend viral murine leukemia".

British Journal of Cancer *February 1975*

G. B. Coombes, R. R. Hall, D. J. R. Laurence and A. M. Neville: "Urinary carcinoembryonic antigen (CEA)-like molecules and urothelial malignancy: A clinical appraisal".

G. A. Currie and T. J. McElwain: "Active immunotherapy as an adjunct to chemotherapy in the treatment of disseminated malignant melanoma: A pilot study".

R. B. Whitney, J. G. Levy and A. G. Smith: "Studies on the effector cell of anti-tumour immunity in a chemically-induced mouse tumour system".

P. D. J. Holland, O. Browne and R. D. Thornes: "The enhancing influence of proteolysis on E-rosette forming lymphocytes (T cells) *in vivo* and *in vitro*".

A. E. Bray and D. Keast: "Changes in host immunity following excision of a murine melanoma".

D. G. Hopper, M. V. Pimm and R. W. Baldwin: "Methanol extraction residue of BCG in the treatment of transplanted rat tumours".

P. L. Grover, P. Sims, B. C. V. Mitchley and F. J. C. Roe: "The carcinogenicity of polycyclic hydrocarbon epoxides in newborn mice".

C. J. Grubbs and R. C. Moon: "Effect of age and parity upon the uptake of 9, 10-dimethyl-1, 2-benzanthracene-9-^{14}C by mammary parenchymal cells of the rat".

E. F. Rose and N. D. McGlashan: "The spatial distribution of oesophageal carcinoma in the Transkei, South Africa".

Y. Ishimaru, H. Ishihara and H. Hayashi: "An electron microscopic study of tumour cell adhesiveness induced by aggregation-promoting factor from rat ascites hepatoma cells".

R. J. Berry, A. H. Laing and J. Wells: "Fresh explant culture of human tumours in vitro and the assessment of sensitivity to cytotoxic chemotherapy".

L. L. Schenken, L. Poulakos and R. F. Hagemann: "Response of an experimental solid tumour to irradiation: A comparison of modes of fractionation".

J. F. Kelly, M. E. Snell and M. C. Berenbaum: "Photodynamic destruction of human bladder carcinoma".

S. Mondal and C. Bresch: "*Short communication.* Transformed liver cells obtained in culture from hepatectomized rats treated with dimethylnitrosamine (DMN)".

L. Cercek and B. Cercek: "Short communication. Changes in the SCM response ratio (RR_{SCM}) after surgical removal of malignant tissue".

L. Cercek and B. Cercek: "Apparent tumour specificity with the SCM test".

"Letter to the Editor"—New approach to anti-cancer drug development by R. C. Landes.

Report on the Ninth Paterson Symposium on mechanisms of mutagenesis in somatic mammalian cells.

B.A.C.R. Autumn Meeting—Abstracts of Papers.

International Journal of Cancer *March 1975*

B. C. Veit and J. D. Feldman: "Cell mediated cytotoxicity to Moloney sarcoma in syngeneic and allogeneic rats".

L. Tomatis, J. Hilfrich and V. Turusov: "The occurrence of tumours in F_1, F_2, and F_3 descendants of BD rats exposed to N-nitrosomethylurea during pregnancy".

J. Yata, C. Desgranges, T. Nakagawa, M. C. Favre and G. de Thé: "Lymphoblastoid transformation and kinetics of appearance of viral nuclear antigen (EBNA) in cord blood lymphocytes infected by Epstein-Barr virus (EBV)".

J. E. de Vries, M. Meyering, A. van Dongen and P. Rümke: "The influence of different isolation procedures and the use of target cells from melanoma cell lines and short term cultures on the non-specific cytotoxic effects of lymphocytes from healthy donors".

H. H. Sedlacek, H. Meesmann and F. R. Seiler: "Regression of spontaneous mammary tumors in dogs after injection of neuraminidase-treated tumor cells".

H. B. Vogel and R. G. Berry: "Chorioallantoic membrane heterotransplantation of human brain tumors".

R. A. Knight, N. A. Mitchison and G. R. Shellam: "Studies on a Gross virus-induced lymphoma in the rat. II. The role of cell membrane associated and serum p30 antigen in the antibody and cell-mediated response".

H. Bartsch, C. Malaveille and R. Montesano: "Human, rat and mouse liver-mediated mutagenicity of vinyl chloride in *S. typhimurium* strains".

R. Kearney, A. Basten and D. S. Nelson: "Cellular basis for the immune response to methylcholanthrene induced tumors in mice. Heterogeneity of effector cells".

L. S. Richardson, R. C. Ting, R. C. Gallo and A. M. Wu: "Effect of cordycepin on the replication of type-C RNA tumor viruses".

D. Vaillier, M. Donner, J. Vaillier and C. Burg: "Stimulation *in vivo* de la croissance de cellules tumorales en chambres à diffusion implantées chez des souris portant des tumeurs chimioinduites".

S-K. Liao and A. A. Axelrad: "Erythropoietin-independent erythroid colony formation *in vitro* by hemopoietic cells of mice infected with Friend virus".

K. S. S. Chang, L. W. Law and E. Appella: "Distinction between tumor specific transplantation antigen and virion antigens in solubilized products from membranes of virus-induced leukemic cells".

M. S. Hirsch, D. A. Ellis, A. P. Kelly, M. R. Proffitt, P. H. Black, A. P. Monaco and M. L. Wood: "Activation of C-type viruses during skin graft rejection in the mouse. Interrelationships between immunostimulation and immunosuppression".

D. J. Moss and J. H. Pope: "EB virus-associated nuclear antigen production and cell porliferation in adult peripheral blood leucocytes inoculated with the QIMR–WIL strain of EB virus".

K. M. Pozharisski, Yu. M. Kapustin, A. J. Likhachev and J. D. Shaposhnikov: "On the mechanism of carcinogenic action of 1.2-dimethylhydrazine (SDMH) in rats".

A. H. Fieldsteel, C. Kurahara and P. J. Dawson: "Helper specificity for retrieval of defective Friend virus".

British Journal of Cancer *March 1975*

J. F. Bithell and A. M. Stewart: "Pre-natal irradiation and childhood malignancy: A review of British data from the Oxford survey".

J. M. MacSween and R. A. Fox: "Carcinoembryonic antigen: Characterisation of binding with insoluble lectins".

L. J. Peters: "Enhancement of syngeneic murine tumour transplantability by whole body irradiation. A new immunological phenomenon".

B. Unsgaard and C. O'Toole: "The influence of tumour burden and therapy on cellular cytotoxicity responses in patients with ocular and skin melanoma".

D. Beeby, G. C. Easty, J. C. Gazet and A. M. Neville: "An assessment of the effects of hormones on short term organ cultures of human breast carcinomas".

D. J. Kirkland, C. Armstrong and R. J. C. Harris: "Spontaneous and chemically-induced transformation of rat embryo cell cultures".

P. M. Naha: "Temperature sensitive cells in the study of carcinogenic transformation".

A. M. Potter and C. W. Potter: "Transformation of human cells by SV40 virus".

S. Chiyoda, H. Mizoguchi, F. Takaku, K. Kosaka and Y. Miura: "*Short communication.* Influence of leukaemic cells on the colony formation of human bone marrow cells *in vitro*".

T. E. Sadler and J. E. Castro: "*Short communication.* The lack of immunological and antitumour effects of orally administered corynebacterium in mice".

S. Muldal, M. A. Mir, C. B. Freeman and C. G. Geary: "*Short communication.* A new translocation associated with the LP' chromosome and an acute course of chronic granulocytic leukaemia".

V. Covelli, P. Metalli and B. Bassani: "*Short communication.* Decreased incidence of reticulum cell sarcoma in lethally irradiated and bone-marrow shielded mice".

G. Th. A. M. Bots and R. G. J. Willighagen: "*Short communication.* Tumours of the mammary gland induced in Lewis rats by intravenous methylnitrosurea".

Book Review. Molecular studies in viral neoplasia by A. C. Allison.

Europ. J. Cancer Vol. 11, pp. 217–224. Pergamon Press 1975. Printed in Great Britain

Immunological Responsiveness Against Two Primary Antigens in Untreated Patients with Hodgkin's Disease*

G. C. DE GAST, M. R. HALIE and H. O. NIEWEG
Division of Haematology, Department of Medicine, University of Groningen, Groningen, The Netherlands

Abstract—*The immune responses against two primary antigens, dinitrochlorobenzene (DNCB) and α-haemocyanin of* Helix pomatia *(α-HPH) were studied in untreated, meticulously-staged patients with Hodgkin's disease and compared with those in control subjects of the same age group. Significantly more patients with the localized and the disseminated form, stages II, III and IV, and patients with nodular sclerosis, mixed cellularity and lymphocytic depletion had low DNCB sensitization grades (DNCB-scores). Antibody production to α-HPH was normal in all stages except in 2 out of 6 patients with stage IV, while significantly more patients with Hodgkin's disease showed reduced lymphocytic stimulation by α-HPH following immunization.*

A close correlation was observed between the DNCB-score and in vitro *lymphocyte stimulation by α-HPH following immunization. This may indicate that inability to produce sensitized T-lymphocytes plays an important role in the impairment of cell mediated immunity in Hodgkin's disease. Although DNCB sensitization and* in vitro *lymphocyte stimulation by α-HPH were impaired in patients with stages II, III and IV as a group, several patients even with stages III and IV or with the disseminated form of Hodgkin's disease showed normal cell-mediated immunity with these parameters.*

INTRODUCTION

A NUMBER of reports have been published on the impairment of cell-mediated immunity in Hodgkin's disease [1]. In many studies, DNCB has been used as a primary antigen for the induction of cell-mediated immunity, but with lack of a quantitation of the test results by expression in "positive" or "negative", only an unrealistic and crude distinction can be made. It has been argued that a decrease of the sensitization dose of DNCB increases the sensitivity of the test and that even in patients with stage I Hodgkin's disease an impaired cell mediated immunity can be demonstrated with that method [2]. A second possibility of increasing the sensitivity of the test is to quantitate the degree of sensitization to DNCB [3]. As the whole chain of the cell-mediated immune response is tested by sensitization to DNCB, a possible defect may be localized in any part of the chain, i.e., the afferent, the central, or the efferent branch of the cell-mediated immune response. Although the reported impaired lymphocyte stimulation by phytohaemagglutinin (PHA), shown to be present in stage I patients [4], points to a defect in the capacity of the T (thymus dependent)-lymphocyte to blast transformation, the localization and the nature of the defect in cell-mediated immunity in Hodgkin's disease is far from clarified.

Accepted 12 December, 1974.

*This work was supported by a grant from the "Koningin Wilhelmina Fonds, Nederlandse Organisatie voor de Kankerbestrijding."

Correspondence: G. C. de Gast, University Hospital, Department of Medicine, Oostersingel 59, Groningen, The Netherlands.

Finally, antibody production against secondary antigens has been reported to be normal by several authors [5–8], but no unanimity exists about antibody production against primary antigens [6, 7, 9–11]. The purpose of our study is to quantitate the immune responses to two primary antigens in untreated patients with Hodgkin's disease with several *in vivo* and *in vitro* parameters in order to gain insight into the localization of the immunological defect.

MATERIAL AND METHODS

Patients studied and staging

Thirty untreated patients with Hodgkin's disease, proved through lymph node biopsy, were studied during the time needed for the determination of the clinical stage. The evaluation included medical history, physical examination, blood and urine profiles, investigation of leucocyte concentrates, liver function tests, chest roentgenograms, lymphograms, bone marrow biopsy, liver and spleen scans and, except in patients with otherwise proven stage IV disease, laparotomy with splenectomy, liver and abdominal lymph node biopsy. Based on the findings of the peripheral blood leucocyte concentrates and the histology of the spleen, the patients were divided into either a group with localized disease or a group with haematogenous dissemination of the disease [12, 13]. In addition, the patients were classified on the basis of the clinical examination and the microscopic study of the biopsied organs according to the recommendations made in the Report of the Committee on the staging of Hodgkin's disease [14].

Histology: The classification proposed at the Rye conference [15] was used to define the histology of all biopsied material of lymph nodes and spleen.

Sensitization to 2,4-*dinitrochlorobenzene* (*DNCB*)

Sensitization was performed in the Department of Dermatology, University of Groningen, as previously described [3] with 2000 μg of DNCB (British Drug House), recrystallized twice, which was applied topically within an area of a 2 cm polythene ring on the volar aspect of the right arm. Challenges were performed by patch testing the patients on their backs with 30, 10 and 3 μg of DNCB in acetone two weeks later. The reactions were graded 1 to 4 plus according to common practice in allergology: 1+reactions = erythema, 2+ = erythema plus induration, 3+ = erythema, induration and vesiculation, 4+ = ibidem plus ulceration. The sum of the reactions on the three concentrations was taken as a measure for sensitization to DNCB, designated as DNCB-score (0+ to 12+). Because the DNCB-scores of normal controls decrease after the age of forty, the DNCB-scores of the patients were compared to those of the appropriate age group of the controls. The DNCB-score was arbitrarily classified low if it was less than the mean minus 1 S.D. of the age group, and normal or high if it was within 1 S.D. or exceeded the mean for the age group by more than 1 S.D. (Table 1).

Immunization with α-haemocyanin of Helix pomatia (*α-HPH*)

This was performed as previously described [16]. One mg α-HPH was administered s.c. in the deltoid region. Antibody-titres were determined by passive haemagglutination before and three weeks after immunization with α-HPH. IgM and IgG antibodies were divided on the basis of sensitivity to 2-mercaptoethanol (2-ME). *In vitro* lymphocyte stimulation by α-HPH was performed three weeks after immunization. This procedure could be completed in 15 patients.

Table 1. *Age-correlated DNCB-score* (*quantitative measurement of sensitization to DNCB*) *and* in vitro *lymphocyte stimulation by PHA of a normal control population*

Age group (yr)	DNCB-score			Lymphocyte stimulation by PHA*		
	No. of subjects	Mean score ± 1 SD†	Normal range‡	No. of subjects	Mean ± 1 SD	Normal range‡
15–40	45	9·6 ± 2·6	7+–12+	30	20,685 ± 5,101	15,584–25,786
41–60	22	7·4 ± 3·1	4+–11+	16	18,457 ± 6,600	11,857–25,057
61	12	4·7 ± 2·8	2+–7+	10	16,215 ± 5,806	10,409–22,021

*Expressed as disintegrations per minute.
†S.D. of mean.
‡Mean ± 1 S.D.

Lymphocyte cultures

Lymphocytes were isolated from defibrinated blood by Isopaque–Ficoll gradient centrifugation [17]. 3×10^5 lymphocytes were cultured in 12×75 mm Falcon tubes in 1 ml MEM-Eagle with 20 % autologous inactivated serum (30 min at 56°C), supplemented with penicillin 100 i.u./m) and streptomycin 100 μg/ml. The cultures with phytohaemagglutinin (PHA, Burroughs Wellcome) 5 μl/ml were incubated for 3 days at 37°C with 5 % CO_2 in air, while control cultures without antigen and cultures with α-HPH 15 μg/ml culture medium were incubated for 6 days.

Twenty hours before harvesting, 0·075 μCi [14C]-thymidine (Radiochemical Centre, Amersham, specific activity 56 mCi/M-Mole) was added. The cells were harvested by filtration through a glass fiber filter and washed twice with distilled water. Radioactivity in the dried filter was counted in a Packard liquid scintillation counter and expressed as disintegrations per minute (dis/min) per culture. The cultures were carried out in triplicate. The arithmetical mean and S.D. were calculated. Lymphocyte stimulation by α-HPH was expressed as ratio of stimulation:

$$\frac{\text{dis/min per culture in cultures with α-HPH}}{\text{dis/min per culture in cultures without antigen}}$$

Lymphocyte stimulation by α-HPH of the patients was classified as reduced if it was less than the mean minus 1 S.D. of the control group (Table 4). Lymphocyte stimulation by PHA was expressed as dpm per culture and compared with that of the appropriate age group of the controls. It was classified as reduced, normal or high in the same way as the DNCB-score (Table 1).

Blood group iso-haemagglutinin titres

Blood group iso-haemagglutinin titres were determined by agglutination in saline.

Serum immunoglobulin titres

Serum immunoglobulin titres were determined by radial immunodiffusion [18].

Statistical analysis

The number of patients with a low DNCB-score and with reduced lymphocyte stimulation by PHA or by α-HPH were evaluated by Chi square analysis.

RESULTS

The data on age, sex, stage and histology of the disease, together with the results of DNCB sensitization and *in vitro* lymphocyte stimulation by PHA of the patients are summarized in Table 2. As a group, significantly more patients with Hodgkin's disease had a low DNCB-score and reduced lymphocyte reactivity to PHA than did the controls. When the DNCB-score was correlated with the clinical stage and the histology and compared with the control population, significantly more patients with the localized and the disseminated form of the disease ($P < 0{\cdot}01$), with stages II, III and IV ($P < 0{\cdot}01$) and patients with nodular sclerosis, mixed cellularity and lymphocytic depletion ($P < 0{\cdot}01$) had a low DNCB-score (Table 3). When lymphocyte reactivity to PHA was correlated with the clinical stage and the histology, and compared with the control population, significantly more patients with the disseminated form ($P < 0{\cdot}01$) with stage III ($P < 0{\cdot}05$), stage IV ($P < 0{\cdot}01$), and patients with nodular sclerosis, mixed cellularity and lymphocytic depletion ($P < 0{\cdot}01$) had reduced lymphocyte reactivity to PHA.

Following immunization with α-HPH (Table 4), only 2/6 patients in stage IV showed a decreased rise in total antibody titre, while all patients in stage II (4 patients) and in stage III (5 patients) had a normal or high rise in total antibody titre. As with the controls, the antibodies were mainly 2-ME sensitive. In contrast with the normal antibody production to α-HPH significantly more patients in stage II, III and IV had reduced lymphocyte stimulation by α-HPH (11/15) compared with the control population (1/15, $P < 0{\cdot}01$). The same 2 patients in stage IV who showed decreased antibody production to α-HPH, had low blood group iso-haemagglutinin titres, but normal serum immunoglobulin levels (Table 4). Five of the 9 patients in stages II and III had an elevated serum IgM level, while the serum IgG level was elevated in 2/15 and the serum IgA level in 3/15 patients. None of the patients showed low serum immunoglobulin levels.

Correlation between the immune response to DNCB, to α-HPH and lymphocyte stimulation by PHA

A close correlation was observed between the results of sensitization to DNCB and *in vitro* lymphocyte stimulation by α-HPH following immunization: 91 % of the patients (10/11) with reduced lymphocyte stimulation by α-HPH also showed a low DNCB-score. Such a close

Table 2. Clinical data, DNCB-score and lymphocyte stimulation by PHA of each patient

Patient	Sex	Age	Staging B.M.J. 1972*	Staging Rye	Histology†	DNCB-score	Lymphocyte stimulation by PHA‡
1. F–M	f	38	L	I	LP	3+	30,495
2. H–K	f	27	L	I	LP	11+	33,526
3. S–B	f	24	L	I	NS	4+	23,334
4. D	m	25	L	I	NS	7+	23,407
5. vdL–vL	f	64	L	I	LD	6+	23,246
6. H–M	f	41	L	II	MC	1+	35,084
7. W–P	f	25	L	II	NS	1+	3,856
8. vA–R	f	29	L	II	NS	5+	18,533
9. G	m	49	L	II	LP	5+	21,375
10. vL	m	43	L	II	LP	9+	11,825
11. dV	m	25	L	II	MC	6+	16,907
12. R	m	26	D	II	NS	1+	4,631
13. D	f	24	D	II	LD	6+	23,506
14. T	m	15	D	II	LD	5+	31,690
15. B–D	f	59	D	III	LD	0+	13,990
16. H	m	53	D	III	LD	1+	10,992
17. H	m	30	D	III	MC	0+	1,774
18. M	m	33	D	III	LD	12+	10,062
19. vG	m	19	D	III	MC	9+	35,943
20. vdW	m	22	D	III	LD	1+	43,868
21. V	m	37	D	III	NS	11+	23,405
22. vdA	m	23	D	III	LP	2+	11,513
23. D–R	f	24	D	III	MC	1+	4,505
24. L–T	f	59	D	IV	MC	0+	1,862
25. KL–B	f	27	D	IV	NS	0+	37
26. B	m	27	D	IV	MC	10+	35,772
27. P	m	58	D	IV	LD	0+	3,585
28. vR	m	57	D	IV	LD	7+	11,286
29. P	m	61	D	IV	MC	0+	1,759
30. B–G	f	63	D	IV	LD	1+	8,495

British Medical Journal (Reference 12).

*L = localized D = disseminated.

†LP = lymphocytic predominance, MC = mixed cellularity,
LD = lymphocytic depletion, NS = nodular sclerosis.

†Underlined: low DNCB score and reduced lymphocyte stimulation by PHA = less than 1 S.D. of the mean for the appropriate age group of Controls.

‡Expressed as dis/min per culture.

correlation was not observed between lymphocyte stimulation by PHA and DNCB-score or lymphocyte stimulation by α-HPH. Fifty-five per cent of the patients (11/20) with a low DNCB-score and 73% of the patients (8/11) with reduced lymphocyte stimulation by α-HPH also showed reduced lymphocyte stimulation by PHA. As can be noted from Table 4 no correlation existed between *in vitro* lymphocyte stimulation by α-HPH and the rise in total antibody titre or in the 2-ME resistant antibody titre.

DISCUSSION

Recently, impaired cell mediated immunity in patients with stage I Hodgkin's disease

Table 3. Age-correlated DNCB-score and in vitro *lymphocyte stimulation by PHA of 30 untreated patients with Hodgkin's disease compared with the results in controls*

Stage or histology	DNCB-score			Lymphocyte stimulation by PHA		
	No.	Per cent low*	Per cent normal or high†	No.	Per cent low*	Per cent normal or high†
I	5	40	60	5	0	100
II	9	78§	22	9	33	67
III	9	67§	33	9	56‡	44
IV	7	71§	29	7	86§	14
Local	11	55§	45	11	18	82
Disseminated	19	73§	27	19	63§	37
LP¶	5	40	60	5	40	60
NS	7	71§	29	7	43‡	57
MC	8	75§	25	8	50‡	50
LD	10	70§	30	10	50‡	50
Total	30	67§	33	30	47§	53
Controls	79	16	84	56	16	84

* < 1 S.D. of mean of controls.
† ± 1 S.D. and > 1 S.D. of mean of controls.
‡Values differ significantly from controls $P < 0{\cdot}05$ by χ^2 analysis.
§Values differ significantly from controls $P < 0{\cdot}01$ by χ^2 analysis.
¶LP = lymphocytic predominance NS = nodular sclerosis
MC = mixed cellularity LD = lymphocytic depletion.

could be demonstrated [2] by limiting the sensitizing dose of DNCB (0·5 mg instead of the usual dose of 2·0 mg), but the control group consisted only of healthy, young-adult subjects and the degree of sensitization has been demonstrated to be age-dependent [3].

In our study, significantly more patients with the localized and disseminated forms of the disease and patients with stage II, III and IV had low DNCB-scores compared with controls of the same age. The number of patients with stage I may have been too low to demonstrate statistically an impaired cell-mediated immunity in that stage. Measuring the degree of sensitization, as we did, is advantageous in that quantitation of the sensitization is possible in an individual patient. It must be emphasized that there existed a great variation in DNCB-scores of our patients and that several patients, even in stages III and IV showed intact cell-mediated immunity, as judged not only by DNCB sensitization, but also by lymphocyte stimulation by α-HPH and PHA. As a consequence, measurement of cell-mediated immunity apparently has little value for staging.

An observation of some interest was the close correlation between the DNCB-score and lymphocyte stimulation by α-HPH following immunization. A low DNCB-score may be due not only to depletion or impaired function of T (thymus dependent) lymphocytes but also to depletion or impaired function of monocytes, which probably play a role in the afferent as well as in the efferent branch of the cell-mediated immune response [19]. Concerning T-lymphocyte function, both the capacity of blast transformation and of lymphokine production may be impaired. In *in vitro* lymphocyte stimulation by antigens, a possible lymphocytopenia is corrected and only the capacity of blast transformation is tested. From recent experiments with isolated T and B lymphocytes it appeared that in antigen-induced lymphocyte stimulation, primarily sensitized T-lymphocytes start to proliferate, while B-lymphocytes are secondarily recruited in the blast transformation [20].

Because the presence of monocytes is required in antigen-induced lymphocyte stimulation [21], depletion of monocytes or impaired monocyte function may influence the results in this test.

The close correlation between the DNCB-score and *in vitro* lymphocyte stimulation by α-HPH indicates that the impairment in cell-

Table 4. Results of immunization with α-haemocyanin, the blood group iso-haemagglutinin titres and the serum immunoglobin levels of 15 patients

Patient	Stage	Anti α-haemocyanin antibody titre (reciprocal)					Lymphocyte stimulation by α-haemocyanin‡	Blood group iso-haemagglutinin titer (reciprocal)		Serum Ig-titre (mg%)		
		Before imm.		3 weeks after imm.		Rise in titre		Anti A	Anti B	M	G	A
		Total	2-MER*	Total	2-MER*							
6. H-M	II	8	neg.	512	8	2^6	1·1¶	32	4	130	1476	176
7. W-P	II	32	neg.	2048	32	2^6	0·1	128	128	270	2050	321
8. vA-R	II	8	neg.	2048	8	2^8	5·3	—	32	183	1705	194
12. R	II	8	neg.	2048	512	2^8	1·4	64	16	237	1701	318
15. B-D	III	neg.	neg.	512	32	2^9	1·4	64	32	180	1092	413
16. H	III	4	neg.	1024	128	2^8	1·2	256	256	210	1722	310
17. H	III	16	neg.	1024	32	2^6	0·7	4	8	185	1323	132
18. M	III	8	neg.	2048	neg.	2^9	22·2	16	—	222	1496	320
19. vG	III	32	neg.	4096	64	2^7	3·9	16	16	264	1606	344
24. L-T	IV	neg.	neg.	128	neg.	2^7	1·0	—	128	118	1134	171
25. KL-B	IV	16	neg.	256	32	2^4	1·8	128	16	210	1239	183
26. B	IV	neg.	neg.	2048	neg.	2^{11}	2·7	8	4	186	1558	354
27. P	IV	8	neg.	16	neg.	2^1¶	0·8	—	2	120	1533	393
28. vR	IV	4	neg.	16	neg.	2^2	5·8	—	2	86	741	103
29. P	IV	2	neg.	512	16	2^8	1·1	8	8	77	2747	1025
Controls N = 15		Mean ± 1 S.D.				$6·2 \pm 1·9$†	$12·7 \pm 9·6$			♂32–187§	735–1806§	40–370§
		Normal range				2^4–2^8	3·1–22·3	8–64	4–32	♀59–214		

*2-mercaptoethanol resistant antibody titer.
†expressed as ^{2}log.
‡Expressed as ratio of stimulation: $\frac{\text{dpm per culture in cultures with } \alpha\text{-haemocyanin}}{\text{dis/min per culture in cultures without } \alpha\text{-haemocyanin}}$.
§Results of 200 Controls in different age groups, as mean ± 2 S.D.
¶Underlined: less than 1 S.D. of the mean for the control group.

mediated immunity as revealed by DNCB sensitization may be due to the inability to produce sensitized T-lymphocytes which start to proliferate after contact with the appropriate antigen. It does not exclude, however, the possible involvement of other parts of the chain of the cell-mediated immune response. Lymphocyte stimulation by PHA, measured with [14C] thymidine incorporation into DNA, which is considered to primarily represent stimulation of T-lymphocytes [22], was normal in patients with stages I and II Hodgkin's disease in our study. Recently, however, PHA-induced lymphocyte stimulation has been reported reduced in patients with stage I as well, if the stimulation of protein synthesis of the lymphocytes was measured by a limiting dose of PHA [4]. In contrast to the impaired lymphocyte stimulation by α-HPH, a normal or high production of antibodies to this antigen was demonstrated by the majority (13/15) of our patients with Hodgkin's disease. This is intriguing because in Hodgkin's disease, impaired antibody production to a closely related primary antigen, keyhole limpet haemocyanin (KLH) has been reported [11].

Antibody production to KLH seems to be thymus-dependent [23], and antibodies against KLH in the primary response are mainly of the IgG class [24]. While the thymus dependency of α-HPH is not known, antibodies in the primary response are mainly of the IgM class [16]. The IgM response even to thymus-dependent antigens, however, is less thymus-dependent than the IgG response [25].

The observed difference in antibody production to KLH and α-HPH may therefore be due to depletion or impaired function of T-lymphocytes, which act as helper cells in antibody production.

The different results of Brown *et al.* [10] and Saslaw *et al.* [9], obtained with tularaemia antigen may be explained in the same way. Brown *et al.* reported normal antibody production measured 2 weeks after immunization, probably mainly IgM, while Saslaw *et al.* reported impaired antibody production measured 3 to 6 weeks after immunization, probably mainly IgG antibodies. The primary immune response to brucella antigen [26] and tetanus toxoid [6, 7], which may be thymus-dependent antigens, were also found to be impaired. Antibody production to known thymus-independent antigens as pneumococcal polysaccharide type 2 and type 7, however, was found to be normal [27].

As a consequence, the results reported in the literature on primary antibody production in Hodgkin's disease also suggest depletion or impaired function of T-lymphocytes as the site of the immunological defect in Hodgkin's disease.

Acknowledgements—We wish to thank Dr. E. Bleumink and Dr. J. P. Nater, Department of Dermatology, University of Groningen, for performing the DNCB skin tests.

REFERENCES

1. H. S. Kaplan, *Hodgkin's Disease*. p. 184. Harvard University Press, Cambridge (1972).
2. J. R. Eltringham and H. S. Kaplan, Impaired delayed-hypersensitivity responses in 154 patients with untreated Hodgkin's disease. *Nat. Cancer Inst. Monograph* **36,** 107 (1973).
3. E. Bleumink, J. P. Nater, H. Schraffordt Koops and T. H. The, A standard method for DNCB sensitization testing in patients with neoplasmas. *Cancer* **6,** 1911 (1974).
4. R. Levy and H. S. Kaplan, Impaired lymphocyte function in untreated Hodgkin's disease. *New Engl. J. Med.* **290,** 181 (1974).
5. W. W. Schier, A. Roth, G. Ostroff and M. H. Schrifft, Hodgkin's disease and immunity. *Amer. J. Med.* **20,** 94 (1956).
6. R. Greenwood, H. Smellie, M. Barr and A. C. Cunliffe, Circulation antibodies in sarcoidoses. *Brit. Med. J.* **1,** 1388 (1958).
7. M. Barr and G. H. Fairley, Circulating antibodies in reticuloses. *Lancet* **1,** 1305 (1961).
8. G. H. Fairley and R. J. Akers, Antibodies to blood group A and B substances in reticuloses. *Brit. J. of Haemat.* **8,** 375 (1962).
9. S. Saslaw, N. H. Carlisle and B. Bouroncle, Antibody responses in haematologic patients. *Proc. Soc. exp. Biol. Med.* (N.Y.) **106,** 654 (1961).

10. R. S. Brown, H. A. Haynes, H. T. Foley, H. A. Godwin, C. W. Bernard and P. P. Carbone, Hodgkin's disease: Immunological, clinical and histologic features of 50 untreated patients. *Ann intern. Med.* **67,** 291 (1967).
11. E. M. Hersh, J. E. Curtis, J. E. Harris, C. McBride, R. Alexanian and R. Rossen, Host defense mechanisms in lymphoma and leukemia. In *Leukemia–Lymphoma.* Edited by M. D. Anderson Hospital, Houston, Texas, p. 149. Year Book Medical Publishers, Inc., Chicago (1970).
12. M. R. Halie, R. Eibergen and H. O. Nieweg, Observations on abnormal cells in the peripheral blood and spleen in Hodgkin's disease. *Brit. Med. J.* **2,** 609 (1972).
13. M. R. Halie, J. J. Seldenrath, H. C. Stam and H. O. Nieweg, Curative radiotherapy in Hodgkin's disease: Significance of haematogenous dissemination established by examination of peripheral blood and spleen. *Brit. Med. J.* **2,** 611 (1972).
14. S. A. Rosenberg, Report of the committee on the staging of Hodgkin's disease. *Cancer Res.* **26,** 1310 (1966).
15. R. J. Lukes, L. F. Craver, T. C. Hall, H. Rappaport and P. Ruben, Report of the nomenclature committee. *Cancer Res.* **26,** 1311 (1966).
16. G. C. de Gast, T. H. The and J. A. M. Snijder, The human immune response to α-Haemocyanin of *Helix pomatia. Acta Med. scand.* **194,** 303 (1973).
17. A. Böyum, Separation of leucocytes from blood and bone marrow. *Scand. J. clin. Lab. Invest.* **21,** 77 (1968).
18. G. Mancini, A. O. Carbonara and J. F. Heremans, Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem* **2,** 235 (1965).
19. J. H. L. Playfair, Cell cooperation in the immune response. *Clin. exp. Immunol.* **8,** 839 (1971).
20. R. S. Geha, E. Schneeberger, F. S. Rosen and E. Merler, Interaction of human thymus-derived and non-thymus-derived lymphocytes *in vitro.* Induction of proliferation and antibody synthesis in B lymphocytes by a soluble factor released from antigen-stimulated T lymphocytes. *J. exp. Med.* **138,** 1230 (1973).
21. E. M. Hersh and J. E. Harris, Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leucocytes. *J. Immunol.* **100,** 1184 (1968).
22. R. S. Geha, F. S. Rosen and E. Merler, Unresponsiveness of human B lymphocytes to phytohaemagglutinin. *Nature (Lond.)* **284,** 426 (1974).
23. J. Krüger and R. K. Gershon, Immune response to hemocyanin in normal, thymus-deprived and reconstituted mice. *J. Immunol.* **106,** 1065 (1971).
24. J. E. Curtis, J. E. Harris, C. McBride and E. J. Freireich, The human primary immune response to keyhole limpet haemocyanin: Interrelationships of delayed hypersensitivity, antibody response and *in vitro* blastogenesis. *Clin. exp. Immunol.* **6,** 473 (1970).
25. R. B. Taylor and H. H. Wortis, Thymus dependency of antibody response: Variation with dose of antigen and class of antibody. *Nature (Lond.)* **220,** 927 (1968).
26. J. N. Dubin, Poverty of immunological mechanism in patients with Hodgkin's disease. *Ann. Med.* **27,** 898 (1947).
27. A. C. Aisenberg and A. Leskowitz, Antibody formation in Hodgkin's disease. *New Engl. J. Med.* **268,** 1269 (1963).

Europ. J. Cancer Vol. 11, pp. 225–230. Pergamon Press 1975. Printed in Great Britain

Characterization of the Hyperlipidemia in Mice Bearing the Ehrlich Ascites Tumor*

DOUGLAS E. BRENNEMAN, SATYA N. MATHUR and ARTHUR A. SPECTOR
Departments of Biochemistry and Medicine, University of Iowa, Iowa City, Iowa 52242, *U.S.A.*

Abstract—*CBA mice bearing the Ehrlich ascites carcinoma developed hyperlipidemia within* 5 *days after inoculation of the tumor. The hyperlipidemia was maximal between days* 6 *and* 10 *of tumor growth. It was characterized as a hypertriglyceridemia resulting from an increase in plasma very low density lipoproteins. This was accompanied by a* 50% *decrease in the triglyceride and cholesterol content of the plasma high density lipoprotein fraction. Large amounts of cholesterol and triglycerides also were present in the tumor ascites fluid, mostly in the form of very low density lipoproteins. The concentrations of lipids in the blood plasma, however, always were greater than those in the ascites fluid. Fasting for* 16 *hr did not reduce the lipid content of either the ascites plasma or blood plasma of the tumor-bearing mouse. Electrophoretic analysis on discontinuous gradient gels revealed a number of similarities between the ascites fluid and the blood plasma lipoproteins.*

These results are compatible with the view that the tumor plasma lipoproteins probably are derived from the blood plasma of the host and that they may be involved in transporting lipids to the tumor cells.

INTRODUCTION

HYPERLIPIDEMIA and changes in the composition of the plasma lipoproteins occur in animals bearing a number of different tumors [1–7]. Crenin and Narayan have reported changes in the plasma lipid and lipoprotein content of CF_1 mice bearing the Ehrlich ascites carcinoma, one of the most widely studied transplanted tumors [6]. They found an elevation in plasma total lipids as well as an increase in plasma low density lipoproteins (LDL) and a decrease in plasma high density lipoproteins (HDL). We are using the Ehrlich ascites carcinoma grown in CBA mice as a model for studies of lipid transport in tumors. We have observed that the ascites plasma in which the Ehrlich cells are bathed in the peritoneal cavity contains free fatty acids and lipoproteins [8, 9]. The predominant lipoproteins in the ascites plasma are triglyceride-rich very low density lipoproteins (VLDL) of density $<1{\cdot}006$ [9, 10]. Because of this observation, we considered it important to reinvestigate the lipoprotein content of the blood plasma of the tumor-bearing mouse, particularly since there is no mention of VLDL or triglycerides in the previous report concerning the hyperlipidemia induced by the Ehrlich ascites tumor [6]. On the other hand, increases in neutral lipids and VLDL have been observed in the plasma of rats bearing the Walker carcinosarcoma 256 [2, 3]. The present studies indicate that there is also a very large increase in triglyceride-rich VLDL in the blood of CBA mice bearing the Ehrlich ascites carcinoma.

Accepted 11 December, 1974.
*Supported by research grants from the National Heart and Lung Institute, National Institutes of Health (HL-14, 781 and HL-14, 388) and by a grant-in-aid from the American Heart Association, supported in part by funds contributed by the Iowa Heart Association (74–689).

MATERIAL AND METHODS

Tumor

The Ehrlich ascites tumor was obtained in

1963 from Drs. D. Burk and M. Woods, National Institutes of Health, Bethesda, Maryland, U.S.A. and serially transplanted by us in male CBA mice. Transplantation was carried out by i.p. injection of 3×10^6 cells into mice weighing between 18 and 25 g. Under these conditions, the LD_{50} of the tumor varies from 15–16 days. The earliest deaths occur on day 14 after transplantation, and all of the inoculated mice die within 19 days. Little tumor can be obtained prior to day 8, and the maximum tumor size, $10{\cdot}1 \pm 1{\cdot}5 \times 10^8$ cells (mean $\pm$SE, 4 mice) occurs on day 13. The mice were fed *ad libitum* on animal chow pellets (Lab Blox Chow, Allied Mills, Inc., Chicago, Illinois U.S.A.).

Lipoprotein preparation

Lipoproteins were isolated from blood and tumor plasma by preparative ultracentrifugation [9, 10] into 3 fractions; VLDL (density $<1{\cdot}006$), LDL (density 1·006–1·063) and HDL (density 1·063–1·21). Chylomicrons, which were present only in negligibly small amounts, were removed by centrifugation at 30,000 $g \times$ 30 min in some experiments. Each of the 3 lipoprotein fractions was washed once by flotation through a salt solution of the appropriate density. When subjected to agarose gel electrophoresis [11], each of the 3 lipoprotein fractions migrated as a single band.

Polyacrylamide gel electrophoresis

A modification of the procedure of Feliste *et al.*, a discontinuous concentration gradient electrophoresis, was employed for separation of the plasma lipoproteins on polyacrylamide gels [12]. The 3% gel was prepared by mixing 1 part of solution A of Davis [13] with 2 parts of 12% acrylamide and 1·2% N,N′-methylene bisacrylamide, 1 part of 4 mg % riboflavin and 4 parts of 20% sucrose. Polymerization of the gel was induced by exposure of this mixture to fluorescent light for 30 min, and the 3% gel did not contain ammonium persulfate. The 3·5 and 7% gels were prepared by mixing suitable amounts of solutions A and C of Davis [13], 150 mg% ammonium persulfate solution and water. The final concentration of the ammonium persulfate in these gels was 75 mg%. These gels were polymerized in the dark for 45 min. The lipoproteins were prestained with Sudan Black-B dissolved in ethylene glycol before electrophoresis [14].

Chemical measurements

Plasma triglycerides were measured by the automated method of Kessler and Lederer [15]. In this procedure, 1 vol of plasma was added to 19 vol of isopropanol in order to extract the lipids. To this mixture, 200 mg/ml of Zeolite were added to remove phospholipids, and the Zeolite was removed by centrifugation. The triglycerides in the extract were saponified in a 50°C heating bath which was part of the automated system. In a second automated heating bath, acetylacetone reagent was added, producing oxidation and condensation reactions. The fluorescence of the product was recorded automatically as the solution passed through the fluorometer. Triolein was employed as the standard for this procedure. Total cholesterol was determined by the method of Abell, Levy, Brodie and Kendall [16]. Cholesterol ester was measured by a slight modification of the Schoenheimer and Sperry method [17].

RESULTS

Lipid analyses

Figure 1A shows the changes in blood plasma triglyceride concentration from 4 hr to 14 days after transplantation of the Ehrlich ascites tumor into CBA mice. Figure 1B shows the corresponding changes in the plasma cholesterol concentration. Because of the difficulties in obtaining large amounts of mouse blood plasma and the quantity of material required for these assays, it was necessary to pool the plasma from 5 mice for each determination. Therefore, the 9 data points shown for each series of animals actually represent plasma obtained from 45 mice. A large increase in plasma triglycerides was observed between days 6 and 14 following transplantation of the intact tumor. A similar increase was noted when isolated, washed Ehrlich cells were inoculated rather than the intact tumor. By contrast, there was no increase in plasma triglycerides when 0·3 ml of the cell-free tumor plasma or 0·3 ml of 0·154 M NaCl was injected i.p. into the mice. Although the cholesterol concentration also appeared to increase slightly in the plasma of the tumor bearing mice, the changes were too small to be considered significant in view of the dispersion of these data.

Table 1 shows the cholesterol and triglyceride contents of the 3 blood plasma lipoprotein fractions isolated from control mice and from mice bearing the Ehrlich ascites tumor on day 10 following transplantation. In the control animals, 70% of the plasma cholesterol and 61% of the plasma triglycerides were contained in HDL. In the tumor-bearing animals, only 23% of the plasma cholesterol and 3% of the

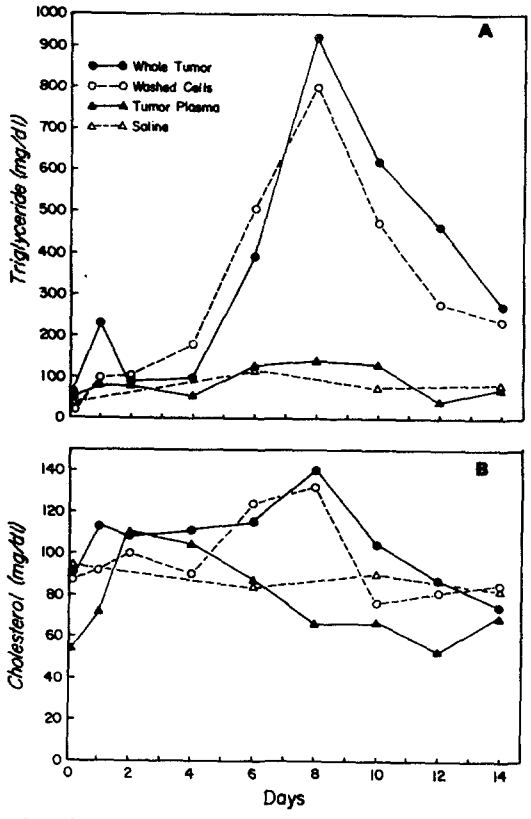


Fig. 1. *Changes in the blood plasma triglyceride and cholesterol concentrations during growth of the Ehrlich ascites tumor in CBA mice.*

Animals inoculated with either intact tumor or isolated, washed tumor cells were injected with the same number of cells, $3{\cdot}5 \times 10^6$. *Equal volumes,* 0·3 *ml, of either* 0·154 M *NaCl or cell-free tumor plasma were injected into the control animals. Each data point represents the value derived from a pooled sample of blood obtained from* 5 *mice. Triglyceride concentrations are shown in the upper graph (A), cholesterol concentrations in the lower graph (B).*

plasma triglycerides were present in HDL. Moreover, the absolute quantities of HDL cholesterol and triglycerides were reduced relative to the control animals, a finding that is in agreement with the reduction in HDL lipids noted by Crenin and Narayan [6]. There also was a decrease in the triglyceride content of the LDL in the tumor-bearing animals. The VLDL, however, contained 66% of the cholesterol and 94% of the triglycerides in the tumor-bearing mice, and all of the increase in plasma lipids in these animals was accounted for by the increases in VLDL.

A comparison of the lipid concentrations in the blood and tumor ascites plasma on days 11–14 of tumor growth is presented in Table 2. Since much larger volumes of plasma could be obtained from the ascites, analyses were made on samples from 4 individual mice, and mean values ± SE are presented. Because of small sample sizes, however, the blood plasma values again represent a single value from a pooled sample of plasma obtained from the 4 mice. On each day, the concentration of cholesterol, cholesterol esters and triglycerides was much greater in the blood plasma than in the tumor plasma. In agreement with the data presented in Fig. 1, there was a decrease in the blood plasma lipid concentrations between days 11 and 14. The ascites plasma triglyceride concentration also decreased during this period, but its cholesterol concentration remained fairly constant. However, a much larger fraction of the total ascites plasma cholesterol was esterified on day 14 (66%) as compared to day 11 (44%). Likewise, there was an increase in the percentage of cholesterol that was esterified in the blood plasma on day 14 (51%) as compared to day 11 (32%).

Table 3 shows the effects of a 16 hr fast on the lipid content of the ascites plasma. Fasting did not produce any statistically significant changes in the cholesterol or triglyceride contents of either the intact tumor plasma or the isolated lipoprotein fractions. In fact, the mean values for VLDL in the fasting mice

Table 1. *Lipoprotein triglyceride and cholesterol concentrations in the blood plasma of normal mice and in mice bearing the Ehrlich ascites carcinoma**

Lipoprotein fraction	Cholesterol (mg/dl)		Triglyceride (mg/dl)	
	Normal	Tumor-bearing†	Normal	Tumor-bearing
Whole plasma	75	125	157	766
VLDL	12	83	17	723
LDL	11	13	45	17
HDL	52	29	95	26

*Because of difficulties in obtaining sufficient quantities of mouse blood plasma for ultracentrifugal isolation of the individual lipoprotein classes, the blood plasma from 30 mice were pooled for each determination.

†The blood plasma from the tumor-bearing mice was collected 10 days after the tumor was transplanted.

Table 2. Daily changes in triglyceride, total cholesterol and cholesterol ester content of blood plasma and tumor plasma

Days after transplantation	Cholesterol (mg/dl)		Cholesterol Ester (mg/dl)		Triglyceride (mg/dl)	
	Blood plasma*	Tumor plasma†	Blood plasma	Tumor plasma	Blood plasma	Tumor plasma
11	178	81 ± 3	57	36 ± 2	1622	484 ± 53
12	139	84 ± 5	70	44 ± 2	627	336 ± 55
13	112	83 ± 1	53	43 ± 2	414	254 ± 50
14	91	59 ± 8	47	39 ± 5	316	126 ± 23

*Each of the daily samples were obtained by pooling the blood plasma of 4 tumor-bearing mice.

†The ascites plasma values were obtained from the same animals utilized for the corresponding blood plasma values. Each value is the mean ± S.E. of 4 determinations.

*Table 3. The effect of fasting on the lipid composition of ascites plasma lipoproteins**

Lipoprotein	Cholesterol (mg/dl)		Triglyceride (mg/dl)	
	Fasting	Non-fasting	Fasting	Non-fasting
Whole Plasma	74 ± 7	64 ± 7	369 ± 67	318 ± 64
VLDL	55 ± 7	47 ± 8	351 ± 67	299 ± 63
LDL	9 ± 0.7	7 ± 0.4	10 ± 0.5	10 ± 1.0
HDL	10 ± 0.8	10 ± 0.8	8 ± 0.1	9 ± 0.6

*This study was done on the 12th day after transplantation of the tumor. One group of 4 mice was fasted for 16 hr prior to sacrifice, and another group of 4 mice was given free access to food until the time of sacrifice. Each value is the mean ± S.E. of 4 determinations.

were slightly higher, suggesting that the VLDL elevation is not induced by food consumption in the several hours prior to the time that the tumor is harvested. This conclusion is supported by the fact that almost no chylomicrons were present in the ascites plasma of the tumor-bearing animals. A 16 hr fast increased the blood plasma triglyceride concentration in the tumor bearing mice from 770 to 1020 mg/dl. In the fasting mice, 97% of the triglycerides were recovered in VLDL. Fasting decreased the cholesterol concentration in the blood plasma of these mice from 125 to 95 mg/dl. In the fasting animals, 66% of the cholesterol was recovered in VLDL, 14% in LDL and 20% in HDL.

Electrophoretic analyses

Polyacrylamide gel electrophoretic patterns of the VLDL, LDL and HDL fractions isolated and concentrated from the ascites plasma are shown in Fig. 2. The VLDL appear as two bands in the 3% gel. LDL are present as two major bands in the 3·5% gel and a single minor band in the 3% gel. Ultracentrifugally isolated HDL appear to migrate as a broad band in the 7% gel.

A comparison of the lipoprotein patterns in the blood plasma of normal mice and in the blood and ascites plasma of tumor-bearing mice is shown in Fig. 3. In addition to the lipoprotein bands seen in Fig. 2, albumin also appears in each of these plasma specimens as a lightly stained band in the 7% gel, having a slightly greater mobility than HDL. The lipropoteins present in these three types of plasma give a very similar pattern in these discontinuous gradient gels. The normal mouse blood plasma contains little VLDL or LDL, but a very large quantity of HDL. Although isolated HDL

appears as a broad single band in the 7% gel, in intact plasma samples it resolves into three bands in the 7% gel and one faint band located at the junction of the 3·5 and 7% gels. The blood plasma of a tumor-bearing mouse contains more VLDL and much less HDL than that of a control mouse. Likewise, the tumor ascites plasma also has large amounts of VLDL and relatively little HDL. These electrophoretic differences are in agreement with the differences in the lipid content of these plasma specimens noted in Tables 1 and 2.

DISCUSSION

These findings confirm the observation that hyperlipidemia occurs during the growth of the Ehrlich ascites tumor [6]. The hyperlipidemia in CBA mice resulted mainly from an elevation in triglyceride-rich VLDL. Large quantities of VLDL also were contained in the ascites plasma present in the peritoneal cavity. Like the blood, the ascites plasma contained LDL and HDL. At all of the times tested, the lipid concentrations were considerably higher in the blood plasma than in the ascites plasma. These results are compatible with the view that the ascites plasma lipoproteins are derived from those present in the blood of the tumor-bearing host. This conclusion is supported by two additional findings. First, the lipid concentrations in the blood plasma were highest on days 6–8, a time when there were relatively few tumor cells in the peritoneal cavity. The plasma lipid concentrations actually decreased as the number of tumor cells increased, between days 10 and 14. Second, we have not been able to demonstrate lipoprotein secretion by the Ehrlich cells during *in vitro* incubations [9]. We suggest that the presence of the tumor leads to secretion of triglycerides in the form of VLDL, probably from the liver, and that these VLDL enter the peritoneal cavity. Recent studies have demonstrated that the triglycerides and cholesterol contained in the ascites plasma VLDL can be taken up and utilized by Ehrlich cells during *in vitro* incubation [10, 18]. Therefore, it is reasonable to speculate that the function of the VLDL may be to transport lipids from host tissues to the growing tumor cells.

We have reported previously that free fatty acids are present in the ascites plasma and that they are an important substrate for the Ehrlich cells [8]. It was assumed that the free fatty acids were derived from the blood plasma of the host. Recent studies, however, indicate that the flux of free fatty acids from the mouse blood plasma to the ascites fluid can account for only a very small fraction of the free fatty acid turnover in the ascites plasma [19]. This has led to a search for alternative routes for free fatty entry into the peritoneal cavity. If a lipase were present in the tumor, some of the triglycerides entering the ascites fluid as a part of VLDL might be hydrolyzed, forming free fatty acids. This might account at least in part for the free fatty acids made available to the tumor. Although a soluble triglyceride lipase has not been reported in the Ehrlich ascites tumor, an enzyme similar or identical to lipoprotein lipase has been extracted from the Walker carcinosarcoma 256 [20]. Therefore, it is possible that the triglyceride-rich VLDL may serve as a transport vehicle for delivery of free fatty acids to the tumor cells.

There is now considerable evidence that lipid transport abnormalities occur in the tumor-bearing animals [1–7]. When the present results are compared to those observed in other tumors, however, it becomes evident that no single, uniform plasma lipid abnormality is produced by the presence of a tumor. For example, Grigor *et al.* noted a decrease in plasma VLDL and triglycerides in Buffalo rats bearing the Morris hepatoma 7777 [7], which is a result completely opposite from the present findings with the Ehrlich ascites tumor. Although Grigor *et al.* did observe an elevation in cholesterol and cholesterol esters, this was due to an increase in LDL of density 1·019–1·060 [7]. In the present study, the cholesterol elevation is secondary to the VLDL increase, and there is no increase in LDL (Table 1). LDL elevations also have been observed in rats bearing tumors induced by 9,10-dimethyl-1,2-benzanthracene [3] and N-2-fluorenylacetamide [4], and in CF_1 mice bearing the Ehrlich ascites tumor [6]. Another major difference among the tumors studied is that HDL are elevated in rats bearing tumors produced by N-2-fluorenylacetamide [4] or the Morris hepatoma 7777 [5], whereas they are decreased in rats bearing tumors induced by 9,10-dimethyl-1, 2-benzanthracene [3], the Walker carcinosarcoma 256 [3] and in the present study (Table 1). The only system exhibiting the same lipoprotein changes observed in the present study, a decrease in HDL together with a large increase in triglyceride-rich VLDL, is the Wistar rat bearing Walker carcinosarcoma 256 [2, 3]. In this context, it should be noted that no consistent lipid abnormality has been noted in the various forms of human cancer that have been investigated [21]. Therefore, it appears that the nature of the plasma lipid response to cancer is quite

variable and must be examined individually for each tumor.

The present findings with the Ehrlich ascites carcinoma are in partial agreement with those of Crenin and Narayan [6]. Both studies indicate that HDL is reduced and the lower density lipoproteins elevated in mice bearing this tumor. Crenin and Narayan, however, did not subfractionate the lower density lipoproteins so that the precise nature of the lipoprotein elevation is not apparent from their data [6]. In the present study, these lipoproteins were separated by preparative ultracentrifugation as well as electrophoresis, and the results demonstrate that the increase is in the triglyceride-rich VLDL, not the LDL fraction.

REFERENCES

1. J. F. Henderson and G. A. LePage, The nutrition of tumors. A review. *Cancer Res.* **19,** 887 (1959).
2. G. L. Frederick and R. W. Begg, A study of hyperlipidemia in the tumor-bearing rat. *Cancer Res.* **16,** 548 (1956).
3. M. Barclay, V. P. Skipski, O. Terebus-Kekish, P. L. Merker and J. P. Cappuccino, Serum lipoproteins in rats with tumors induced by 9,10-dimethyl-1, 2-benzanthracene and with transplanted Walker carcinosarcoma 256. *Cancer Res.* **27,** 1158 (1967).
4. K. A. Narayan, Serum lipoproteins of rats fed an essential fatty acid-deficient diet and N-2-fluorenylacetamide. *Cancer Res.* **30,** 1185 (1970).
5. K. A. Narayan and H. P. Morris, Serum lipoproteins of rats bearing transplanted Morris hepatoma 7777. *Int. J. Cancer* **5,** 410 (1970).
6. H. L. Crenin and A. K. Narayan, Effect of Ehrlich ascites tumor cells on mouse plasma lipoproteins. *Z. Krebsforsch.* **75,** 93 (1971).
7. M. R. Grigor, M. L. Blank and F. Snyder, Cholesterol metabolism in rats bearing Morris hepatoma 7777. *Cancer Res.* **33,** 1870 (1973).
8. A. A. Spector, The importance of free fatty acid in tumor nutrition. *Cancer Res.* **27,** 1580 (1967).
9. A. A. Spector and D. E. Brenneman, Role of free fatty acid and lipoproteins in the lipid nutrition of tumor cells. *Tumor Lipids, Biochemistry and Metabolism.* Edited by R. Wood, p. 1. Amer. Oil Chem. Soc., Champaign (1973).
10. D. E. Brenneman and A. A. Spector, Utilization of ascites plasma very low density lipoprotein triglycerides by Ehrlich cells. *J. Lipid Res.* **15,** 309 (1974).
11. F. T. Hatch, F. T. Lindgren, G. L. Adamson, L. C. Jensen, A. W. Wong and R. I. Levy, Quantitative agarose gel electrophoresis of plasma lipoproteins: a simple technique and two methods of standardization. *J. Lab. clin. Med.* **81,** 946 (1973).
12. R. Feliste, N. Dousset and L. Douste-Blazy, Separation of human plasma lipoproteins by polyacrylamide mixed gel electrophoresis *Clin. Chim. Acta* **47,** 329 (1973).
13. B. J. Davis, Disc electrophoresis—II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121,** 404 (1965).
14. N. Ressler, R. Springgate and J. Kaufman, A method of lipoprotein electrophoresis. *J. Chromatog.* **6,** 409 (1961).
15. G. Kessler and H. Lederer, Fluorometric measurement of triglycerides. *In* Automation in Clinical Chemistry, Technicon Symposium, Edited by L. T. Skeggs, Jr., p. 341. Mediad, Inc., New York (1966).
16. L. L. Abell, B. B. Levy, B. B. Brodie and F. E. Kendall, A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. biol. Chem.* **195,** 357 (1952).
17. R. Schoenheimer and W. M. Sperry, A micromethod for the determination of free and combined cholesterol. *J. biol. Chem.* **106,** 745 (1934).
18. D. E. Brenneman, R. McGee and A. A. Spector, Cholesterol metabolism in the Ehrlich ascites tumor. *Cancer Res.* **34,** 2605 (1974).
19. P. Mermier and N. Baker, Flux of free fatty acids among host tissues, ascites fluid and Ehrlich ascites carcinoma cells. *J. Lipid Res.* **15,** 339 (1974).
20. M. Barclay, E. Garfinkel, O. Teberus-Kekish, E. B. Shah, M. deGuia, R. K. Barclay and V. P. Skipski, Properties of lipoprotein lipase extracted from livers of normal rats and livers and tumors of rats bearing Walker carcinosarcoma 256. *Arch. Biochem. Biophys.* **98,** 397 (1962).
21. E. T. Mays, Serum lipids in human cancer. *J. surg. Res.* **9,** 273 (1969).

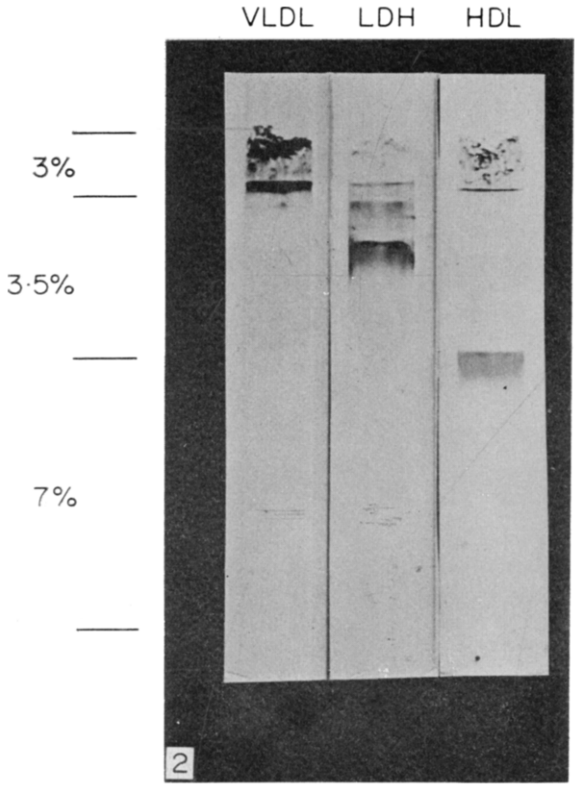


Fig. 2. Polyacrylamide gel electrophoretic patterns obtained using washed lipoprotein fractions isolated from Ehrlich ascites tumor plasma. The polyacrylamide concentration of the three segments of the gel are listed on the left side. Each of the lipoprotein fractions was stained with Sudan Black-B prior to electrophoresis.

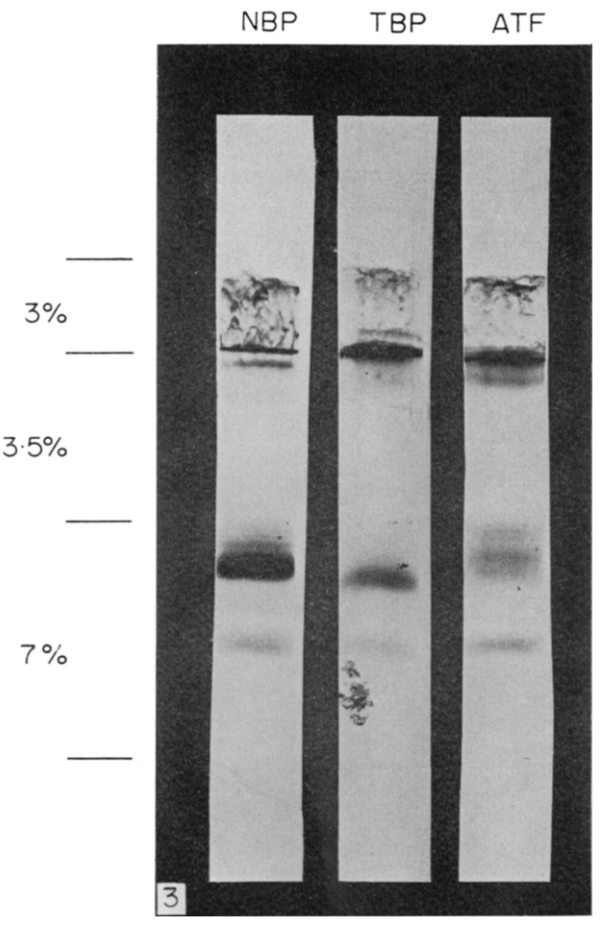


Fig. 3. Comparison of the lipoprotein patterns of normal mice and those bearing the Ehrlich ascites tumor. Abbreviations: NBP, blood plasma from a normal mouse; TBP, blood plasma from a tumor-bearing mouse; and ATF, plasma of the Ehrlich ascites tumor. The plasma was stained with Sudan Black-B prior to electrophoresis. Equal volumes of plasma, 30 μl, were electrophoresed in each of the 3 specimens.

(to face p. 228)

Erop. J. Cancer Vol. 11, pp. 231–240. Pergamon Press 1975. Printed in Great Britain

Carcinostatic Activity of 4-hydroxy-2-pent-en-1-al against Transplantable Murine Tumour Lines*

P. J. CONROY, J. T. NODES, T. F. SLATER and G. W. WHITE
Brunel University, Biochemistry Department, Kingston Lane, Uxbridge, Middlesex, UB8 3PH. England.

Abstract—*The carcinostatic activity of 4-hydroxy-2-pent-en-1-al (HPE) was studied with the following transplantable murine tumours in the ascitic form: Ehrlich carcinoma, Gardner lymphosarcoma, and Sarcoma* 180. *A bioassay system was used involving treatment of tumour cell suspension* in vitro *with* 0·04–1·28 *mM HPE for* 60 *min at* 37°C *and re-injection of treated cells into groups of mice. Significant increases in survival time were observed with concentrations of HPE greater than* 0·16 *mM* (16·8%–91·5%) *and "indefinite survivors" (surviving beyond day* 60 *following transplantation) were obtained using HPE* (0·64–1·28 *mM) in all three tumour lines. The* ID_{50} (0·095–0·153 *mM) and* ID_{90} (0·131–0·315 *mM) values obtained demonstrate that HPE is a relatively potent carcinostatic agent* in vitro *in the tumour lines tested in this study. Results obtained with HPE under conditions* in vitro *do not distinguish between a direct cytotoxic effect of HPE on the ascitic tumour cells or a possible extension in the generation time of these cells. Treatment of these ascitic cell lines* in vivo *with multiple i.p. injections of HPE* (4–128 *mg/kg body wt/day) on days* 3 *and* 4 *following transplantation resulted in no significant increase in survival time compared to controls for both Ehrlich and Gardner. Sarcoma* 180 *responded with a moderate inhibition of tumour growth of* 24% *and* 35% *at* 64 *and* 128 *mg/kg/day of HPE.*

INTRODUCTION

THE ANTI-TUMOUR properties of some keto-aldehydes, aliphatic hydroxy-aldehydes and related compounds have been reported in a number of previous publications. French and Freedlander [1–4] described the effectiveness of 3-ethoxy-2-oxo-butanal (Kethoxal) and its related bis-derivatives against a variety of tumour lines; Szent-Györgyi and co-workers [5] have ascribed a role to a glyoxal derivative that functions as an electron—accepting growth inhibitor regulating cell division in both bacterial and mammalian cell systems. More recently, Alarcon [6] suggested acrolein as a candidate for this hypothesis. Certainly the work of Apple, Greenberg and co-workers [7–12] has provided valuable, though not conclusive, support for the "electron acceptor" idea by reporting the inhibitory effects of normal metabolites such as propanals, on the growth of murine tumours. In general, such compounds have been reported to have strong inhibitory effects on protein synthesis [13–18]. Schauenstein [19], in the first of numerous reports, has shown that the peroxidation of poly-unsaturated fatty acids leads to the production of water-soluble products of low molecular weight, some of which have powerful inhibitory effects on cell division in mammalian cell lines in culture [20–22]. Two of the most active compounds in this respect were 4-hydroxy-2,

Accepted 19 November, 1974.

*This investigation was supported by the Cancer Research Campaign, London.

3-*trans*-oct-en-l-al (HOE) and 4-hydroxy-2, 3-*trans*-pent-en-l-al (HPE), which have broadly similar biological properties. Moderate to good inhibition of tumour growth has been observed using the following subcutaneously implanted lines: Ehrlich [23, 24], Nemeth-Kellner lymphoma [25], Sarcoma 180 [26] after treatment with HPE at 30–40 mg/kg body weight with peritumoural injections.

Bickis *et al.* [21] have reported that HPE strongly inhibits the oxidation of tricarboxylic acids, glycolysis and the incorporation of radioactive percursors into DNA, RNA, protein and lipids in ascites tumour cells. All the observed reactions can be prevented by the addition of reduced glutathione (GSH) to the incubation medium. Certain of these observations have been confirmed in this laboratory [27]. In general these effects are more pronounced with tumour cell lines, since the production of similar results with normal tissues requires up to four times the corresponding dose required with the tumour cell lines. The double bond of HPE reacts avidly with non-protein thiol compounds (NPSH) to give cyclic semi-acetals [23, 28]. HPE inhibits the activity of several SH-containing enzymes including glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase [21] and the inhibition can be reversed by the addition of cysteine (CySH) or GSH. The relative resistance of normal cells to HPE treatment may be the result of a higher content of low molecular weight, SH-compounds providing a better protection of functional-SH groups of essential enzymes. Nohammer *et al.* [29] have shown that the treatment of Ehrlich ascites tumour cells with 5 mM HPE reduces the protein-bound SH (PSH) content by 30%. Rindler and Schauenstein [30] report that the loss of PSH is higher in certain transplantable animal tumours after treatment with 5 mM HPE than in normal liver (40–50% compared to 1%). Respiratory inhibition in these tumours was practically 100% whilst that of liver was almost zero.

From 1966 onwards, Burlakova and colleagues have published many reports suggesting that an inverse correlation occurs between the extent of lipid peroxidation and the rate of cell division in various experimental systems [31–35]. This work is consistent with the hypothesis that products of lipid peroxidation have cytostatic activity.

Sparkes and Kenny [36, 37] have shown that a bacterial growth inhibitor released from HeLa cells in culture was a ketoaldehyde, 4-hydroxy-2-keto-butyraldehyde. This substance profoundly depressed the growth of *S.aureus* in culture even though the nutrient levels were optimal for growth. A similarity may well exist between the biological activity of this compound on *S.aureus* and the effects of substances such as HOE and HPE on experimental tumour lines.

The purpose of the present investigation was to examine, using a semi-quantitative method, the extension in survival time produced by geometrically increasing doses of HPE added *in vitro* to three experimental murine tumour lines in the ascitic form.

The report will demonstrate that HPE is a relatively potent carcinostatic agent *in vitro* but has only weak activity *in vivo*. A possible approach to understanding the reasons for such differences between the results *in vitro* and *in vivo* is discussed.

MATERIAL AND METHODS

4-hydroxy-2, 3-trans-pent-en-l-al (HPE) was synthesized and purified as described below. Tissue culture medium 199 (TC 199) and horse serum 5 (inactivated) (HS 5) were purchased from Wellcome Reagents Ltd., Beckenham, Kent, England. Isoton and "Zapoglobin" were purchased from Coulter Electronics Ltd., Dunstable, Beds., England. Female CBA/CA mice were obtained from S.A.C.I. Ltd., Braintree, Essex, England.

HPE was synthesized from acrolein by the method of Esterbauer and Weger [38]. For biological testing, the final product was purified by multiple-distribution between chloroform and water [39] using a Craig counter-current distribution apparatus. Pure HPE, isolated from the aqueous solution by saturation with sodium chloride and extraction with chloroform, exhibited infra-red and nuclear magnetic resonance spectra in good agreement with authentic spectra kindly provided by Dr. H. Esterbauer.

The following experimental tumour lines were maintained in female CBA/CA mice (20–25 g) in the Ascites form: Ehrlich carcinoma, Gardner lymphosarcoma and Sarcoma 180. Transplantation and harvesting occurred on the 7th day following i.p. injection of 2×10^6 cells per animal.

In vitro *bioassay method*

The procedure followed was essentially that of Ball *et al.* [40]. Sterile precautions were employed throughout. Ascites tumour cells

were harvested from the peritoneal cavity into sterile 0·9% saline containing 0·1 ml heparin. The cells were then washed with sterile "lysing buffer" (7·47 g NH_4Cl; 2·06 g Tris–HCl in 1 l. brought to pH 7·2 with conc. HCl) to remove erythrocytes and sedimented at 700 g_{av} for 5 min. The process was repeated until an erythrocyte-free pellet was produced. The pellet was then gently resuspended in 5 ml sterile 0·9% saline and a count taken on a Coulter Model D Particle Counter. The cells were then resuspended in tissue culture medium (TC 199/HS 5 [inact.] 6:4 v/v) to a final concentration of 5×10^6/ml. Aliquots (9·9 ml) of the tumour cell suspension were removed into sterile, graduated 10 ml stoppered tubes and equilibriated at 37°C for 30 min. Treated cell suspensions received 0·1 ml of a sterile HPE solution (in 0·9% saline) to give a final concentration of 0·04–1·28 mM in 10 ml. Control cell suspensions received 0·1 ml sterile saline. All tubes were gently inverted every 5 min over their total incubation time at 37°C. Since tumour cell suspensions are relatively fastidious in their nutritional requirements, we attempted to eliminate the possibility that incubation in the tissue culture medium itself contributed to an increase in survival time in the following manner. "Time controls" were performed using cells without added HPE to give total incubation times of 120, 180 and 240 min at 37°C. Following the above treatments, groups of animals received 0·2 ml of cell suspensions containing 1×10^6 cells, and the survival times of the animals were recorded.

In vivo *carcinostatic activity method*

Ascites tumour cells were removed from the peritoneal cavity into heparinized 0·9% sterile saline on the 7th day following transplantation. A cell count was taken on a Coulter Model D particle counter. For counting purposes, erythrocytes present in samples were removed by lysis using 3–4 drops of a commercial saponin solution, "Zapoglobin". The cell concentration was then adjusted by serial dilution so that 0·2 ml. of cell suspension contained either 2×10^6 (Ehrlich) or 1×10^6 (Gardner and Sarcoma 180) tumour cells. These were then injected into groups of 40 female CBA/CA mice (20–25 g) per tumour line. Within each group, 10 animals served as "controls" with the remainder divided into 6 groups of 5 animals each. Each group of 5 mice received, on days 3 and 4 following transplantation, 4 doses of HPE in 0·9% saline spaced at 2-hr intervals. The dose levels employed were 4, 8, 16, 32, 64 and 128 mg/kg body wt./day. In female CBA/CA mice, this dose regime gives an LD_{50} for HPE of 181 mg/kg/day. Following the above treatments the survival times of the animals were recorded.

Cell number—Survival time method

As above, ascites tumour cells were removed from the peritoneal cavity into heparinized 0·9% sterile saline on the 7th day following transplantation and a cell count taken. The cell concentration was adjusted so that 0·2 ml. of cell suspension contained between 1×10^7 and 1×10^2 tumour cells. These were then injected into groups of 5 animals and the survival times recorded.

RESULTS

Before assessing the activity of HPE (*in vitro*) on the three murine tumour lines using the bioassay method, a cell number–survival time experiment was necessary for each line (see Material and Methods). Groups of 5 CBA/CA ♀ mice (20–25 g) were injected with 1×10^7–1×10^2 cells per animal and the survival times recorded. The results are expressed in Tables 1–3 and a representative plot of $\log_{10}$ cells injected against survival time is given in Fig. 1. A linear plot was obtained with each tumour line after injecting 1×10^7–5×10^4 cells per animal. At approx. 10^4 cells injected, the survival plots deviate from linearity and the percentage take declines. Since none of the experimental tumour lines used was totally syngeneic with the host, the failure to take successfully below 10^4 cells probably results from an immune response mounted by the host.

Table 1. *Cells injected against survival time in female CBA/CA mice* (20–25 *g*)
(*A*) *Ehrlich carcinoma*

Cells inj./mouse	Day of Death	Av.Day Death	Range	% Take
1×10^7	11,11,11,11,12	11.2	11–12	100
5×10^6	12,13,13,13,14	13.0	12–14	100
2×10^6	14,14,15,15,16	14.8	14–16	100
1×10^6	16,16,16,17,18	16.6	16–18	100
5×10^5	17,18,18,19,20	18.6	17–20	100
1×10^5	22,23,23,23,24	23.0	22–24	100
5×10^4	24,25,25,26	25.0	24–26	80
1×10^4	—	—	—	0
1×10^3	—	—	—	0
1×10^2	—	—	—	0

Table 2. Cells injected against survival time in female CBA/CA mice (20–25 g)
(B) Gardner lymphosarcoma

Cells inj./mouse	Day of Death	Av.Day Death	Range	% Take
1×10^7	8,9,9,10,10	9.2	8–10	100
2×10^6	12,13,14,14,14	13.4	12–14	100
1×10^6	14,14,14,15,16	14.6	14–16	100
5×10^5	15,16,16,17,17	16.2	15–17	100
1×10^5	19,20,20,21,21	20.2	19–21	100
5×10^4	21,23,23,23,23	22.6	21–23	100
1×10^4	24,26,28 ——	26.0	24–28	60
1×10^3	——	——	——	0
1×10^2	——	——	——	0

Table 3. Cells injected against survival time in female CBA/CA mice (20–25 g)
(C) Sarcoma 180

Cells inj./mouse	Day of Death	Av.Day Death	Range	% Take
1×10^7	11,12,12,12,12	11.8	11–12	100
5×10^6	12,13,13,13,14	13.0	12–14	100
2×10^6	13,14,14,14,15	14.0	13–15	100
1×10^6	13,14,15,15,15	14.4	13–15	100
5×10^5	15,16,16,16,17	16.0	15–17	100
1×10^5	17,18,18,19,20	18.6	17–20	100
5×10^4	21,22,22,23 --	22.0	21–23	80
1×10^4	——	——	——	0
1×10^3	——	——	——	0

Bio-assay results

For each tumour line investigated, 10 animals served as controls receiving 1×10^6 cells following a 60-min incubation at 37°C. For the "time controls" incubation times of 120, 180 and 240 min were employed. Inspection of Tables 4, 5 and 6 indicates that a 120-min incubation time in the absence of HPE produced no significant increase in survival time (zero "cell kill") with Ehrlich carcinoma and Gardner lymphosarcoma; Sarcoma 180 cells withstood incubations of up to 180 min. Thereafter only Ehrlich proved relatively sensitive to incubation at 37°C, there being a 5% increase in survival time at 180 min and an 11·1% increase at 240 min. These values correspond to 5% and 35% "cell kill".

Using a 60-min incubation at 37°C (concentration above 0·16 mM) produced significant increases in survival time in animals injected with each of the three tumour lines tested; indefinite survivors were observed when the HPE concentration exceeded 0·32 mM (Tables 4, 5 and 6).

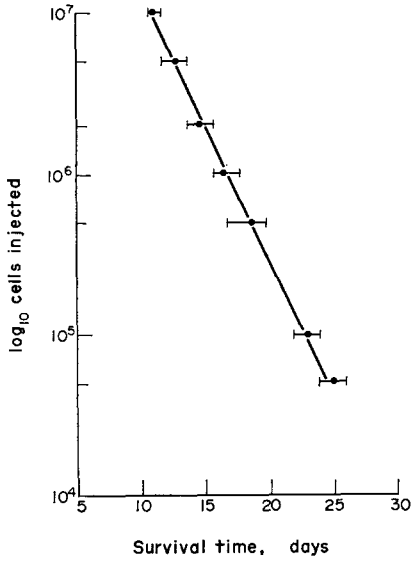


Fig. 1. Ehrlich carcinoma in ♀ CBA/CA mice. Plot of $\log_{10}$ cells injected against survival time.

In the present bio-assay system we define percentage "cell kill" as:

$$\frac{a-b}{a} \times 100\,\%$$

where a = No. cells injected per animal.
b = No. cells theoretically injected to give the survival times obtained with animals receiving untreated tumour cells (as taken from Fig. 1).

This is not an absolute figure since the method precludes viable cell counts during the survival time of treated animals and only extends to the linear portion of the $\log_{10}$ cells injected–survival plot. However, it is a useful comparative index.

A representative plot of percentage "cell kill" against $\log_{10}$ dose for Ehrlich carcinoma is shown in Fig. 2. It indicates a sigmoidal response of the tumour cell population to an increasing level of HPE (0·04–1·28 mM). Plotting the intercepts at 50% and 90% "cell kill" gives the ID_{50} and ID_{90} values quoted in Table 7.

The results shown in the tables do not enable us to distinguish between a direct cytotoxic effect of treatment with HPE *in vitro* or an

Table 4. In vitro *Bioassay data for Ehrlich carcinoma in female CBA/CA mice* (20–25 *g*). *One million cells injected per mouse following a* 60*-min incubation with HPE* (*final conc.* 0·04–1·28 *mM*) *at* 37°*C*

HPE Final conc.(mM)	Day of Death	Av.Day Death	Range	% Take	% Increase in survival time	% Cell Kill
Controls (60 min)	15,16,16,16,16 16,16,17,17,17	16.2	15–17	100	——	——
(120 min)	15,16,16,17,17	16.2	15–17	100	0	0
(180 min)	16,16,17,17,17	17.0	16–17	100	+ 5.0	5.0
(240 min)	16,17,17,20,20	18.0	16–20	100	+11.1	35.0
0.04	15,16,16,16,17	16.0	15–17	100	- 1.3	0
0.08	17,17,17,18,18	17.4	17–18	100	+ 7.3	17.5
0.16	18,18,19,23,23	20.2	18–23	100	+24.6	72.5
0.32	21,23,23,23,25	23.0	21–25	100	+41.9	91.0
0.64	26,26,29 * *	27.0	26–29	160	> 66.3	> 95.0
1.28	* * * * *	——	——	0	——	100.0

* = Indefinite Survivors : arbitrarily defined as those animals surviving past Day 60 after implantation.

Table 5. In vitro *Bioassay data for Gardner lymphosarcoma in female CBA/CA mice* (20–25 *g*). *One million cells injected per mouse following a* 60*-min incubation with HPE* (*final conc.* 0·04–1·28 *mM*) *at* 37°*C*

HPE Final conc.(mM)	Day of Death	Av.Day Death	Range	% Take	% Increase in survival time	% Cell Kill
Controls (60 min)	13,13,14,14,14 14,14,15,15,15	14.0	13–15	100	----	---
(120 min)	14,14,14,15,15	14.4	14–15	100	+ 2.8	0
(180 min)	14,14,14,15,17	14.8	14–17	100	+ 5.7	5.0
(240 min)	14,14,15,15,15	14.6	14–15	100	+ 4.2	5.0
0.04	13,14,14,14,14	13.8	13–14	100	- 1.5	0
0.08	14,14,15,15,15	14.6	14–15	100	+ 4.2	5.0
0.16	24,26,27,27,30	26.8	24–30	100	+91.5	99.0
0.32	24, * * * *	24.0	——	20	>71.5	> 98.0
0.64	* * * * *	---	---	0	----	100.0
1.28	* * * * *	---	---	0	----	100.0

* = Indefinite Survivors : arbitrarily defined as those animals surviving past Day 60 after implantation.

Table 6. In vitro *Bioassay data for Sarcoma* 180 *in female CBA/CA mice* (20–25 *g*). *One million cells injected per mouse following a* 60 *min incubation with HPE* (*final conc.* 0·04–1·28 *mM*) *at* 37°*C*

HPE Final conc.(mM)	Day of Death	Av.Day Death	Range	% Take	% Increase in survival time	% Cell Kill
Controls (60 min)	13,13,13,14,14 14,14,14,14,14	13.7	13–14	100	——	——
(120 min)	13,14,14,14,15	14.0	13–15	100	+ 2.1	0
(180 min)	13,14,14,14,15	14.0	13–15	100	+ 2.1	0
(240 min)	14,15,15,15,16	15.0	14–16	100	+ 9.5	0
0.04	14,14,15,15,16	14.8	14–16	100	+ 8.0	0
0.08	15,15,15,15,16	15.2	15–16	100	+11.0	14.0
0.16	15,16,16,16,17	16.0	15–17	100	+16.8	50.0
0.32	22,23,23,27,—	23.5	22–27	80	>71.4	> 95.0
0.64	* * * * *	—	—	0	—	100.0
1.28	* * * * *	—	—	0	—	100.0

* = Indefinite Survivors : arbitrarily defined as those animals surviving past day 60 after implantation.

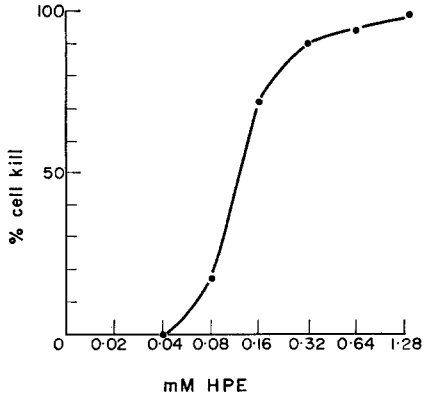


Fig. 2. *Ehrlich carcinoma Bio-assay. One million cells injected per mouse* (♀ *CBA/CA*) *following a 60-min incubation with HPE* (*final conc. 0·04–1·28 mM*) *at 37°C. Percentage cell kill against mM HPE.*

Table 7. *From the plot of percentage cell kill against* log_{10} *final concentration of HPE* (0·04–1·28 *mM*), *the following* ID_{50} *and* ID_{90} *values are obtained*

Tumour	ID_{50} (mM)	ID_{90} (mM)
Ehrlich carcinoma	0.120	0.315
Sarcoma 180	0.153	0.300
Gardner lymphosarcoma	0.095	0.131

extension of the generation time of the tumour cells investigated in this report. The end result measured in an increase in survival time would be similar in both cases, *i.e.*, if HPE treatment depressed the tumour cell population below the level required for a "take" or if the mitotic index was changed.

HPE injected i.p. (4–128 mg/kg body wt/day) in 4 divided doses into animals bearing the ascitic form of Gardner lymphosarcoma or Ehrlich carcinoma produced no significant increases in survival time compared to untreated controls (Tables 8, 9). However, CBA/CA mice transplanted with Sarcoma 180, responded with a moderate increase in survival time on treatment with HPE at dose levels of 64 and 128 mg/kg/day (days 3 and 4). This treatment resulted in increases in survival time compared to untreated controls of 24% and 35% respectively (Table 10).

We can conclude that the attempt to overcome the disadvantage of a relatively short half-life *in vivo* be repeated injections of HPE has not succeeded. These poor results obtained under conditions *in vivo* are in marked contrast to the effects of added HPE *in vitro* to these same cell lines using the 'bio-assay' system described above. In this sense, the data is consistant with the reports of Schauenstein *et al.* [23–26] in which close peritumoural

Table 8. In vivo *carcinostatic data for Ehrlich carcinoma in female CBA/CA mice* (20–25 *g*). *Two million cells injected i.p. per mouse. Treated animals received* 4–128 *mg/kg body wt./day i.p. HPE in saline on days* 3 *and* 4 *following transplantation. The total daily dose consisted of* 4 *equal doses spaced at* 2*-hourly intervals*

Total daily dose of HPE in mg/Kg body wt./day.	Day of Death	Range	Av.day of death	% Increase in Survival Time
Untreated Controls	14,15,15,16,16 16,17,17,17,17	14–17	16.0	—
4	15,15,16,17,17	15–17	16.0	0
8	15,16,16,17,17	15–17	16.2	+ 1.1
16	14,15,16,16,17	14–17	15.6	- 2.5
32	15,15,18,18,19	15–19	17.0	+ 6.2
64	15,15,16,16,16	16–16	15.6	- 2.5
128	16,16,16,16,16	—	16.0	0

Table 9. In vivo *carcinostatic data for Gardner lymphosarcoma in female CBA/CA mice* (20–25 *g*). *One million cells injected i.p. per mouse. Treated animals received* 4–128 *mg/kg body wt./day i.p. HPE in saline on days* 3 *and* 4 *following transplantation. The total daily dose consisted of* 4 *equal doses spaced at* 2*-hourly intervals*

Total daily dose of HPE in mh/Kg body wt./day.	Day of Death	Range	Av.day of death	% Increase in Survival Time
Untreated Controls	15,16,16,16,16 16,16,16,16,16	15–16	15.9	—
4	15,15,17,17,17	15–17	16.2	+ 2.0
8	15,15,17,17,17	15–17	16.2	+ 2.0
16	14,16,16,16,16	14–16	15.6	- 1.9
32	17,17,17,17,17	—	17.0	+ 7.0
64	14,17,17,17,17	14–17	16.5	+ 3.8
128	14,14,14,17,17	14–17	15.2	- 3.4

injections of HPE were employed against several subcutaneously implanted murine tumour lines and improvements in survival time were observed.

DISCUSSION

Relatively little work has been reported on the influence of hydroxy- and keto-aldehydes on mammalian cell division. Klamerth [41] has indicated that glyoxal at concentrations greater than 0·5 mM is cytotoxic for human

Table 10. In vivo *carcinostatic data for Sarcoma* 180 *in female CBA/CA mice* (20–25 *g*). *One million cells injected i.p. per mouse. Treated animals received* 4–128 *mg/kg body wt./day i.p. HPE in saline on days* 3 *and* 4 *following transplantation. The total daily dose consisted of* 4 *equal doses spaced at* 2*-hourly intervals*

Total daily dose of HPE in mg/Kg body wt./day.	Day of Death	Range	Av.day of death	% Increase in Survival Time
Untreated Controls	14,15,15,15,15 16,16,16,16,16	14–16	15.4	—
4	15,15,15,15,17	15–17	15.4	0
8	14,14,15,17,17	14–17	15.4	0
16	15,15,15,17,17	15–17	15.8	+ 2.5
32	14,15,16,17,17	14–17	15.8	+ 2.5
64	18,19,19,19,21	18–21	19.2	+24.0*
128	14,20,23,23,24	14–24	20.8	+35.0†

* $P \leqslant 0{\cdot}01$ † $P \leqslant 0{\cdot}05$ (2 tailed *t*-test).

fibroblasts in culture primarily through blocking DNA and protein synthesis. Gregg [42] reported on the effects of methyl-glyoxal and propyl-glyoxal on mammalian cell lines growing exponentially in suspension culture. Neither compound induced synchronous growth in the lines employed. Both compounds acted as general inhibitors of protein synthesis at the 0·03–0·2 mM level. Scaife [22] has reported that HPE is a more potent mitotic inhibitor than either methyl-glyoxal or Kethoxal. Cytotoxicity is reduced when cultured cells are in the non-proliferating phase of the cell cycle. Using synchronized human kidney T-cells, HPE was 7 times as effective on a molar basis as Kethoxal (0·005–0·1 mM). The cytotoxicity could be diminished but not prevented by the addition of 5 mM CySH or cysteamine. However, that HPE acts as a differential cytotoxic agent in this system remains to be demonstrated.

In the present study, we report that in the 3 murine tumour systems investigated, cytotoxicity starts at the 0·08–0·16 mM level with indefinite survivors occurring in the 0·64–1·28 mM region *in vitro*. ID_{50} values in the 0·1–0·15 mM range represent quite good *in vitro* tumour cytotoxicity and compare well with the lower level of 0·5 mM reported by Klamerth [41] for the effect of glyoxal on human fibroblasts in culture. Bickis *et al.* [21] and more recently Slater *et al.* [27] have reported that the incorporation of ^{3}H-thymidine into certain murine tumour cells *in vitro* is severely depressed by HPE treatment at the level of

0·16–0·20 mM. Incorporation of the corresponding precursors into RNA and protein at the same level was only moderately reduced. This value coincides with the lower level of "cell kill" in this investigation. Necrosis did not occur in monkey kidney and liver cells treated with up to 10 mM of the higher homologue of HPE, HOE [19]. Necrosis does occur at the 2·0–3·0 mM level in Ehrlich ascites tumour cells, with inhibition of glycolysis at 0·3 mM and cessation of respiration at 2·0 mM. The indefinite survivors reported in the current work lie in the 0.54–1·28 mM region and represent close to a 100% "cell kill." Even with the reservations already outlined concerning the term "cell kill", the reported levels confirm that HPE is a rather potent carcinostatic agent under the conditions used here *in vitro*.

Both HOE and HPE have relatively short half-lives *in vivo;* following an injection i.p. of a 5 mM solution of HOE, 33% remains after 5 min, 12% after 10 min. This gives a $t_{\frac{1}{2}}$ of 3–4 min. This short $t_{\frac{1}{2}}$ produces difficulties in relation to studies *in vivo* but in the investigations *in vitro* presented here this feature is not of significance. Tissue culture medium contains a smaller quantity of low molecular weight thiol compounds (such as CySH and GSH) which can form cyclic semi-acetals with HPE at neutral pH, than is present in the whole animal. The absence of large surface areas such as gut epithelia which can rapidly reduce the level of free HPE by combination with free SH must contribute to a higher intrinsic activity of HPE under conditions *in vitro*.

The poor *in vivo* carcinostatic activity of HPE presumably could be improved markedly by an increase in the half-life under conditions *in vivo*. Slater *et al.* [27] have shown that HPE (0·16–0·20 mM) *in vitro* severely depressed ^{3}H-thymidine incorporation into DNA in all three tumour lines used here; ^{3}H-uridine and ^{3}H-leucine into RNA and protein were considerably less affected. Preliminary work indicates that the addition of cysteine before treatment of tumour cells with HPE provides partial protection against these effects [43]. HPE reacts readily with thiol groups at neutral pH. One approach to increasing the $t_{\frac{1}{2}}$ of HPE *in vivo* may be via the formation of an adduct of HPE and cysteine. Assuming such an adduct is much less toxic than the free aldehyde injected i.p., then a sustained release of HPE may occur with time, thus improving its *in vivo* anti-tumour activity. Such a procedure is under investigation.

Acknowledgements—We are grateful to Dr. T. A. Connors of the Chester Beatty Research Institute, London for his generous donation of the original tumour cell lines and unfailing help and criticism; to Dr. H. Esterbauer of the Institute of Biochemistry, Graz, Austria for the provision of spectra and for his helpful comments on the synthesis of HPE; and to Dr. E. Schauenstein for helpful advice and samples of HPE and HOE for comparative purposes.

REFERENCES

1. F. A. French and B. L. Freedlander, Carcinostatic action of polycarbonyl compounds and their derivatives II. Glyoxal bis-(guanylhydrazone) and derivatives. *Cancer Res.* **18,** 360 (1958).
2. B. L. Freedlander and F. A. French, Carcinostatic action of polycarbonyl compounds and their derivatives I. 3-ethoxy-2-ketobutyraldehyde and related compounds. *Cancer Res.* **18,** 172 (1958).
3. F. A. French and B. L. Freelander, Carcinostatic action of polycarbonyl compounds and their derivatives IV. Glyoxal bis (thiosemicarbazone) and derivatives. *Cancer Res.* **18,** 1290 (1958).
4. F. A. French, B. L. Freedlander, A. Hosking and J. French, Carcinostatic activity of some dicarbonyl compounds and their bis-hydrazones. *Acta. Un. int. Cancer* **16,** 614 (1960).
5. A. Szent-Györgyi, *Bioelectronics, a Study in Cellular Regulation, Defense and Cancer.* Academic Press. N.Y. (1968).
6. R. A. Alarcon, Acrolein, a component of a Universal Growth Regulatory system: A Theory. *J. theor. Biol.* **37,** 159 (1972).
7. M. A. Apple and D. M. Greenberg, Arrest of cancer in mice by therapy with normal metabolites I. 2-oxo-Propanal. *Cancer Chemother. Rep.* **51** (7), 455 (1967).
8. M. A. Apple and D. M. Greenberg, Arrest of cancer in mice by therapy with normal metabolites II. Indefinite survivors among mice treated with mixtures of 2-oxo-propanal and 2, 3-dihydroxy-propanal. *Cancer Chemother. Rep.* **52** (7), 687 (1968).

9. M. A. Apple and D. M. Greenberg, Inhibitory effects of DL-2-Mercapto-3-hydroxypropanal on growth of transplantable cancers in mice. *Cancer Chemother. Rep.* **53** (3), 195 (1969).
10. M. A. Apple and D. M. Greenberg, A cancer growth inhibitory intermediate of the amino-acetone cycle with a direct action on Deoxyribonucleic acid. *Proc. Amer. Assoc. Cancer Res.* **10,** 3 (1969).
11. M. A. Apple, F. C. Ludwig and D. M. Greenberg, Selective cancer growth inhibition in mice by dihydroxypropanal without concomitant inhibition of bone-marrow or other normal tissue. *Oncology* **24,** 210 (1970).
12. M. A. Apple and M. J. Cline, Anti-cancer effects of Propanals: comparison between human and animal tumour systems. *Nat. Cancer Inst. Monogr.* **34,** 161 (1971).
13. G. G. Guidotti, A. Fonnesu and E. Ciaranfi, Inhibition of amino-acid incorporation into protein of Yoshida ascites hepatoma cells by Glyceraldehyde. *Cancer Res.* **24,** 900 (1964).
14. G. G. Guidotti, L. Loretti and E. Ciaranfi, Studies on the anti-tumour activity of aliphatic aldehydes, I. The mechanism of inhibition of amino-acid incorporation into protein of Yoshida hepatoma ascites cells. *Europ. J. Cancer* **1,** 23 (1965).
15. E. Ciaranfi, L. Loretti, A. Borghetti and G. G. Guidotti, Studies on the anti-tumour activity of aliphatic aldehydes II. Effect on survival of Yoshida ascites hepatoma-bearing rats. *Europ. J. Cancer* **1,** 147 (1965).
16. L. Loretti, M. E. Ferioli, G. C. Gazzola and G. G. Guidotti, Studies on the anti-tumour activity of aliphatic aldehydes III. Formation of Thiazolidine-4-carboxylic acid in tissues. *Europ. J. Cancer* **7,** 281 (1971).
17. T. Jerzykowski, W. Matuszewski, N. Otrzonsek and R. Winter, Antineoplastic activity of Methyl-Glyoxal. *Neoplasma* **17** (1), 25 (1970).
18. A. Perin, G. Sessa, G. Scalabrino, A. Arnaboldi and E. Ciaranfi, Studies on the anti-tumour activity of aliphatic aldehydes V. Preferential inhibition of protein synthesis in normal or neoplastic tissues in relation to molecular structure. *Europ. J. Cancer* **8,** 111 (1972).
19. E. Schauenstein, Autoxidation of polyunsaturated esters in water: Chemical structure and biological activity of the products. *J. Lipid. Res.* **8,** 417 (1967).
20. E. Schauenstein, H. Verdino and M. Taufer, The inhibitory mechanism induced in tumour cells by 4-hydroxy-2-pentenal (HPE). *Mh. Chem.* **10** (4), 1189 (1970).
21. I. J. Bickis, E. Schauenstein and M. Taufer, Effects of Hydroxypentenal on the metabolism of normal and malignant cells. *Mh. Chem.* **100** (3), 1077 (1969).
22. J. F. Scaife, Modification of the cytotoxic action of Hydroxypentenal on cultured mammalian cells. *Naturwissenschaften* **57** (5), 251 (1970).
23. E. Schauenstein, B. Wunschmann and H. Esterbauer, The selective *in vivo* destruction of sub-cutaneously implanted Ehrlich ascites tumour cells by 4-hydroxy-enales and especially by 4-hydroxypentenal. *Z. Krebsforsch.* **71,** 21 (1968).
24. E. Schauenstein, B. Wunschmann and H. Esterbauer, Experiments on the therapeutic effect of hydroxypentenal on the Ehrlich ascites solid tumour of mice. *Z. Krebsforsch.* **72,** 325 (1969).
25. E. Schauenstein, M. Zollner, M. Ernet and H. Esterbauer, Experiments on the therapeutic effect of 4-hydroxypentenal III. Inhibition of the growth of solid Nemeth-Kellner lymphoma. *Z. Krebsforsch. Klin. Onkol.* **76** (2), 140 (1971).
26. E. Schauenstein, M. Ernet, H. Esterbauer and H. Zollner, Experiments on the therapeutic effect of 4-hydroxypentenal II. Inhibition of the growth of Sarcoma 180. *Z. Krebsforsch.* **75** (2), 90 (1971).
27. T. F. Slater, P. J. Conroy, H N. Fraval, P. J. Jose, D. C. H. McBrien, J. T. Nodes, B. C. Sawyer and G. W. White, Biochemical and anti-tumour properties of hydroxy- and keto-aldehydes. *2nd Meeting Europ. Ass. Cancer Res., Heidelberg* 182 (1973).
28. H. Esterbauer, Kinetics of the reaction of sulphydryl compounds with α, β-unsaturated aldehydes in an aqueous system. *Mh. Chem.* **101,** 782 (1970).

29. G. Nohammer, E. Schauenstein and P. Weber, Quantification of selective binding of 4-hydroxypentental to structural protein thiols in Ehrlich ascites tumour cells. *J. Histochem. Cytochem.* **21,** 12, 1082 (1973).
30. R. Rindler and E. Schauenstein, On the effect of 4-hydroxypentenal on the content of protein-bound and non-protein bound SH-groups in normal and tumour cells. *Z. Naturf. B.* **25B**, 7, 739 (1970).
31. E. B. Burlakova, V. D. Gaintseva and N. M. Emanuel, Relationship between the radioprotective and anti-neoplastic properties of radical processes. *Izv. Akad. Nauk SSR. Ser. Biol.* **4,** 511 (1966).
32. E. B. Burlakova, The possible regulation of cell multiplication by a free radical mechanism. *Biofizika* **12** (1), 82 (1967).
33. E. A. Baglei and E. B. Burlakova, Changes in the antioxidative activity of liver lipids and tumours during the development of Zeidel's ascitic hepatoma and treatment with anti-tumour agents *Biofizika* **13** (1), 76 (1968).
34. S. K. Dubrina, Y. P. Kozlov and E. B. Burlakova, A study of free radical changes in tissue lipids of animals with implanted tumours. *Zh. Otd. Vyp. Obshch. Vop. Patol. Onkol.* **8** (53), 580 (1970).
35. A. V. Alesenko, E. B. Burlakova and A. A. Vainson, Change of the antioxidant activity of lipids by stages in the cell cycle in a HeLa cell culture. *Dokl. Akad. Nauk. SSR. Ser. Biol.* **202** (1), 208 (1972).
36. B. G. Sparkes and C. P. Kenny, Identification of a bacterial growth inhibitor from HeLa cells: a Keto-aldehyde. *Proc. nat. Acad. Sci. (Wash.)* **64** (3), 920 (1969).
37. B. G. Sparkes and C. P. Kenny, A growth inhibitor from human cells: identification and synthesis. *Int. J. clin. Pharmacol.* **3** (3), 223 (1970).
38. H. Esterbauer and W. Weger, Synthesis of homologous 4-hydroxy-alkenals. *Mh. Chem.* **98,** 1944 (1967).
39. H. Esterbauer, Synthesis of some 4-hydroxy-2-alkenals. *Mh. Chem.* **102,** 824 (1971).
40. C. R. Ball, T. A. Connors, J. A. Double, V. Ujhazy and M. F. Whisson, Comparison of nitrogen-mustard sensitive and resistant Yoshida sarcomas. *Int. J. Cancer* **1,** 319 (1966).
41. O. L. Klamerth, Influence of Glyoxal on cell function. *Biochim. Biophys. Acta.* **155,** 271 (1968).
42. C. H. T. Gregg, Inhibition of Mammalian cell division by Glyoxals. *Exp. Cell Res.* **50,** 65 (1968).
43. P. J. Conroy, J. T. Nodes, T. F. Slater and G. W. White, Effects of 4-hydroxy-2-pent-en-1-al on the *in vitro* incorporation of radioactive precursors in transplantable murine tumour lines. *9th Meeting FEBS.* Budapest, 417, (1974).

Europ. J. Cancer Vol. 11, pp. 241–246. Pergamon Press 1975. Printed in Great Britain

Ultraviolet Light Irradiation as Initiating Agent in Skin Tumor Formation by the Two-stage Method*

F. STENBÄCK

Department of Pathology, University of Oulu,
90220 *Oulu, Finland, and Eppley Institute for Research in Cancer,*
University of Nebraska Medical Center, Omaha, Nebraska 68105, *U.S.A.*

Abstract—*The biological and morphological characteristics of neoplastic progression in mouse skin induced by the two-stage method, using ultraviolet light as an initiator and croton oil as a promoter, were studied by biological and histological methods. Ultraviolet light proved to be an effective initiator in skin tumor formation, when followed by repeated applications of croton oil. In comparison with experiments using* 7,12-*dimethylbenz(a)anthracene (DMBA) as an initiator, the average latent period was longer and the number of tumor-bearing animals and of tumors was lower. Morphologically, an orderly epidermal hyperplasia, progressing through papillomatous hyperplasia and ending in extroverted squamous cell papillomas, occurred in only a few animals. The proliferation of epithelial cells induced by multiple u.v. irradiation was disorderly with distinct cytological and histological abnormalities. A few squamous cell carcinomas developed from the borders of ulcers induced by repeated u.v. light applications. Efforts to increase the neoplastic response, by giving either u.v. light* 10 *times prior to promotion or croton oil immediately after each of* 16 *u.v. irradiations, did not increase the total number of tumors but did induce some malignant ones. These studies emphasize the specificity of initiation and promotion in skin tumor formation and the different actions of physical and chemical carcinogens on animal skin, depending upon the combination of progression and persistence of destructive and proliferative actions specific to each agent.*

INTRODUCTION

THE TWO stage concept in skin tumor formation, proposed by Berenblum and Shubik [1–3], contained as the first stage, the formation of dormant tumor cells by a single carcinogen application. These latent tumor cells were provoked by subsequent application of a promoter to form visible tumors. In his studies on skin carcinogenesis by u.v. light, Blum [4] indicated that skin tumor induction by light is a continuous process which begins to develop from the first exposure and progresses to ultimate tumor formation. Blum's conclusions were based on experiments under which he [5] and Rusch *et al.* [6] could not produce tumors unless exposures were carried out over a minimum of 2½ months, regardless of the energy used. Blum's determinations suggested the shorter exposure periods did not accelerate tumor formation to visible manifestations within the lifetime of the experimental animal. Epstein and Roth [7], using a single u.v. light application as an initiator and croton oil as a promoter, concluded that croton oil stimulates tumor formation, the characteristics of which were established by the exposure to u.v. light. The results of these researchers were significantly different from those encountered when a chemical carcinogen was used as the initiator [8].

Accepted 19 November 1974.

*Supported by the United States Public Health Service contract PH 43-NCI-E-68-959 from the National Cancer Institute.

The following studies were performed to elucidate the occurrence of initiation and promotion in skin carcinogenesis, using physical and chemical tumor-provoking agents to study the morphological characteristics of the neoplastic response and the specificity and persistence of initiation.

MATERIAL AND METHODS

Eight-week-old, random bred Swiss mice of both sexes from the Eppley colony were used, housed in plastic cages (10 per cage) and given Rockland diet pellets and water *ad libitum*. The light source was a Westinghouse FS40T12 sunlamp emitting a total energy of $5{\cdot}1 \times 10^7$ erg/cm^2 in a 3 hr period at a distance of 15 in. Light energy was measured with an International Light IL-335 exposure meter. The carcinogen in the control experiments was 7, 12-dimethylbenz(a)anthracene (DMBA) (Aldrich Chemical Company, Milwaukee, Wis.) dissolved in acetone. Used as promoter was a 2·5% solution of croton oil in reagent grade (Fisher Scientific Co. Rocklawn, Ill.).

The chemicals were applied twice weekly, 0·02 cm^3 per time, on the skin between the flanks, which were shaved regularly. Group 1 received u.v. irradiation once ($5{\cdot}5 \times 10^7$ erg/cm^2), group 2, croton oil 0·02 cm^3 of a 2·5% acetone solution 2 ×/week for lifetime. Group 3 received u.v. light, $5{\cdot}5 \times 10^7$ erg/cm^2 once, followed after a 10 day interval by croton oil, 0·02 cc of a 2·5% acetone solution 2 ×/week for lifetime, and group 4, the same treatment except that the interval between u.v. irradiation and start of croton oil treatment was 6 months. Group 5 received u.v. irradiation 5 times a week for 2 weeks, a total of $5{\cdot}5 \times 10^8$ erg/cm^2 and Group 6 the same treatment followed 10 days later by croton oil treatment, 0·02 cm^3 of a 2·5% solution twice a week for life. Group 7 received u.v. irradiation $5{\cdot}5 \times 10^7$ erg/cm^2 per application twice a week for 8 weeks and Group 8, the same amount of irradiation, $5{\cdot}5 \times 10^7$ erg/cm^3 twice a week for 8 weeks as well as croton oil 0·02 cm^3 1 hr after each irradiation, these groups were followed for 30 weeks. Groups 9 and 10 received DMBA, 50 μg once, Group 10 also received after a 10 day interval, croton oil 0·02 cm^3 of a 2·5% solution for 50 weeks. Treatments of the various groups are further described in Table 1.

The animals were examined weekly and tumor occurrences checked. They were killed when moribund or were left to die spontaneously. Sections from all skin lesions and organs showing gross abnormalities were studied. Formalin-fixed sections were embedded in paraffin and stained with hematoxylin-eosin and other stains, when necessary. The statistical methods used are described by Armitage [9].

RESULTS

The results show that u.v. light is effective as a tumor-inducer in mouse skin. However, tumor numbers and morphological characteristics depended significantly upon the type of treatment. Details of the treatment, its duration, number of animals, application frequency, dose, etc. and the ensuing tumorigenic response are given Table 1. Ultraviolet treatment once at $5{\cdot}5 \times 10^7$ erg/cm^2 (Group 1), produced necrosis of the superficial epidermis and accumulation of inflammatory cells in the dermis the first week after treatment. One week later, a hyperplastic epithelium around the ulceration was a prominent feature, as well as accumulations of thickened collagen fibers in the dermis. The epidermal hyperplasia and fibrosis gradually subsided and at the time of the animals' deaths, no significant differences by comparison with untreated animals were seen. Croton oil, given repeatedly for life (Group 2), produced a slight hyperplasia in some animals. Papillomas, composed of regularly proliferating squamous epithelium, were seen after a very long latent period in 2 animals; however, both ultimately regressed.

Initiation with u.v. light and promotion with croton oil (Group 3) produced a regular hyperplasia, in several animals, which progressed to a papillomatous nature and included the suprainfundibular portions of the hair follicles. Later, a small number of tumors occurred (Fig. 1) after a long latent period (at an average of 54 weeks), and only 1–2 tumors per animal (Table 1). The papillomas were regularly structured with many features of normal epidermis remaining (Fig. 2). Hyper- para- and hyperorthokeratosis were predominant features. In some papillomas the stromal proliferation was prominent, comprising the bulk of the tumor, while the epidermal surface remained virtually unchanged (Fig. 2).

In many animals a fibroblastic proliferation occurred, originating from an increased number of fibroblasts in a thickened, hyalinized stroma. In a few animals, progression to cellular fibromas composed of tightly arranged elongated cells with small nuclei and abundant cytoplasm as well as of thickened collagen fibers was encountered, though the number remained small; 4 altogether (Table 1).

Efforts were made to modify the initiation-

Table 1. *Tumors resulting from initiation by u.v. light and from DMBA with promotion by croton oil*

Group	Treatment (initiation-promotion)	Duration of experiment	Total no. animals	Total no. skin tumors	TBA*	%	T/TBA average	Latent period (weeks) average	Papillomas	Keratoacanthomas	Fibromas	Fibrosarcomas	Squamous cell carcinomas	Regressing tumors
1.	Ultraviolet light $5{\cdot}5 \times 10^7$ erg/cm^2 once	lifetime	30	0	0	0	0	0	0	0	0	0	0	0
2.	Croton oil 0·02 cm^3 2×/week	lifetime	30	2	2	6	1	45	0	0	0	0	0	2
3.	Ultraviolet light $5{\cdot}5 \times 10^7$ erg/cm^2—10 days later croton oil 0·02 cm^3 2×/week	lifetime	60	11	7	11·7	1·6	54	4	0	4	0	0	3
4.	Ultraviolet light $5{\cdot}5 \times 10^7$ erg/cm^2—6 mo. later croton oil 0·02 cm^3 2×/week	lifetime	30	4	4	13·3	1	39	2	0	0	0	0	2
5.	Ultraviolet light $5{\cdot}5 \times 10^8$ erg/cm^2 in 2 weeks	lifetime	30	13	9	30	1·4	12	3	0	3	3	1	3
6.	Ultraviolet light $5{\cdot}5 \times 10^8$ erg/cm^2—10 days later croton oil 0·02 cc 2×/week	lifetime	30	12	8	27	1·5	6	3	0	3	2	2	2
7.	Ultraviolet light $5{\cdot}5 \times 10^7$ erg/cm^2 2×/week for 8 weeks	30 weeks	30	8	6	20	1·3	9	3	0	1	0	2	2
8.	Ultraviolet light $5{\cdot}5 \times 10^7$ erg/cm^2 and croton oil 0·02 cm^3 1 hr after each irradiation 0·02 cm^3 2×/week for 8 weeks	30 weeks	30	7	5	17	1·4	15	3	0	0	0	2	2
9.	DMBA 50 μg once	50 weeks	30	0	0	0	0	0	0	0	0	0	0	0
10.	DMBA 50 μg once 10 days later croton oil 0·02cm^3 2×/week for 50 weeks	50 weeks	30	46	18	60	2·6	31	32	2	0	0	1	11
11.	Untreated control	lifetime	100	1	1	1	1	0	0	0	0	0	0	0

*TBA = tumor-bearing animals.

promotion process. For example, to determine the persistence of the initiation, u.v. light was given 6 months before the start of the croton oil applications (Group 4). In this case, the neoplastic response was weaker (Table 1), and the latent period was shorter with only 4 tumors, 2 of which regressed. The remaining tumors were papillomas, morphologically similar to those of Group 3.

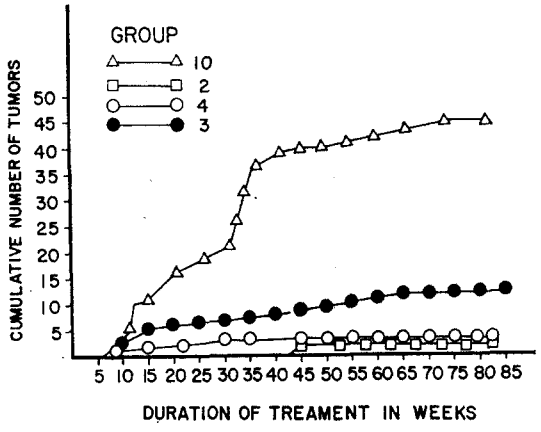


Fig. 1. Cumulative number of tumors induced by u.v. light ($5{\cdot}5 \times 10^7$ erg/cm²) and DMBA followed by croton oil. Group 2 received croton oil alone, Group 3, u.v. light, 10 days later followed by croton oil; Group 4, u.v. light, 6 months later followed by croton oil; and Group 10, DMBA followed by croton oil.

Prolonged u.v. treatment, 10 irradiations of 3 hr each to increase the effect of the initiating agent (Group 6), alone was sufficiently tumorigenic (Group 5), as shown in Table 1. The tumors were similar in both groups. The papillomas, while in many aspects like those of the previous experiments, showed cytological irregularities with cells containing large irregular hyperchromatic nuclei, scanty cytoplasm, disturbed stratification and polarisation (Fig. 3) progressing to the formation of a squamous cell carcinoma in 1 animal. The fibroblastic cellular accumulations were prominent, progressing to the formation of fibromas in 3 animals and fibrosarcomas in 3 animals (Table 1).

Ultraviolet light given twice weekly for 2 months (Group 7) produced a response similar to that of the previous group, resulting in the formation of papillomas, a fibroma and 2 squamous cell carcinomas (Table 1). Efforts to enhance the neoplastic potential of u.v.-irradiatted skin by applying croton oil after each irradiation (Group 8) did not increase the number of tumors (Fig. 4). Morphologically, the tumors were also the same as those of groups 5 and 6, showing papillomas with dysplastic features, and 2 squamous cell carcinomas. The lesions began as a regular hyperplasia around u.v.-induced epidermal ulcers. Grossly, the tumors were indistinguishable from benign ulcers. Ultimately, irregular islands of malignant cell sheets and cords penetrating the dermis were seen.

Skin tumor formation in the control groups (Group 10), using a conventional chemical carcinogen, consisted of a number of tumors with a significantly shorter latent period ($P < 0{\cdot}05$) (Table 1). DMBA alone was non-effective (Group 9), but when followed by repeated applications of croton oil, it induced significant biological and morphological alterations. A regular hyperplasia gradually became papillomatous in character, ultimately forming a number of acantho- and fibropapillomas. Regression were common and multiple tumors, as many as 4 or 5, could be seen on the same animal. Keratoacanthomas, composed of a proliferating epithelium on a cup-shaped base, were seen in 2 animals, along with 1 squamous cell carcinoma (Table 1).

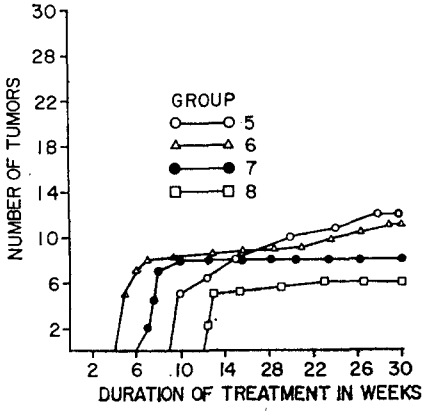


Fig. 4. Cumulative number of tumors induced by u.v. light ($5{\cdot}5 \times 10^8$ erg/cm²) and croton oil. Group 5 received u.v. light alone; Group 6, u.v. light followed by croton oil; Group 7, u.v. light ($5{\cdot}5 \times 10^7$ erg/cm²) twice weekly for 8 weeks and Group 8, the same dose of u.v. light followed by croton oil after each irradiation.

The untreated animals failed to show any significant alterations in the skin; in only one animal was a papilloma found (Table 1).

DISCUSSION

The results of these experiments show that u.v. light is effective as an initiator in two-stage carcinogenesis in mouse skin. However, when comparing the type of neoplastic progression observed and the number and types of tumors,

etc. to results obtained by the use of chemical carcinogens such as DMBA as the initiators, several significant differences are discernible.

The number of tumors in animals receiving u.v. light and croton oil was significantly smaller by comparison with those treated using DMBA as an initiator, both in terms of total tumor-bearing animals and total tumors, as well as the number of tumors per animal. Epstein and Roth [7] obtained only 1 tumor per mouse in their experiments using repeated u.v. light applications and the number of tumors was also considerably smaller, than in corresponding experiments employing repeated carcinogen applications [8]. Blum considered the cause of the limited tumor yield in certain animals due to their short life spans, which prevented ultimate detection of the tumor.

Another aspect of tumor progression is the latent period. In experiments using u.v. light as the initiator, the latent period was significantly longer. The mean in these experiments was about 54 weeks, as compared with animals receiving DMBA and croton oil, whereby the same number of tumors were already discernible by 10 weeks. The same phenomena were reported in the experiments of Epstein and Roth [7]. Based upon studies using different doses of initiators and promoters, Berenblum and Shubik [2] concluded the latent period is exclusively a function of the promoting agent. In the present studies, however, the same promoter, croton oil, produced tumors with a significantly longer latent period, when u.v. light was used as the initiator.

Experiments employing a prolonged u.v. treatment (Groups 5 and 6)showed a neoplastic response, essentially different from that of previous groups, because a significant number of malignant tumors were seen. Nakahara [10], Baba *et al.* [11], and Druckrey [12, 13] concluded that initiation and promotion are only two of a number of stages and that skin carcinogenesis is basically a summation phenomenon. They used, however, a prolonged initiation treatment which, as shown here, produces a completely different result when compared to a conventional two-stage experiment using the initiating agent only once. The specificity of initiation and promotion in two-stage skin carcinogenesis is further emphasized by the results of our experiments, showing that croton oil treatment after each irradiation did not increase tumor yield. The proponents of the summation concept [13] considered initiating and promoting agents as acting by summation of the carcinogenic activity of the two. Blum [4] suggested that chemical carcinogens and u.v. light act independently of one another. These hyptheses do not, however, account for the fact that croton oil after each sub-carcinogenic dose of u.v. light fails to affect tumorigenesis as seen in these experiments.

The morphology of the tumors as well as the events leading to the formation of neoplastic lesions also differed depending upon the initiating agent. In these studies, u.v. light, once followed by repeated percutaneous applications of croton oil, induced an orderly progression of neoplastic transformation and a regular epidermal hyperplasia. The papillomas seen in these animals were similar to those induced by a single application of a chemical carcinogen and repeated applications of croton oil, but in certain aspects different from lesions reported in studies using a single application of a chemical carcinogen [14–16] as well as in studies where a single u.v. irradiation was administered prior to initiation-promotion treatment [17]. The disorderly proliferation of epidermal cells seen when repeated u.v. irradiation was used as initiating agent was related more closely to the neoplastic progression induced by repeated carcinogen applications [8, 16, 18] and repeated irradiation with u.v. light. [19].

REFERENCES

1. I. Berenblum and P. Shubik, A new, quantitative, approach to the study of the stages of chemical carcinogenesis in the mouse's skin. *Brit. J. Cancer* **1,** 383 (1947).
2. I. Berenblum and P. Shubik, The role of croton oil applications associated with a single painting of a carcinogen, in tumour induction of the mouse's skin. *Brit. J. Cancer* **1,** 379 (1947).
3. I. Berenblum and P. Shubik, An experimental study of the initiating stage of carcinogenesis, and a re-examination of the somatic cell mutation theory of cancer. *Brit. J. Cancer* **3,** 109 (1949).
4. H. F. Blum, *Carcinogenesis by Ultraviolet Light.* Princeton University Press, Princeton N.J. (1959).
5. H. F. Blum, H. G. Grady and J. S. Kirby-Smith, Relationships between dosage and rate of tumor induction by ultraviolet radiation. *J. nat. Cancer Inst.* **3,** 91 (1943).

6. H. P. Rusch, B. E. Kline and C. A. Baumann, Carcinogenicity by ultraviolet rays with reference to wavelength and energy. *Arch. Path.* **31,** 135 (1941).
7. J. H. Epstein and H. L. Roth, Experimental ultraviolet light carcinogenesis: A study of croton oil promoting effects. *J. invest. Derm.* **50,** 387 (1968).
8. F. Stenbäck, Promotion in the morphogenesis of chemically inducible skin tumors. *Acta path. microbiol. scand. Suppl.* **208,** 1 (1969).
9. P. Armitage, *Statistical Methods in Medical Research.* Blackwell, Oxford, England (1971).
10. W. Nakahara, Critique of carcinogenic mechanism. In *Progress in Experimental Tumor Research,* (Edited by F. Homburger) Vol. 2, p. 158. Karger, Basel (1969).
11. T. Baba, K. Aoki and M. Ishii, Relation between the so-called two-phase theory and summation theory in carcinogenesis. *Gann* **58,** 161 (1967).
12. H. Druckrey, Beiträge zum Mechanismus der Carcinogenese. *Acta Un. int. Cancer* **10,** 29 (1954).
13. H. Druckrey and D. Schmähl,Die Summationswirkung. *Med. Klin.* **55,** 648 (1960).
14. B. Terracini, P. Shubik and G. Della Porta, A study of skin carcinogenesis in the mouse with single applications of 9,10-dimethyl-1,2-benzanthracene at different dosages. *Cancer Res.* **20,** 1538 (1960).
15. V. Turusov, N. Day, L. Andrianov and D. Jain, Influence of dose on skin tumors induced in mice by single applications of 7,12-dimethylbenzanthracene. *J. nat. Cancer Inst.* **47,** 105 (1971).
16. F. Stenbäck, H. Garcia and P. Shubik, Present states of the concept of promoting action and cocarcinogenesis in skin. In *The Physiopathology of cancer.* Biology and Biochemistry. (Edited by F. Homburger) Vol. I, p. 155. Karger, Basel (1974).
17. F. Stenbäck, H. Garcia and P. Shubik, Studies on the influence of ultraviolet light on initiation of skin carcinogenesis. *J. invest. Derm.* **61,** 101 (1973).
18. F. Stenbäck and P. Shubik, Carcinogen induced skin tumorigenesis in mice: Enhancement and inhibition by ultraviolet light. *Z. Krebsforsch.* **79,** 234 (1973).
19. F. Stenbäck, Manifestation of neoplastic transformation in uv light skin carcinogenesis as compared to chemically induced neoplasia: A stepwise process. *Abst. XI int. Cancer Congr.* Firenze. 659 (1974).

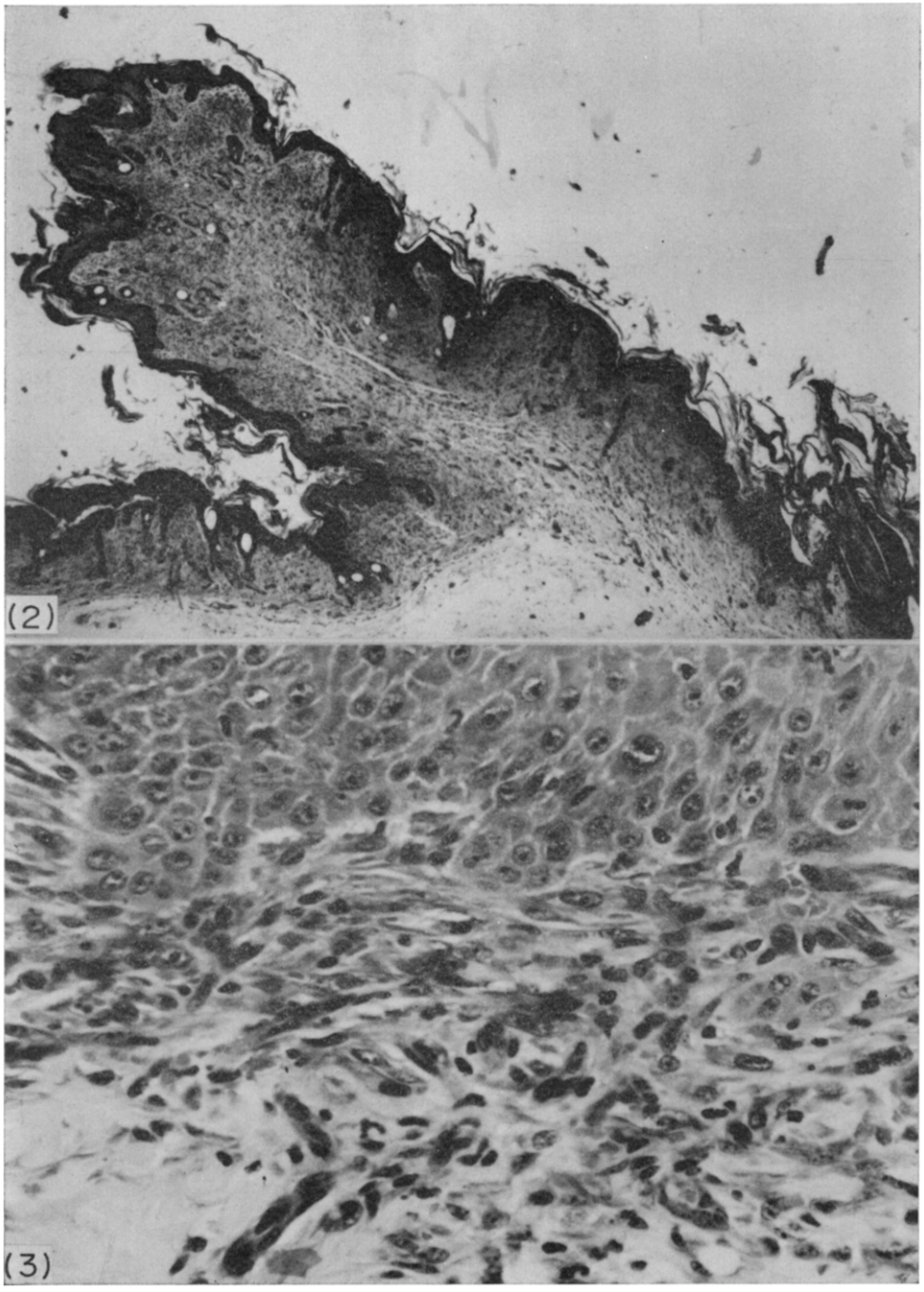


Fig. 2. *Fibroepithelial papilloma composed of a fibrous stalk covered by a regular epidermis induced by u.v. light once and repeated applications of croton oil in mouse skin. H & E* × 28.

Fig. 3. *Dysplastic alterations in squamous epithelium of mice treated with u.v. light* 10 *times and croton oil repeatedly, showing epidermal cells with enlarged hyperchromatic nuclei, disorganised stratification and polarisation as well as dermal fibroblastic proliferation. H & E* × 350.

(to face p. 244)

Europ. J. Cancer Vol. 11, pp. 247–250. Pergamon Press 1975. Printed in Great Britain

Failure to Obtain Transfection with XC Mitochondrial DNA*

JAN SVOBODA,† IVO HLOŽÁNEK,† JAN KORB‡ and OTAKAR MACH§

†*Institute of Experimental Biology and Genetics, Czechoslovak Academy of Sciences;*
‡*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences,*
§*Institute of Haematology and Blood Transfusion, Prague, Czechoslovakia*

Abstract—*The transfecting activity of DNA obtained from mitochondria of XC virogenic rat cells transformed with the Prague strain of RSV was tested. Both mitochondrial DNA isolated with EthBr, containing circular molecules, and total mitochondrial DNA were inefficient in transfecting sensitive chicken fibroblasts in spite of the fact that doses of mitochondrial DNA up to two orders higher than the minimum efficient dose of total XC DNA were used. These findings support the view that a DNA transcript of the RSV genome is not integrated in mitochondrial DNA.*

INTRODUCTION

THE FINDINGS of Hill and Hillová [1, 2], showing that DNA isolated from XC virogenic rat cells transformed with the Prague strain of Rous sarcoma virus (PR RSV) [3, 4] effectively transfects chicken fibroblasts have opened the possibility of testing the subcellular localization of biologically active proviral DNA of RSV. This paper describes the results of experiments in which defined circular and total mitochondrial XC DNA was used for transfection of chicken fibroblasts.

MATERIAL AND METHODS

Transfection assay

The transfecting activity of XC DNA—isolated from either whole cells or nuclei or mitochondria—was tested as published previously [5].

Preparation of mitochondria by zonal centrifugation and isolation of mitochondrial DNA in the presence of ethidium bromide (EthBr) and isolation of control nuclear DNA

The cells were suspended in 3 volumes of a medium containing 0·1 M sucrose, 20 mM Tris–HCl, 5 mM EDTA, pH 7·4. This suspension was homogenized in a Turmix using microscopic control. After disruption of 85% of the cells the suspension was diluted 5 times and sucrose was immediately added to a concentration of 0·25 M. After centrifugation for 10 min at 1000 *g*, the supernatant was separated and recentrifuged twice under identical conditions. The mitochondrial fraction was obtained by centrifugation of the final supernatant for 30 min at 20,000 *g*. The sediment was resuspended in the same buffer and purified by zonal centrifugation. The suspension was placed on the top of a sucrose gradient (15–50% sucrose in 20 mM Tris–HCl, 5 mM EDTA, pH 7·4) and centrifuged for 60 min at 30,000 rev/min in a BXIV rotor of the MSE 65 centrifuge. The fraction of purified mitochondria was diluted and pelleted. All the operations were done at 2°C.

Mitochondrial DNA was isolated by the method described previously [6]. Centrifugation in the CsCl–EthBr gradient was done at 35,000 rev/min for 40 hr at 20°C in a Spinco SW 41 rotor. The bands containing circular DNA were separated and EthBr was removed by dialysis against buffer (0·15 M NaCl, 0·015 M sodium citrate, 0·001 M EDTA, pH 7·2) at 4°C for 2–3 days [7] or with a Dowex 50 resin column [8].

Control nuclear DNA was prepared from the sediment of the cell homogenate after centrifugation at 1,000 *g* for 10 min (above). The

Accepted 19 November 1974.

*This investigation was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research (USA).

pellet was resuspended in a buffer (0·15 M NaCl, 0·1 M EDTA, 0·02 M Tris–HCl of pH 8·0), mixed thoroughly and sodium dodecyl sulphate was added to 1%. The mixture was incubated for 20 min at 20°C. The DNA was isolated by the same procedure as described for isolation of mitochondrial DNA.

EthBr treatment of total XC DNA

Total XC DNA prepared according to Marmur [9] was treated with 200 μg of EthBr per one ml of DNA solution and the solution was dialysed as described for mitochondrial DNA.

Electron microscopy

Electron microscopic control of the isolated DNA molecules was done according to Korb [6].

Preparation of mitochondria by differential centrifugation and isolation of mitochondrial DNA and control nuclear DNA

Mitochondria were prepared according to the method of Wilson and Cascarano [10]. Briefly, a 10% suspension of XC tumour tissue was prepared in 0·25 M sucrose—0·1 mM EDTA—10 mM Tris–HCl buffer, pH 7·4, using a Potter–Elvehjem glass homogenizer and a motor driven Teflon pestle. The homogenate was centrifuged for 10 min at 400 *g* and the supernatant was recentrifuged for 5 min at 400 *g*. The second supernatant was then centrifuged at 7,800 *g* for 10 min. The mitochondrial pellet was washed twice by resuspending it in buffered sucrose solution and recentrifuging it at 7,800 *g* for 10 min. The resulting mitochondrial pellet was used for the isolation of XC DNA as already described [5].

As in our previous experiments [5], nuclei were obtained by the method of Schneider [11]. The pellets obtained after centrifugation of XC homogenates at 400 *g* (see above) were pooled and after two washes in sucrose–EDTA–tris solution, were used for isolation of XC nuclear DNA by the same procedure as described for isolation of mitochondrial DNA.

RESULTS

The transfecting activity of circular DNA isolated with EthBr from XC mitochondria obtained by zonal centrifugation was tested in three experiments performed with a total of 19 dishes. No signs of transfection were observed with doses of DNA ranging from 0·17 μg to 28 μg (Table 1).

Groups of dishes, on which total or nuclear XC DNA was applied, were used as controls. In each group, some of the dishes showed positive transfection. Out of a total of eighteen control dishes, six were successfully transfected. The frequency of transfection in these experiments was lower than that in the experiments tabulated in Table 2. Larger doses of DNA did not produce higher transfecting activity. The efficiency of transfection was found to differ

Table 1. Attempts to transfect chicken fibroblasts with mitochondrial XC DNA prepared by EthBr gradient centrifugation

Dose of XC DNA (μg)	Number of dishes positive in transfection/ Total number of experimental dishes
Mitochondrial DNA	
17–28	0/3
4–15	0/6
1·5	0/6
0·17	0/4
Total DNA	
150	0/1
28	0/1
15	0/2
1·5	2/2
Total DNA + ethidium bromide	
75	1/2
15	1/2
1·5	1/2
Nuclear DNA	
150	0/2
15	1/2
1·5	0/2

Table 2. Attempts to transfect chicken fibroblasts with mitochondrial XC DNA prepared in the absence of EthBr

Dose of XC DNA (μg)	Number of dishes positive in transfection/ Total number of experimenta dishes
Mitochondrial DNA	
150–175	0/3
15	0/6
1·5	0/6
Total DNA	
150	4/4
15	4/6
1·5	4/6
Nuclear DNA	
150	8/8
15	4/4
1·5	4/4

between different experiments. No dose dependence was observed with doses of XC DNA ranging from 1·5 to 150 μg [12].

Mitochondrial DNA preparations from CsCl–EthBr gradient contained before dialysis about 85% circular molecules, the amount of which dropped to 40% after dialysis. The remaining DNA represents opened DNA molecules. The density equilibrium sedimentation profile of XC mitochondrial DNA in CsCl–EthBr gradient is shown in Fig. 1. An electron microscopic picture showing circular molecules of XC mitochondrial DNA is presented in Fig. 2.

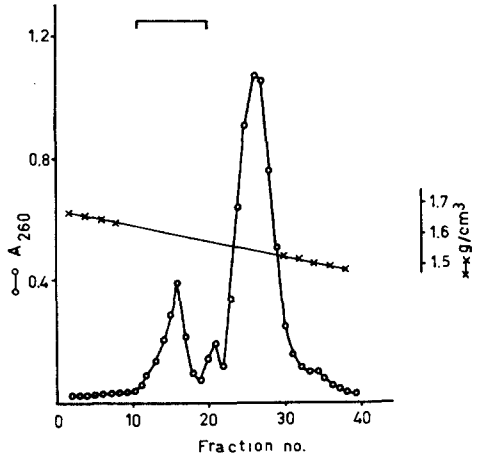


Fig. 1. Density equilibrium sedimentation profile of mitochondrial DNA from XC cells in CsCl–ethidium bromide gradient. Fractions 10–18 *contained supercoiled DNA, fractions* 19–22 *contained opened circular DNA and fractions* 23–32 *contained linear DNA. Brackets indicate fractions which were used as mitochondrial DNA for testing the transfecting activity.*

It should be noted that treatment of total XC DNA with EthBr under conditions used for the isolation of mitochondrial DNA does not destroy its biological activity. Similarly, nuclear DNA isolated in the presence of EthBr remains active in transfection (Table 1).

Two additional experiments were performed with doses of 1·5 to 175 μg of mitochondrial DNA isolated in the absence of EthBr from XC mitochondria obtained by differential centrifugation (Table 2). Again, all 15 dishes treated with such preparation gave negative results in transfection. Control experiments with total or nuclear XC DNA prepared from the same batch of cells used for obtaining mitochondrial DNA gave positive results in 28 out of 32 dishes.

DISCUSSION

The lack of transfecting activity of XC mitochondrial DNA observed in our experiments is in agreement with the previous finding that XC nuclear DNA exhibits the same degree of transfecting activity as total cellular DNA [5, 13]. Similarly, Montagnier and Vigier [14] found no transfecting activity in the fraction containing low molecular weight DNA, which had been prepared according to Hirt [15]. This method, however, does not guarantee the isolation of circular DNA molecules. Finally, Donner *et al.* [16] did not obtain RSV rescue after fusion of enucleated virogenic cells with indicator chicken fibroblasts. This is in agreement with the data discussed above, which indicate the absence of proviral RSV DNA in the cytoplasm of virogenic cells.

It can be argued that circular mitochondrial molecules do not penetrate effectively into recipient cells. Although incorporation of such molecules was not measured in our experiments, it has clearly been shown that double- and single-stranded circular DNA of SV40 and polyoma viruses penetrate and produce a lytic infection in their respective permissive cells [17]. It should, however, be taken into account that mitochondrial DNA is three times bigger than the viral DNAs mentioned above.

Because mitochondrial DNA represents only a small fraction of the total cell DNA—about 0·1%—some activity of mitochondrial DNA could have escaped detection in the experiment in which transfection activity of total and nuclear DNA was compared. Therefore, large doses of mitochondrial DNA (up to 170 μg), two orders higher than the least active amount of total XC DNA (1·5μg), were used in an attempt to transfect chicken fibroblasts. However, no transfection has been obtained which eliminates the possibility of the presence of rare active DNA transcripts of the RSV genome in the mitochondria. This agrees well with molecular hybridization data which indicate the absence of DNA copies of the avian myeloblastosis virus (AMV) genome in the circular DNA molecules from chicken leukaemic myeloblasts [18].

REFERENCES

1. M. Hill and J. Hillová, Production virale dans les fibroblastes de poule traités par l'acide désoxyribonucléique de cellules XC de rat transformées par le virus de Rous. *C.R. Acad. Sci. Paris* **272,** 3094 (1971).

2. M. Hill and J. Hillová, Virus recovery in chicken cells tested with Rous sarcoma cell DNA. *Nature New Biol.* **237,** 35 (1972).
3. J. Svoboda, Presence of chicken tumour virus in the sarcoma of the adult rat inoculated after birth with Rous sarcoma tissue. *Nature (Lond.)* **186,** 980 (1960).
4. J. Svoboda, The tumorigenic action of Rous sarcoma in rats and the permanent production of Rous virus by the induced rat sarcoma XC. *Folia biol. (Praha)* **7,** 46 (1961).
5. J. Svoboda, I. Hložánek, O. Mach, A. Michlová, J. Říman and M. Urbánková, Transfection of chicken fibroblasts with single exposure to DNA from virogenic mammalian cells. *J. gen. Virol.* **21,** 47 (1973).
6. J. Korb, Mitochondrial DNA of avian leukaemic myeloblast: Isolation and electron-optical characterization. *Neoplasma* **18,** 337 (1971).
7. M. M. K. Nass, Mitochondrial DNA. II. Structure and physicochemical properties of isolated DNA. *J. mol. Biol.* **42,** 529 (1969).
8. R. Radloff, W. Bauer and J. Vinograd, A dye-buoyant-density methods for the detection and isolation of closed circular duplex DNA in HeLa cells. *Proc. nat. Acad. Sci. (Wash.)* **57,** 1521 (1967).
9. J. Marmur, A procedure for the isolation of deoxyribonucleic acid from microorganisms. In *Methods in Enzymology.* (Edited by S. P. Colowick and N. O. Kaplan) Vol. VI, 726. Academic Press, New York (1963).
10. M. A. Wilson and J. Cascarano, Biochemical heterogeneity of rat liver mitochondria separated by rate zonal centrifugation. *Biochem. J.* **129,** 209 (1972).
11. W. C. Schneider, Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. *J. biol. Chem.* **176,** 259 (1948).
12. J. Svoboda, I. Hložánek, O. Mach and S. Zadražil, Problems of RSV rescue from virogenic mammalian cells. *Proc. of the Cold Spring Harbor Symp. on Quantitative Biology* 39 (1974).
13. G. Goubin and M. Hill, Infectiosité de l'acide désoxyribonucléique extrait des noyaux purifiés des cellules non permissives du sarcome de Rous. *C.R. Acad. Sci. Paris* **278,** 685 (1974).
14. L. Montagnier and P. Vigier, Un intermédiaire ADN infectieux et transformant du virus du sarcome de Rous dans les cellules de poule transformées par ce virus. *C.R. Acad. Sci. Paris* **224,** 1977 (1972).
15. B. Hirt, Selective extraction of polyoma DNA from infected mouse cell cultures. *J. mol. Biol.* **26,** 365 (1967).
16. L. Donner, L. Turek and J. Svoboda, Absence of RSV production in enucleated RSV-transformed virogenic mammalian cells fused with chick embryo fibroblasts. *Int. J. Cancer* **14,** 657 (1974).
17. R. Dulbecco, The state of the DNA of polyoma virus and SV40 in transformed cells. *Proc. of the Cold Spring Harbor Symp. on Quantitative Biology* **33,** 777 (1968).
18. P. D. Markham and M. A. Baluda, Integrated state of oncornavirus DNA in normal chicken cells and in cells transformed by avian myeloblastosis virus. *J. Virol.* **12,** 721 (1973).

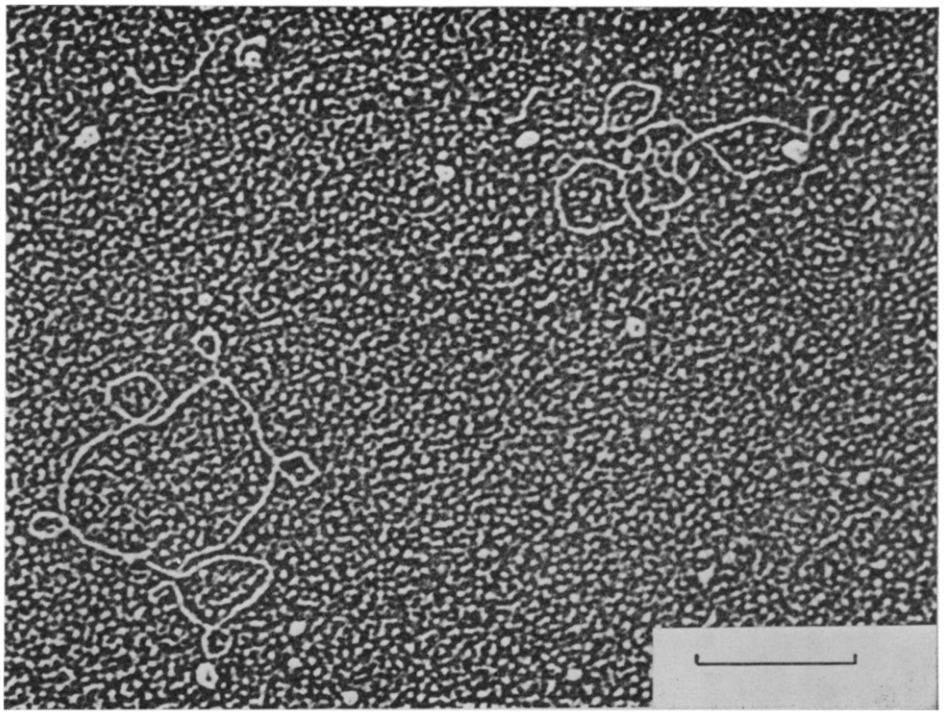


Fig. 2. Electron microscopic picture of circular molecule of mitochondrial XC DNA. The bar represents 0·5 *μm.*

Europ. J. Cancer Vol. 11, pp. 251–266. Pergamon Press 1975. Printed in Great Britain

Recent Trends in the Medical Treatment of Hodgkin's Disease

GIANNI BONADONNA, CARLO USLENGHI and ROBERTO ZUCALI
Istituto Nazionale Tumori, Milan, Italy.

Abstract—*The results of recent trials with chemotherapy and combined chemotherapy and radiotherapy for advanced Hodgkin's disease are reviewed. The results of three, four and five drug combinations designed during the past four years by deletion, substitution or addition of various components of MOPP regimen (nitrogen mustard, vincristine, procarbazine, prednisone) failed to improve both the incidence and the duration of complete response. Therefore, MOPP remains the simplest, most effective and safest drug therapy to be used in clinical practice. At present, there are no sufficient data indicating which is the optimum maintenance regimen. Furthermore, there is not yet sufficient evidence to state that maintenance therapy is really of significant benefit once complete remission has been achieved after 6–8 cycles of intensive combination chemotherapy. The goals for future chemotherapy probably would be in the use in sequence of two effective non-cross resistant combinations which differ in their drug content. The preliminary results of a controlled study randomizing MOPP with adriamycin, bleomycin, vinblastine and imidazole carboxamide (ABVD) are presented. Complete remission was observed in 77% of patients after MOPP and in 83% after ABVD. No cross resistance was observed between the two regimens in a limited number of patients. Another possibility for improving the disease-free survival lies in the appropriate use of combined treatment modalities, where chemotherapy and radiotherapy can become ideal partners to achieve eradication of Hodgkin's disease. Although the results of some pilot studies appear encouraging, timing and dosage of chemotherapy and radiotherapy deserve more accurate evaluation.*

INTRODUCTION

THOUGH the incidence of Hodgkin's disease is limited compared to that of other solid tumors, the importance of this disease in the progress of cancer treatment stems from its almost unique responsiveness to both radiotherapy (RT) and chemotherapy. For more than two decades, Hodgkin's disease has represented the most effective model for trials with local, regional and extensive irradiation as well with all available chemotherapeutic agents. Much of the progress achieved with the control of this disease has derived from aggressive treatment modalities designed specifically with curative intent. The impressive results obtained with RT developed the concept of cure through the analysis of relapse-free interval. The more recent data on long-term disease-free survival after combination chemotherapy seem to follow a qualitatively similar curve. In fact, the relapse rate is highest during the first two years after achieving complete remission and levels off thereafter.

As in acute leukemia, the treatment of Hodgkin's disease generated innumerable therapeutic trials. The interpretation of most recent studies dealing primarily with the medical treatment of advanced disease appears somewhat difficult for a number of reasons. Some reports include patients staged according to the Rye system, others with the Ann Arbor classi-

Accepted 29 October, 1974.

Presented in part at the 15th International Congress of Hematology, Jerusalem (September 1–6, 1974).

fication, and within this recent staging system, different efforts are made to define the anatomical extent of disease (clinical methods, peritoneoscopy, laparotomy); most published series deal with untreated and previously treated patients; and finally, the criteria adopted to define complete (CR) or partial (PR) remission vary among investigators. All the above mentioned facts inhibit an adequate comparison of treatment data from various centers.

The problems arising from the modern chemotherapeutic approach to Hodgkin's disease are numerous. First of all, we still do not know when to stop treatment once complete remission has been obtained. Furthermore, it is not sufficiently clear whether chemotherapy and radiotherapy combined is technically feasible in all patients, and whether this approach is really more effective than either of these two techniques employed independently. Finally, one must take into serious consideration the potential long-term detrimental effects of treatment.

We shall outline first the new drugs which were found to be effective recently in advanced and refractory Hodgkin's disease. Then we shall review the most important results obtained with different drug combinations and analyze the role of maintenance treatment. Finally, we will discuss briefly some of the new combined treatment modalities both in advanced and in localized disease.

NEW EFFECTIVE DRUGS

The response rates of the most effective drugs in advanced Hodgkin's disease are reported in Table 1. Most data are derived from the review articles of Carter *et al.* [1, 8]. The response rate of established agents is well known through several uncontrolled [1, 8, 9] and controlled [10–12] studies carried out over the past twenty years. The results of the experience achieved with single agent chemotherapy culminated in the design of the 4-drug combination known as MOPP [13]. During the past six years new agents were found effective in Hodgkin's disease. Those producing a promising objective response rate were adriamycin (ADM), bleomycin (BLM), nitrosourea derivatives (BCNU, CCNU, SZT), 4-dimethyl-epipodophyllotoxin derivative (VM-26) and dimethyl triazeno imidazole carboxamide (DTIC). Most of these drugs were tested in patients with disseminated disease refractory to conventional agents. Therefore, available data might underestimate considerably their effectiveness especially in terms of CR and duration of remission. However, these results provide sufficient evidence of lack of cross

Table 1. *Single agents most effective in Hodgkin's disease*

Drug	Response rate (%)		Reference
	Overall	CR	
Alkylating agents			
Mechlorethamine (HN2)	64	13	[1]
Cyclophosphamide (CTX)	54	12	[1]
Chlorambucil	60	16	[1]
Vinca alkaloids			
Vinblastine (VLB)	68	30	[1]
Vincristine (VCR)	58	36	[1]
Antibiotics			
Actinomycin D (Act. D)	50	—	[1]
Adriamycin (ADM)	41	4	[2]
Bleomycin (BLM)	43	7	[3]
Nitrosourea derivatives			
BCNU	50	5	[1]
CCNU	74	21	[4]
Streptozotocin (SZT)	44	—	[5]
Corticosteroids			
Prednisone (PRED)	61	0	[1]
Miscellaneous			
Procarbazine (PCZ)	69	38	[1]
VM-26	39	0	[6]
Imidazole carboxamide (DTIC)	56	6	[7]

resistance between the group of conventional drugs and that of new agents. Interestingly enough, there is no cross resistance between STZ (methyl-nitrosourea) and BCNU (chloroethyl-nitrosourea). The new agents are being incorporated now into treatment plans of several ongoing studies.

In general, with single agent chemotherapy, CR rarely exceeds 30%. Although complete response data are available for only a portion of the patient population [1, 8] this statement is very likely to be true also for the new agents.

Until the late 1960's, chemotherapy of advanced Hodgkin's disease was practically always confined to the use of single agents given in sequence (alkylating agent → vinblastine → procarbazine → corticosteroids or new drug). With this sequential approach, more than half of the patients were maintained in PR or CR for many months, and occasionally for few years. However, the overall median survival from institution of chemotherapy was in the range of 24 months [14]. Survival was longer in patients responding to at least two drugs (40–42 months) compared to those responding less or not at all (9–18 months) [14, 15].

MOPP TREATMENT AND THE ERA OF COMBINATION CHEMOTHERAPY

There is general agreement that MOPP represented a breakthrough in the therapy of advanced Hodgkin's disease. The importance of MOPP as a successful example of optimum combination of active agents without overlapping or undue toxicity goes beyond the treatment of Hodgkin's disease. In fact, the modern approach of intermittent (cyclic) combination chemotherapy with full or nearly full dose regimen as well as the concept of different dose attenuation schedules in the presence of various types and degrees of toxicity and of average percentage of the projected total dose administered for each drug were all developed during the initial trial with MOPP [13]. Actually, the experience gained with MOPP has, during the past five years, stimulated an innumerable number of new studies and treatment plans with multiple drug therapy for practically all types of neoplastic disease.

Remission induction

The results of MOPP in terms of CR are well known, and are summarized for the most consistent series in Table 2. The incidence of CR has been impressively high despite different methods for defining CR and different percentages of previously treated patients present in each series. Once the NCI experience was duplicated, two controlled studies by Moores [28] and Brook [29] were definitely able to demonstrate the superiority of combination chemotherapy (MOPP) in producing the remission induction over single agent (HN2) chemotherapy (CR: 50% vs 19%, and 73% vs 42%, respectively).

As outlined recently by Goldsmith and Carter [1], new trends appeared in clinical studies after the development of MOPP. The first trend involved altering the MOPP combination by using three-, four-, and five-drug regimens. In fact, a number of new combinations was designed by deletion (COP), substitution (MVPP, COPP, CVPP, BCOP, BCVP, MABOP) or addition (MABOP) of various components of MOPP regimen with the intention of improving both the incidence and the duration of complete response. The results of

Table 2. Complete remission and duration with six cycles of three, four and five-drug combinations

Combination	Stage	Patients	CR %	Median duration of CR (months)	Author
CTX–VCR–PRED (COP)	III–IV	107	36	5	Luce [16]
HN2–VCR–PCZ–PRED (MOPP)	III–IV	43	81	36+	De Vita [13, 17, 18]
		64	74	48+	Young [19]
		178	66	see text	Frei [20]
	IV	81	74	26	Moore [21]
	IV	34	56	14+	Rubens [22]
HN2–VLB–PCZ–PRED (MVPP)	II–III–IV	175	77	see text	McElwain [23]
CTX–VCR–PCZ–PRED (COPP)	II–III–IV	102	66	1–29+	Morgenfeld [24]
CTX–VLB–PCZ–PRED (CVPP)	II–III–IV	76	59	data not available	Høst [25]
BCNU–CTX–VCR–PCX (BCOP)	III–IV	31	35	data not available	Durant [26]
BCNU–VLB–CTX–PCZ (BCVP)	III–IV	25	52	data not available	Durant [26]
HN2–ADM–BLM–VCR–PRED (MABOP)	IIB–IIIB–IV	56	63	17+	Bonadonna [27]

some modified MOPP regimens are also summarized in Table 2. It appears evident that none of the reported regimens appears superior when compared retrospectively to MOPP. Even in a controlled trial carried out by the ALGB [30] where 6 cycles of MOPP were compared to VCR + PCZ alternating with VLB + chlorambucil + PRED given for 6 months, the sequential regimen yielded a lesser response rate than that achieved with MOPP (CR 49% vs 61%). Moreover, the unmaintained remission duration after MOPP was significantly longer compared to that obtained with the sequential schedule (27+ months vs 9·3 months). The same significant difference was observed in the relapse rate (75% vs 54%). A more appropriate retrospective comparison between MOPP and modified MOPP combination can be carried out when the percentage of CR for untreated patients is reported separately from that of previously treated cases. As can be seen from data reported in Table 3, the response rate is similar in practically all series of untreated patients regardless of the combination used. It also appears evident that prior irradiation alone does not lower significantly the incidence of CR. On the contrary, patients previously treated with chemotherapy show a poorer response to all types of combinations compared to the other groups. McElwain [23] predicted that the 3-year relapse-free survival for previously untreated patients is 64·2% and 76·6% for those irradiated previously but it is only 7·9% for the group who received previous single agent chemotherapy.

Table 3. Incidence of complete remission in relation to previous therapy

Combination	No prior therapy	Prior radiotherapy	Prior chemotherapy (± radiotherapy)	Reference
MOPP	35/43 (81%)	16/21 (76%)	5/10 (50%)	[13, 31, 32]
	74/93 (80%)	23/31 (74%)	20/54 (37%)	[20]
	17/21 (81%)	43/60 (72%)		[21]
MVPP	35/45 (78%)	54/62 (87%)	45/68 (66%)	[23]
COPP	20/28 (71%)		44/74 (59%)	[24]
BCOP, BCVP	11/17 (65%)		13/39 (33%)	[26]
MABOP	21/30 (70%)	8/14 (57%)	6/12 (50%)	[27]

Remission duration and maintenance treatment

Another trend to improve the medical treatment in advanced Hodgkin's disease was the study of various maintenance regimens to increase the duration of CR and the survival. In the original series of De Vita *et al.* [13] no further therapy was given in the 35 complete responders after 6 cycles of MOPP. The study of unmaintained remission revealed a median duration of response of 36 months, and 43% of patients achieving an initial CR remained free of disease for at least five and up to seven years [17, 18, 33]. A prolonged disease-free survival, though impressive for patients with advanced disease, could be obtained only in about 40% of cases. Since the relapse rate was highest in the first 18 months [13, 17, 18, 33] it seemed logical to try to improve the percentage of long-term complete responders with different forms of maintenance therapy.

The first trial to examine the effects of maintenance therapy after induction of CR with MOPP actually was started by the same NCI group [19]. After entering CR, patients were randomly allocated to no additional therapy, intermittent MOPP, or intermittent BCNU (Table 4). Surprisingly, no significant difference was observed among the three groups in terms of median duration of CR and relapse rate. The only significant difference observed was the mean time to relapse (no maintenance: 6 months, MOPP: 25·8 months, BCNU: 25·5 months).

Table 4 also reports the results of other controlled studies. Frei *et al.* [20] compared the unmaintained remission after MOPP vs. intermittent MOPP every 2 months and noticed that the relapse rate was significantly less in the patients in maintained remission, whether plotted from time of randomization or from end of MOPP treatment. Two years after the end of treatment, 77% of patients receiving 15 cycles of MOPP were still in CR as compared to 56% of those treated with only 6 cycles. However, at 4 years, the analysis of survival showed no difference between the maintained and the unmaintained groups. This was inter-

preted as due largely to more effective secondary treatment in the group of patients relapsing after 6 cycles of MOPP. Results favoring maintenance treatment were reported previously by the same group of investigators employing COP [16]. In another study, Frei, Coltman *et al.* [35] compared three different maintenance therapies (actinomycin D, VLB, MTX) following MOPP induction. No significant difference was found in both relapse rate and median duration of CR among the three groups. However, the authors stated that VLB was better than no maintenance, and maintenance MOPP was better than actinomycin D. In the MABOP study (Stages IIB–IIIB and III_S) a true CR, prolonged maintenance treatment can probably be of benefit by delaying the clinical appearance of relapse. Other factors responsible for the difference in the usefulness of maintenance therapy may also be involved (histopathology, number of previously treated patients, type of staging, etc.).

Drug tolerance and complications of prolonged therapy

With all types of combinations regimens employed, drug tolerance in terms of hematopoietic toxicity was surprisingly good when dose attenuation schedules were employed during remission induction. This is reflected

Table 4. Influence of maintenance treatment upon relapse rate and duration of remission

Induction	Maintenance	Relapse rate	Median duration of CR (months)	Reference
COP (10 cycles)	None		5	[16]
	COP (q. 3 weeks until relapse)		10·5	
MOPP (8 cycles)	None	24%	>48	[19]
	MOPP (q. 3 months × 15 months)	25%	48·2	
	BCNU (q. 3 months × 15 months)	13%	48·2	
MOPP (6 cycles)	None	53%	56% in CR	[20, 34]
	MOPP (q. 2 months × 18 months)	31%	77% in CR	
		after 2 yr from end of therapy		
MOPP (6 cycles)	Act. D (q. 2 months × 9)	35%	20	[35]
	VLB (q. 2 weeks × 39)	29%	31	
	MTX (q. 2 months × 9)	30%	18	
MABOP (6 cycles)*	None		18	[27]
	CTX→VLB→PCZ→MTX+ reinduction × 24 months		29	

*Followed by RT.

after 6 cycles of combination chemotherapy followed by radiotherapy, the unmaintained remission yielded a lesser duration of CR compared to sequential single-agent chemotherapy plus intermittent reinduction with one cycle of MABOP [27].

In conclusion, at present, there are no data indicating which is the optimum maintenance regimen. Furthermore, from data reported in Table 4 there is not enough evidence to state that maintenance therapy is really of any benefit once CR has been achieved after intensive combination chemotherapy. The discrepancies between the results obtained by Young *et al.* [19] and those reported by other investigators are due in part possibly to the different efforts made in defining CR. In fact, only in the NCI study when extralymphatic sites were affected, were biopsies done in accessible areas previously involved to confirm CR also on histophatologic basis. In absence of

by the fact that an average of 83–97% of the projected total dose for each myelosuppressive agent was administered during the first 6 cycles [13, 23, 36, 37]. Previously-treated patients tolerate only a slightly less percentage of the optimal dose.

Prolonged therapy can increase the risk of some complications. Young *et al.* [19] reported that patients on maintenance with either MOPP or BCNU more frequently developed bacterial and viral infections as well as myelofibrosis and amenorrhea. Goffinet *et al.* [38] and Monfardini *et al.* [39] have shown an increased incidence of herpes-zoster varicella infection. Although aggressive therapy can damage the patients' immunologic system, it has been shown that delayed hypersensitivity was not impaired by the MOPP program, once therapy was stopped. Furthermore, in anergic patients entering into CR, delayed hypersensitivity returned to normal [18]. Other long-term

complications involve sterilization [33] and increased incidence of second malignancies [40, 41].

COMBINED TREATMENT MODALITIES

The rationale for combined treatment modalities in Hodgkin's disease stems from the evidence that patients with the disease spread above and below the diaphragm and/or with systemic symptoms at onset will experience a relapse rate between 25 and 60% despite optimal radiotherapy. Presumably, occult foci beyond the fields of total nodal irradiation are responsible for these failures. Even after staging laparotomy and bone marrow biopsy, the absence of microscopic foci of the disease either in the liver or in the marrow cannot be excluded entirely especially in patients with splenic involvement. Since both radiation therapy and chemotherapy proved to be very effective in producing a high percentage of CR, in recent years a combined therapeutic approach was attempted by some investigators to improve the free interval and the survival in high-risk patients.

Uncontrolled studies

Table 5 summarizes the types of treatment employed as well as the essential results. In 1966 Frei *et al.* [42, 43] published the preliminary results of a pilot study where a four-drug combination regimen was given before (1 cycle) and after (2 cycles) radiation therapy to involved lymph node-bearing areas. This study demonstrated the feasibility of combined modality from both toxicological and therapeutic points of view. Subsequently, in Houston, Gamble *et al.* [44] started a sequentially combined treatment with short courses of chemotherapy followed by RT. Their intents were: (1) to obtain in untreated Stage III patients a prompt reduction in node size (especially in regard to mediastinal involvement), (2) to produce an early control of systemic symptoms, (3) to delay tumor growth during the period of prolonged radiotherapy, and (4) to possibly achieve cure or long-term control of disease. Their 5-year survival rate of 68% appears encouraging if one considers that the series included patients with and without constitutional symptoms. Bonadonna, De Lena *et al.* [27, 36] initiated an intensive treatment program with the intention of exploring the possibility of treating malignant lymphomas by a combined-sequential treatment similar to that used successfully in treating acute leukemia. The protocol included an induction phase with 5-drug combination (MABOP) followed by a consolidation phase with high-energy irradiation. Patients with Stages IIB–IIIB–III_S were then randomized to either no further therapy or to maintenance treatment for 2 years with sequential cyclic single-agent chemotherapy (CTX → VLB → PCZ → MTX) periodically interrupted by one cycle of reinduction. Although it is still too premature to properly evaluate the effect of treatment on long-term

Table 5. *Combined chemotherapy (CT)—Radiotherapy (RT) approach in advanced stages uncontrolled studies*

Type of treatment	Stage	Patients	Results	Author
CTX–VCR–MTX–PRED (3 cycles) + RT* between 1st and 2nd cycle	I–IIA IIB–IIIA	4 5	CR for 30+ months (med.) CR for 33+ months (med.)	Frei [42, 43]
CTX–VCR or MOPP (2–3 cycles) + RT†	III (A+B)	33	5 yr. survival: 68%	Gamble [44]
MABOP (6 cycles) → RT† ± maintenance CT	II B IIIB–III_S (A+B)	11 27	CR 64% for 24+ months (med.) CR 89% for 17+ months (med.)	Bonadonna [27]
PCZ–VLB–TEM–PRED → RT† → VLB	IIIB–IV	56	CR 82% (IIIB med. 28·5 months)	Amiel [45]
HN2–VCR–VLB–PCZ–PRED (3 cycles) → RT‡ → CT (2 cycles)	IIIB–IV	25	CR 80% for 12+ months (med.)	Prosnitz [37]
CTX–VCR or CTX–PCZ–PRED + RT† → CTX–VCR	II–IV	49§	CR 82% for 6–58 months	Smith [46]

med: median
*4,000 rads.
†3,000–4,000 rads.
‡1,500–2,500 rads.
§children and adolescents.

results, 64% of Stage IIB patients had a complete remission with a median duration of 24^+ months, and 89% of those with Stage IIIB–III_S, for 17^+ months, respectively [27]. Radiation therapy (3000–4000 rads) was not well tolerated in about half of the patients with Stage III disease and the irradiation program was frequently interrupted because of severe and prolonged myelosuppression (especially thrombocytopenia). Similar results were obtained in France by Amiel *et al.* [45] and Jacquillat *et al.* [47] with the sequence: combination chemotherapy → high dose RT → chemotherapy. In 1973, Tubiana and Mathé [48] updated Amiel's results showing that the median duration of first remission was 29 months for untreated Stage III, and 13 months for Stage IV, respectively. More recently, Prosnitz *et al.* [37] reported the results of a study designed in 1969 where low-dose RT (1500–2500 rads) was delivered in both Stages IIIB and IV to gross areas of tumor involvement after an induction period with a 5-drug regimen (3 cycles). After completion of RT, 2 more cycles of chemotherapy were administered. To our knowledge, this is the first treatment program where RT was planned to be delivered at tolerable doses to all anatomic areas known to be involved with disease before the institution of chemotherapy. As in previously-mentioned studies, about one month rest period elapsed before RT and before subsequent chemotherapy. Considering the stage of patients treated with this program, the results appear satisfactorily as does the degree of myelosuppression. In fact, only occasionally were low platelet levels reached in the fourth and fifth drug cycles. RT was well tolerated generally in almost any area of the body, and following irradiation, only one patient had a platelet count below 80000/mm^3. In children and adolescents, Smith *et al.* [46] achieved similar therapeutic results by concurrent 2 or 3 drug chemotherapy and radiotherapy administered in 12 weeks followed by single agent chemotherapy for 1–2 yr. Despite the simultaneous administration of high-dose RT (3500–4000 rads), the authors state that bone marrow depression presented no major problems during the intensive phase of therapy. This fact could be attributed both to the moderately aggressive chemotherapy used in comparison to 4 and 5 drug regimens, and to the known fact that children usually tolerate higher doses of myelosuppressive treatment than do adults.

Controlled studies

The results of 2 published controlled studies are reported in Table 6. The Stanford group [49, 50] randomized total lymphoid irradiation (TLR) alone vs. TLR followed, 4–8 weeks later, by 6 cycles of MOPP. Relapse occurred in a significantly smaller percentage of patients receiving the sequential therapy than in the group receiving RT alone. However, at the time of analysis, the overall survival was not significantly different for the two groups, although MOPP improved the disease-free survival. Six cycles of MOPP therapy were surprisingly well tolerated after recent TLR, and no cases of severe and prolonged leukopenia and thrombocytopenia were observed during chemotherapy. However, the percentage of calculated total dose was lower compared to the series where MOPP or similar combinations were given either in untreated patients [13, 23, 36, 37], or after previous RT [23, 31], or after previous chemotherapy [32]. The ALGB [51] initiated a 5-arm study in Stage III (A+B) and observed no significant difference in terms of CR, whether RT was given alone or preceeded or followed by chemotherapy. On the contrary, chemotherapy alone

Table 6. Combined chemotherapy (CT)—Radiotherapy (RT) approach in advanced stages randomly controlled studies

Type of treatment	Stage	Results	Author
TLR* vs TLR→MOPP (6 cycles)	IB–IIB IIIB–III_SB	relapses 10/45 (22%) relapses 1/48 (2%)	Moore [48, 49]
VLB for 4 weeks→HN2 (day 29)	III (A+B)	CR 10/22 (45%)	Hoogstraten [50]
same →RT (involved areas)		CR 18/24 (75%)	
same →TNI†		CR 18/20 (90%)	
TNI→HN2+VLB		CR 17/22 (77%)	
TNI		CR 12/16 (75%)	

*TLR: Total lymphoid radiation (4400 rads to nodes + 3000 rads to liver in stage III_SB).
†TNI: Total nodal irradiation (3000–3500 rads).

yielded the lowest response rate. There is no difference in the remission duration of the 4 RT groups, but in the adequately treated patients, the addition of chemotherapy leads to longer remissions (median 35–57 months) compared to the group given RT alone (median 25 months). In symptomatic patients, chemotherapy before RT was significantly better than RT followed by chemotherapy ($P < 0{\cdot}5$).

THE MOPP VS. ABVD PROGRAM

The aim of study

In the last five years, worldwide experience has clearly defined the usefulness and the limits of MOPP therapy. Since only about 40 % of patients entering into CR with MOPP have prolonged disease-free survival, a multiple drug combination designed to include some of the new drugs known to be effective in Hodgkin's disease and not present in the classical MOPP would be very useful. In fact, this new combination could be employed in MOPP failures. Furthermore, it could be tried in appropriate sequence with MOPP to increase both the percentage of CR and the number of long-term survivors free of disease. In April 1973, a new combination was designed in our Institute with ADM, BLM, VLB and DTIC (ABVD). All drugs, including VLB, are known, in humans to be non-cross resistant to those included in the MOPP combination. The purpose of combining these 4 drugs was twofold: 1) to compare in terms of remission induction and duration, and the efficacy of ABVD versus MOPP; 2) to demonstrate the absence of cross resistance between the two combinations [52].

Patients and methods

At the time of this review, a total of 40 patients with Stages IIB–IIIB–III_S and IV were entered into each study arm. However, presently, only 53 patients (MOPP 30, ABVD 23) have completed the first 6 cycles of therapy. Patients were not treated previously with chemotherapy but a total of 13 cases (MOPP 8, ABVD 5) have relapsed to prior irradiation. Patients were staged according to the Ann Arbor classification. Pathologic staging with laparotomy or with peritoneoscopy was performed in a total of 96 % of patients (MOPP 100 %, ABVD 91 %). Bone marrow biopsy was performed in all patients.

In the ABVD program, ADM (25 mg/m^2), BLM (10 mg/m^2) and VLB (6 mg/m^2) were administered on days 1 and 14 of each cycle. DTIC (150 mg/m^2) was given from days 1–5 of each cycle. No treatment was given from days 15 to 28. All drugs were injected i.v. MOPP was administered according to the classical dose schedule as originally reported by De Vita *et al.* [13]. Dose attenuation schedules were used in the presence of toxicity. Patients were allocated randomly to receive 6 cycles of either combination. In presence of relapse, cross over was carried out. Two months after completion of remission induction, patients with Stages IIB–IIIB–III_S in CR or in PR greater than 75 % were given high energy RT to all initially involved (3000–3500 rads) as well as to adjacent lymph node bearing areas (3000 rads). After completion of RT, no further treatment was given. In those with extralymphatic involvement (Stage IV) a low-dose RT (2000–2500 rads) was delivered to all sites of major pretreatment involvement, followed after an 8 week rest period by 6 more cycles of either MOPP or ABVD every 2 months. Assessment of results after 6 cycles of combination chemotherapy included a thorough physical, biochemical, radiological and radioisotopic evaluation of pretreatment sites of disease. In Stage IV patients, a second biopsy of all accessible extralymphatic sites of known involvement (e.g. liver, marrow) was carried out. The tissue sample had to be interpreted as negative to consider the patient in CR.

Results

Tables 7 and 8 update the preliminary therapeutic results published elsewhere [52]. No significant difference was noticed between the two treatment groups in relation to stage and histology. Furthermore, as observed in previously-mentioned studies, prior RT did not affect the incidence of CR. In both groups the last parameter to return to normal was the lymphogram. Most patients treated with ABVD achieved the status of CR between the second and third cycles compared to those given MOPP who were classified in CR between the fourth and the fifth cycles. The incidence of toxic manifestations is reported in Table 9. Nausea and vomiting were more severe during treatment with ABVD than with MOPP. No signs of cardiomyopathy or of lung fibrosis were observed. After 6 cycles of MOPP, the average of the projected total dose was 87 % for HN2 and PCZ, 91 % for VCR and 100 % for PRED.

Table 7. Comparison of response in relation to stage

Stage	MOPP			ABVD		
	No.	CR	CR + PR	No.	CR	CR + PR
IIB	3	2	2	1	1	1
III B–III$_S$ (A+B)	14	11 (79%)	14	12	10 (83%)	10
IV (A+B)	13	10 (77%)	11	10	8 (80%)	10
Total	30	23 (77%)	27 (90%)	23	19 (83%)	21 (91%)

Table 8. Comparison of response in relation to histology and to previous irradiation

	MOPP				ABVD			
	No.	CR	PR	IF*	No.	CR	PR	IF*
Lymphocytic pred.	1	1			1			1
Nodular sclerosis	14	11	3		11	9	1	1
Mixed cellularity	8	7	1		8	7	1	
Lymphocytic depl.	7	4		3	3	3		
No prior radiotherapy	22	17	2	3	18	15	1	2
Prior radiotherapy	8	6	2		5	4	1	

*Induction failure.

Table 9. Comparison of toxicity during primary chemotherapy

	MOPP (%)	ABVD (%)
Hematosuppression	60	67
Leukopenia*	57	67
Thrombocytopenia†	23	9
Stomatitis		5
Paresthesias	70	5
Loss of hair	33	71
Skin changes		48
Amenorrhea	2/11‡	3/5
Infections (All types)	50	19

*Leukocytes < 4000/mm^3.
†Platelets < 130 000/mm^3.
‡Menstruating females.

After ABVD these values were 87% for ADM and VLB, 97% for BLM and DTIC.

So far, a total of 20 patients (MOPP 12, ABVD 8) received RT as the second part of treatment program. In 6/20 patients (Stage IIIB–III$_S$: 4, Stage IV: 2) the irradiation had to be interrupted because of prolonged hematosuppression, mostly due to thrombocytopenia, which required multiple platelet transfusions in 3 patients. In the remaining patients, the irradiation treatment could be completed without significant untoward effects. Total nodal irradiation was carried out within an average of 3·5 months, while involved lymphatic and extralymphatic areas in Stage IV patients received the irradiation program within an average of 4 weeks.

Table 10 presents the essential data observed so far in terms of relapse. At the time of this report, there seems to be a higher incidence of relapses in the MOPP-treated group compared to the ABVD group. Data on therapeutic effects after cross-over are available presently for only 7 patients. After secondary treatment with ABVD, 3/5 patients responded compared to 1/2 after MOPP. Furthermore, in a small series of patients followed aside from this controlled study and relapsing after MOPP, objective response with ABVD was observed in 4/6 (CR in 1 case). The duration of response is not yet available.

In short, the preliminary results of this new treatment program show that ABVD yielded a response rate similar to MOPP. Drug tolerance and relapse rate also seem fairly comparable.

Table 10. *Patterns of relapse after primary treatment*

	MOPP	ABVD
Total relapsed	8/30 (27%)	3/23 (13%)
Median time from starting therapy	5 months (2–10)	5 months (5–8)
Time of relapse		
during induction	5	2
during interval*	1	1
during irradiation	0	0
after completion of therapy	2	0
Site of relapse		
lymphatic sites	4	2
extralymphatic sites	2	0
both sites	2	1
Relapse in relation to stage		
II B	1/3	0/1
III B–III_S	3/14	2/11
IV	4/13	1/9

*Between the end of induction and the beginning of RT.

Also, due to considerable vomiting after each administration of DTIC, this drug will be administered in the future on days 1 and 14. Data on long-term remission as well as on remission rate after cross-over should await a longer period of follow up.

ADJUVANT CHEMOTHERAPY IN EARLY STAGES

Few studies on the value of RT plus single agent chemotherapy in Stage I and Stage II were published over the past 15 yr. The most pertinent findings are summarized in Table 11. From the historical point of view, the first report on the possible usefulness of chemotherapy when added to RT was that of Cook *et al.* [60] who found virtually no difference in the survival rates between the group receiving RT alone and that given RT plus HN2. However, in addition to not being a randomized study, patients receiving chemotherapy were, in general, in poorer condition. Linke [53] did a similar study using the alkylating agent Trenimon. He reported distinctly better results in groups receiving RT plus chemotherapy. However, the author's results with combined therapy were very close to those obtained by V. Peters with RT alone [61]. Similar conclusions were drawn by Karnofsky [54]. Two British controlled studies [56, 57], both employing a single dose of HN2, failed to show a significant difference in both relapse rate and survival in the two treatment groups. The most recent experience was again achieved in Europe. The large controlled study carried out by the European Organization for Research on Treatment of Cancer, (EORTC) employed VLB as adjuvant treatment (10 mg/week) for two consecutive years [48, 58]. This study also failed to show a statistical difference in the

Table 11. *Results of adjuvant single agent chemotherapy in early stages*

Type of treatment	Stage	Patients	Survival	Author
RT vs RT + Trenimon	I	53	at 5 yr: 41·4% vs 75%	Linke [53]
	II	86	at 5 yr: 19·6% vs 68%	
	III	113	at 5 yr: 1·3% vs 17%	
HN2 + RT*	I–II	22	same as RT alone	Karnofsky [54]
RT vs HN2 + RT†	I	67	no difference at 5 yr	Paterson [55]
RT* vs RT + HN2†	I–II	108	no difference at 10 yr	Thompson-Hancock [56, 57]
RT* vs RT → VLB†	I–II	295	no difference at 4 yr	EORTC [48, 58]
RT* vs HN2 + RT → VLB†	I–II	150	same as RT alone	Teillet [59]

*RT to involved plus adjacent areas.
†controlled study.

survival rate between the two groups. On the contrary, there was a significant difference in the disease-free interval in favor of the chemotherapy group. In fact, at 1 yr the relapse rate was 8% in the group treated with RT plus VLB compared to 24% in the group given RT alone. In particular, the difference in favor of VLB was related only to the mixed cellularity and lymphocytic depletion subgroups.

A similar, but uncontrolled study was carried out in France by Teillet *et al.* [59] who prolonged the administration of VLB for 3 yr. The authors observed that both relapse rate and survival were equal to what had been observed by Kaplan [62] with radiation therapy alone.

In short, at present there are no conclusive data showing that adjuvant single agent chemotherapy can be of any benefit in affecting the survival rate in localized Hodgkin's disease, although prolonged administration of VLB can reduce the occurrence of early relapses in patients with unfavorable histology.

DISCUSSION

The review of results published during the past four years clearly shows that treatment for Hodgkin's disease is evolving and improving rapidly. The most important data is probably represented by the fact that potentially curative treatment can be offered today to almost all patients regardless of stage and histopathologic subgroup. However, the analysis of published reports presents many interesting data and few definitive results [63]. Therefore, conclusions are still somewhat premature and practical guidelines difficult to provide.

As far as remission induction is concerned, cyclic multiple drug treatment is now accepted as the most successful therapeutic modality to induce a prompt and complete remission in advanced stages of adults and children. If patients are previously untreated and an optimal percentage of dose for each drug is administered during the remission induction period, CR can be obtained in 70–80%. Since none of the treatment regimens designed in various centers throughout the world yielded better results than those achieved with MOPP, this combination should be regarded at the present moment as the most simple, effective and safe drug therapy to be used in clinical practice. However, MOPP proved unable to provide a long-term control in about 60% of complete responders. Therefore, another effective, tolerable and non-cross resistant drug combination is required to successfully treat MOPP failures. Our preliminary studies indicate that ABVD could be regarded as an example of such a new effective combination. Although considerable nausea and vomiting are produced by DTIC and potentially severe and, to a certain extent, unpredictable side effects may occur from prolonged administration of ADM and BLM, our experience shows that long-term therapy with ABVD was not hazardous and could be administered in nearly full dosage without great difficulty even after combined treatment with RT.

Once CR has been achieved, a difficulty exists in determining the end point of treatment. Present data seem to indicate that different types of maintenance treatment can affect only the relapse-free period rather than survival. Therefore, despite different criteria used in the studies to define CR, it appears evident that a minimum of 6–8 cycles of MOPP or of modified MOPP regimen can produce long-term disease-free survivors in an almost fixed fraction (40–45%) of initially complete responders, regardless of subsequent treatment. As already anticipated by De Vita *et al.* [18], the goals for future chemotherapy would probably be in the use in tandem of two effective non-cross resistant combinations which differ in their drug content. This is what is being done in our Institute with the sequential administration of MOPP and ABVD in stage IV disease with the specific intent to further improve the frequency of complete regressions and, more important, the duration of unmaintained remissions.

Another possibility for improving the disease-free survival of Hodgkin's disease lies in our opinion, in the appropriate use of combined treatment modalities, especially in Stages IIIB–III_S and IV. In Stages IIIB and III_S (A and B) the information obtained by laparotomy is not sufficiently reliable to allow us to deny complementary treatment with irradiation and/or chemotherapy when biopsies are negative [48]. If combination chemotherapy represents the treatment of choice, RT still has its role especially in contributing to the sterilization of the initially involved regions. By properly timing the administration of drugs and RT, patients with either localized lymphoma at high risk of early relapse after treatment with irradiation alone, or with advanced disease should be able to receive the benefits of both effective modalities of treatment in the attempt to eradicate the disease [48, 64]. There is early evidence that 6 cycles chemotherapy followed by irradiation or vice versa can be tolerated,

though at the expense of considerable marrow toxicity. Clearly, in the sequential combined modality, a compromise should be reached in terms of dosage of either chemotherapy or radiotherapy. Peripheral neutropenia after chemotherapy does not necessarely represent a significant damage of bone marrow reserve [65], while following segmental sequential irradiation prolonged suppression of bone marrow reserve persists for 1–2 yr [66]. Therefore, it seems more convenient from the practical point of view to administer drug therapy prior to irradiation when both treatment schedules are intensive and extensive. There are also strategic reasons which favor chemotherapy first even in patients with only lymphatic extent. Combination chemotherapy, in fact, can successfully control the occult disseminated foci located beyond the irradiation fields. Moreover, a partial response in the lymph nodes after chemotherapy can become more easily complete after subsequent irradiation than vice versa [36, 52].

The relapse pattern in Hodgkin's disease after chemotherapy has been studied by Frei *et al.* [20]. These investigators observed an excellent correlation between the site of the largest masses of tumor before treatment and the site of initial recurrence. This is consistent with the hypothesis that relapses result from persisting tumor cells and strongly suggests that, after patients enter CR on chemotherapy, RT to sites of major pretreatment involvement is a rational approach. This is being done by the Southwest Cancer Chemotherapy Study Group after MOPP as well as in our Institute following either MOPP or ABVD [20]. Prosnitz *et al.* [37] advise low dose RT to all involved areas after CR has been achieved with combination chemotherapy. In theoretical considerations regarding the optimum dose of RT to be used in combination with chemotherapy [67], it was calculated that an effective multiple drug regimen can reduce the residual viable cells in the grossly involved area to 10^4–10^5. Since doses of about 4000 rads sterilize masses of Hodgkin's disease containing 10^{11}–10^{12} cells, the predicted dose necessary for sterilization of residual masses of 10^4–10^5 cells is in the range of 1500–2500 rads. Since effective chemotherapy can produce in most patients a clinical CR after 3–4 cycles, the sequence proposed by Prosnitz *et al.* [37], who delivered low dose RT after 3 cycles of combination chemotherapy to be followed by more cycles of the same drug treatment, seems wise and worth of controlled studies.

Even under ideal circumstances there remains a fair proportion of patients with localized Hodgkin's disease who will not be cured by radiotherapy. Adjuvant chemotherapy appears therefore a rational approach in the unfavorable histologic types. Although it is reasonable to assume that in many of such patients the number of neoplastic cells persisting after radiotherapy is small, multiple drug treatment would be probably much more effective in improving the disease-free survival than single agent chemotherapy as employed in the past.

In conclusion, substantial improvement has been made in the medical treatment of advanced Hodgkin's disease during the past 5 yr. Although none of the modified versions of MOPP therapy yielded better results compared to the original quadruple combination, new treatments proved that a significant percentage of CR could be achieved with different drug regimens at the expense of tolerable toxicity. The recent results achieved with the ABVD combination indicate that in research institutions the conservative approach of MOPP therapy can be overcome by devising additional non-cross resistant combination regimens to be compared in a controlled fashion to the currently effective treatments. Future trends will probably lie in both sequential combination chemotherapy and in combined treatment modalities. Appropriate drug sequence and timing between chemotherapy and radiotherapy deserve more accurate evaluation. In combined treatment modalities radiotherapy and chemotherapy can become ideal partners [68] to achieve eradication of neoplastic disease. Prospective controlled studies with more consideration to stratification of patients according to parameters known to influence prognosis are mandatory to ensure validity of new data. The risk of developing second tumors especially after combined chemotherapy and radiotherapy is probably high [40, 41]. Therefore, in designing new trials the potential for long-term complications must be taken into serious consideration although, in our opinion, this potential hazard should not prevent the appropriate use of effective and tolerable combined modalities in good-risk patients. Nevertheless, the problem of possible overtreatment does exist, and must be weighed more heavily in the decision making.

REFERENCES

1. M. A. Goldsmith and S. K. Carter, Combination chemotherapy of advanced Hodgkin's disease: a review. *Cancer (Philad.)* **32,** 1 (1974).
2. R. H. Blum and S. K. Carter, Review of adriamycin, a new anticancer drug with significant clinical activity. *Ann. intern. Med.* **80,** 249 (1974).
3. R. H. Blum and S. K. Carter, A clinical review of bleomycin, a new antineoplastic agent. *Cancer (Philad.)* **31,** 903 (1973).
4. O. Selawry and H. Hansen, Superiority of CCNU over BCNU in the treatment of advanced Hodgkin's Disease. *Proc. Amer. Ass. Cancer. Res.* abstr. No. 182 **13,** 46 (1972).
5. P. Schein, M. O'Connell, J. Blom, S. Hubbard, I. Magrath, P. Bergevin, P. Wiernik and V. de Vita, Clinical antitumor activity and toxicity of streptozotocin. *Proc. Amer. Ass. Cancer Res.* abstr. No. 739 **15,** 168 (1974).
6. European Organization for Research on the Treatment of Cancer, Clinical Screening of 4-dymethyl-epipodophyllotoxin B.D. thenyledene glucoside (VM26) in malignant lymphomas and solid tumors. *Brit. med. J.* **2,** 744 (1972).
7. E. Frei III, J. K. Luce, R. W. Talley, V. K. Vaitkeivicious and H. E. Wilson, 5-(3, 3-dymethyl-1-triazeno) imidazole-4-carboxamide (NSC-45388) in the treatment of lymphoma. *Cancer Chemother. Rep.* **56,** 667 (1972).
8. S. K. Carter and R. B. Livingstone, Single agent therapy for Hodgkin's disease. *Arch intern. Med.* **131,** 377 (1973).
9. R. K. Desser and J. H. Ultmann, The sensitivity of Hodgkin's disease to chemotherapeutic agents administered singly. *Ser. Haemat.* **6,** 152 (1973).
10. P. P. Carbone, C. Spurr, M. Schneiderman, G. Scotto, J. F. Holland and B. I. Shnider, Management of patients with malignant lymphoma. A comparative study with cyclophosphamide and vinca alkaloids. *Cancer Res.* **28,** 811 (1968).
11. J. Solomon, E. M. Jacobs, J. R. Bateman, R. J. Lukes, J. M. Weiner and D. M. Donohue, Chemotherapy of lymphoma with mechloretamine and vinblastine. *Arch. intern. Med.* **131,** 407 (1973).
12. E. Ezdinli and L. Stutzman, Vinblastine versus nitrogen mustard therapy of Hodgkin's disease. *Cancer (Philad.)* **22,** 473 (1968).
13. V. T. de Vita, A. A. Serpick and P. P. Carbone, Combination chemotherapy in the treatment of advanced Hodgkin's disease. *Ann. intern. Med.* **73,** 881 (1970).
14. S. Monfardini, G. Tancini, M. Gasparini and G. Bonadonna, Response and survival in Hodgkin's disease after sequential chemotherapy employing a single agent. *Tumori* **59,** 45 (1973).
15. A. C. Aisenberg and J. M. Goldman, Prolongation of survival in Hodgkin's disease. *Cancer (Philad.)* **27,** 802 (1971).
16. L. K. Luce, J. F. Gamble, H. E. Wilson, R. W. Monto, B. L. Isaacs, R. L. Palmer, C. A. Coltman, Jr., J. S. Hewlett, R. A. Geham, E. Frei, III, et al., Combined cyclophosphamide. vincristine and prednisone therapy of malignant lymphoma. *Cancer (Philad.)* **28,** 306 (1971).
17. V. T. de Vita and P. P. Carbone, Chemotherapeutic implications of staging in Hodgkin's disease. *Cancer Res.* **31,** 1838 (1971).
18. V. T. de Vita, G. P. Canellos and J. H. Moxley III, A decade of combination chemotherapy of advanced Hodgkin's disease. *Cancer (Philad.)* **30,** 1495 (1972).
19. R. C. Young, B. A. Chabner, G. P. Canellos, P. S. Schein and V. T. de Vita, Maintenance chemotherapy for advanced Hodgkin's disease in remission. *Lancet* **1,** 1339 (1973).
20. E. Frei III, J. K. Luce, J. F. Gamble, C. A. Coltman Jr., J. J. Costanzi, R. W. Talley, R. W. Monto, H. E. Wilson, J. S. Hewlett, F. C. Delaney and E. G. Gehan, Combination chemotherapy in advanced Hodgkin's disease. Induction and maintenance of remission. *Ann. intern. Med.* **79,** 376 (1973).
21. M. R. Moore, J. M. Bull, S. E. Jones, S. A. Rosenberg and H. S. Kaplan, Sequential radiotherapy and chemotherapy in the treatment of Hodgkin's disease. *Ann. intern. Med.* **77,** 1 (1972).
22. R. D. Rubens, E. Wiltshaw, E. Boesen and D. A. G. Galton, Multiple-drug therapy in Hodgkin's disease. *Europ. J. Cancer* **8,** 477 (1972).

23. T. J. McElwain, P. F. M. Wrigley, A. Hunter, D. Crowther, J. S. Malpas, M. J. Peckham, D. W. Smithers and G. H. Fairley, Combination chemotherapy in advanced and recurrent Hodgkin's disease. *Nat. Cancer Inst. Monogr.* **36,** 395 (1973).
24. M. Morgenfeld, A. Pavlovsky, L. Isola, N. Somaza, S. Paulovsky, A. Suarez and C. Barros, Treatment of malignant lymphomas with cyclophosphamide, vincristine, procarbazine and prednisone combination. *Proc. XIV Int. Congr. Hemat.*, San Paulo, (abstr. No. 578) (1972).
25. H. Høst and A. F. Abrahamsen, Combination chemotherapy with cyclophosphamide, vinblastine, procarbazine and prednisone in the treatment of malignant lymphomas. *Scand. J. Haemat.* **10,** 170 (1973).
26. J. R. Durant and H. E. Lessner, Development of four-drug BCNU combination chemotherapy regimens. *Cancer (Philad.)* **32,** 277 (1973).
27. G. Bonadonna, M. de Lena, S. Monfardini, G. Beretta and P. Valagussa, Combination chemotherapy with adriamycin in malignant lymphomas. *Second Int. Symp. on Adriamycin,* Bruxelles 1974. In press.
28. R. R. Moores, Comparison of combination therapy versus nitrogen mustard in Hodgkin's disease. *Proc. Amer. Ass. Cancer Res.* (abstr. 227) **11,** 58 (1970).
29. J. Brook and C. Gocka, Comparison of single agent with combination therapy in Hodgkin's disease. *Proc. Amer. Ass. Cancer Res.* (abstract No. 45) **12,** 12 (1971).
30. L. Stutzman and O. Glidwell, Multiple chemotherapeutic agents for Hodgkin's disease. Comparison of three routines: a cooperative study by Acute Leukemia Group B. *J. Amer. med. Ass.* **225,** 1202, (1973).
31. G. P. Canellos, R. C. Young and V. T. de Vita, Combination chemotherapy for advanced Hodgkin's disease in relapse following extensive radiotherapy. *Clin. Pharmacol. Ther.* **13,** 750 (1972).
32. S. Lowenbraun, V. T. de Vita and A. A. Serpik, Combination chemotherapy with nitrogen mustard, vincristine, procarbazine, and prednisone in previously treated patients with Hodgkin's disease. *Blood* **36,** 704 (1970).
33. V. T. de Vita, Combined drug treatment in Hodgkin's disease: Remission induction, remission duration, and survival: an appraisal. *Natl. Cancer Inst. Monogr.* **36,** 373 (1973).
34. E. Hersh, E. Frei III, C. Coltman and J. Luce, Improved combination chemotherapy (MOPP) for remission induction and maintenance in advanced Hodgkin's disease. *Proc. Amer. Soc. clin Oncol.* abstr. No. 28 (1972).
35. C. A. Coltman, E. Frei III, F. C. Delaney, Effectiveness of actinomycin D (A), Methotrexate (MTX) and vinblastine (V) in prolonging the duration of combination chemotherapy (MOPP) induced remission in advanced Hodgkin's disease. (HD). *Proc. Amer. Soc. clin. Oncol.* abstract 78 (1973).
36. M. de Lena, S. Monfardini, G. Beretta, F. Fossati-Bellani and G. Bonadonna, Clinical trials with intensive chemotherapy and radiotherapy in Hodgkin's disease. *Natl. Cancer Inst. Monogr.* **36,** 403 (1973).
37. L. R. Prosnitz, L. R. Ferber, J. J. Fischer and J. R. Bertino, Low dose radiation therapy and combination chemotherapy in the treatment of advanced Hodgkin's disease. *Radiology* **107,** 187 (1973).
38. D. R. Goffinet, E. J. Glatstein and T. C. Merigan, Herpes zoster-varicella infections and lymphoma. *Ann. intern. Med.* **76,** 235 (1972).
39. S. Monfardini, E. Bajetta, C. A. Arnold, R. Kenda and G. Bonadonna, Herpes zoster-varicella infection in malignant lymphomas. Influence of splenectomy and intensive treatment. *Europ. J. Cancer.* In press (1975).
40. J. C. Arsenau, R. W. Sponzo, L. E. Schnipper, H. Bonner, R. C. Young, G. P. Canellos, R. E. Johnson and V. T. de Vita, Non lymphomatous malignancies complicating Hodgkin's disease. Possible association with intensive therapy. *New Engl. J. Med.* **287,** 1119 (1972).
41. G. Bonadonna, M. de Lena, A. Banfi and A. Lattuada, Secondary neoplasms in malignant lymphomas after intensive therapy. *New Engl. J. Med.* **288,** 1242 (1973).
42. E. Frei III, V. T. de Vita, J. H. Moxley III and P. P. Carbone, Approaches to improving the chemotherapy of Hodgkin's disease. *Cancer Res.* **26,** 1284 (1966).
43. J. H. Moxley III, V. T. de Vita, K. Brace and E. Frei III, Intensive combination chemotherapy and X-irradiation in Hodgkin's disease. *Cancer Res.* **27,** 1258 (1967).

44. J. F. Gamble, L. M. Fuller, E. Ibrahim, J. J. Butler and C. C. Schullenberger, Combined chemotherapy-radiotherapy management of stage III Hodgkin's disease, *Arch. intern. Med.* **131,** 435 (1973).
45. J. L. Amiel, G. Mathe, M. Tubiana, J.-R. Schlumberger, J. Rouësse et P. Pouillart, Traitement des maladies de Hodgkin, stades IIIB et IV, par la séquence chimiothérapie radiothérapie, chimiothérapie. *Bull. Cancer (Paris)* **58,** 191 (1971).
46. K. L. Smith, D. Johnson, O. Hustu, C. Pratt, I. Fleming and C. Holton, Concurrent chemotherapy and radiation therapy in the treatment of childhood and adolescent Hodgkin's disease. *Cancer* **33,** 38 (1974).
47. C. Jacquillat, M. Weil, J. P. Desprez-Curely, J. Tanzer, M. F. Gemon, R. Blouin, F. Teillet, J. Delobel, Nikdjou, J.-P. Loisel, M. Boiron, J. Bernard, Chimiothérapie de la maladie de Hodgkin par les association thérapeutiques. *Bull. Cancer (Paris)* **58,** 221 (1971).
48. M. Tubiana and G. Mathe, Combined radiotherapy and chemotherapy in the treatment of Hodgkin's disease. *Ser. Haemat.* **6,** 202 (1973).
49. M. R. Moore, J. M. Bull, S. E. Jones, S. A. Rosenberg and H. S. Kaplan, Sequential radiotherapy and chemotherapy in the treatment of Hodgkin's disease. A progress report. *Ann. intern. Med.* **77,** 1 (1972).
50. S. A. Rosenberg, M. R. Moore, J. M. Bull, S. E. Jones and H. S. Kaplan, Combination chemotherapy and radiotherapy for Hodgkin's disease. *Cancer* **30,** 1505 (1972).
51. B. Hoogstraten and O. Glidwell, Chemotherapy-radiotherapy for stage III Hodgkin's disease. *Proc. Amer. Cancer Res.* abstr. No. 706 **15,** 160 (1974).
52. G. Bonadonna, R. Zucali, S. Monfardini, M. de Lena, C. Uslenghi, Combination chemotherapy of Hodgkin's disease with adriamycin, bleomycin, vinblastine and imidazole carboxamide versus MOPP. *Cancer.* In press (1975).
53. A. Linke, Die Behandlung der Hemoblastosen und malignen Tumoren mit triethyleniminobenrochinon. *Dtsch. Med. Wschr.* **85,** 1928 (1960).
54. D. A. Karnofsky, D. G. Miller and R. S. Phillips, Role of chemotherapy in the management of early Hodgkin's disease. *Amer. J. Roentgenol.* **90,** 968 (1963).
55. E. Paterson, Evaluation of chemotherapeutic compounds in the reticuloses. *Brit. J. Cancer* **12,** 332 (1958).
56. P. E. Thompson-Hancock and E. M. Ledlie, Treatment of early Hodgkin's disease. *Lancet* **1,** 26 (1967).
57. P. E. Thompson-Hancock, D. E. Austin and P. G. Smith, Treatment of early Hodgkin's disease. *Lancet* **1,** 832 (1973).
58. Radiotherapy-Chemotherapy Group of EORTC, A randomized study of irradiation and vinblastine in stages I and II of Hodgkin's disease. Preliminary results. *Europ. J. Cancer* **8,** 353 (1972).
59. F. Teillet, C. H. Weisgerberger, M. Dana, J. P. Desprez-Curely, C. Jacquillat, R. Bousquet, J. Tanzer, M. Boiron and J. Bernard, Traitment de la maladie de Hodgkin. Principes généraux, applications et résultats préliminaires des méthodes utilisées a l'Institut de Recherches sur les Leucémies et les Maladies du Sang. *Bull. Cancer (Paris)* **58,** 203 (1971).
60. J. C. Cook, K. L. Krobbenhoft and T. Leucutia, Combined radiation and nitrogen mustard therapy in Hodgkin's disease as compared with radiation therapy alone. *Amer. J. Roentgenol.* **82,** 651 (1959).
61. M. V. Peters, A study of survivals in Hodgkin's disease treated radiologically. *Amer. J. Roentgenol.* **63,** 299 (1950).
62. H. S. Kaplan, Hodgkin's disease. Modern radiotherapy techniques and their results. *Ser. Haemat.* **6,** 139 (1973).
63. G. Mathe and M. Tubiana, Hodgkin's disease: Considerable progress and more to be achieved. *Ser. Haemat.* **6,** 244 (1973).
64. L. Stutzman, Combined radiotherapy and chemotherapy of lymphomas and other cancers. *Cancer Res.* **31,** 1845 (1971).
65. A. S. Deinard, I. E. Fortuny, A. Theologides, G. L. Anderson, J. Boen and B. Y. Kennedy, Studies on the neutropenia of cancer chemotherapy. *Cancer* **33,** 1210 (1974).
66. P. Rubin, S. Landman, E. Mayer, B. Keller, S. Ciccio, Bone marrow regeneration and extension after extended field irradiation in Hodgkin's disease. *Cancer* **32,** 699 (1973).

67. J. J. FISHER and R. Y. PAPAC, Theoretical considerations in combination of localized and systematic therapy for neoplastic disease. *J. theor. Biol.* **37,** 105 (1972).
68. J. H. BURCHENAL, Chemotherapy and radiotherapy: competitors or partners? *Cancer* **22,** 790 (1968).

Europ. J. Cancer Vol. 11, pp. 267–276. Pergamon Press 1975. Printed in Great Britain

Potentiation and Arming of Lymphocyte Mediated Immunity by Sera from Melanoma Patients*

ROMAIN VANWIJCK†, CLAIRE BOUILLENNE‡ and SHIRIN MALEK-MANSOUR§

†*Department of Plastic Surgery*, ‡*Tissue Typing Laboratory*, §*Department of Dermatology*, *University of Liège, Hôpital de Bavière, Liège, Belgique*

Abstract—*A serum-induced lymphocyte cytotoxicity has been detected in the blood of five out of forty melanoma patients. We found that the sera potentiated the cytotoxic effect of autologous lymphocytes or "arm" (i.e., made cytotoxic) blood lymphocytes from healthy donors on allogeneic melanoma cells. The presence of anti HL-A antibodies in the sera has been determined by a complement-mediated cytotoxic microtest. A multi-specific anti-HL-A immunization has been detected in the serum from one patient. Three of the patients were free of disease for at least a year and a half. The two others were patients submitted to a non-specific immunotherapy by 2–4 dinitrochlorobenzene (DNCB). No absolute correlation could be made between a favorable clinical evolution and the presence of arming and potentiating factors in the serum. In one case, the appearance of the potentiating activity was detected during the immunotherapy by sequential analysis of the serum. Arming and potentiating activities are usually, but not always, associated. Dissociation of the two activities seems to be related mainly to an unresponsiveness of the lymphoid cell populations of certain healthy individuals.*

The demonstration of serum-dependent lymphocyte activity against melanoma cells provides an additional insight into malignant melanoma.

INTRODUCTION

HUMORAL antibodies to antigens on the surface of target cells can induce cytotoxicity of lymphoid cells from non-immune donors ("arming"), as well as potentiate cytotoxicity of lymphocytes from immune donors. This antibody-induced cytotoxicity has been studied primarily in hetero-immune cases, [1] but it has also been observed in transplantation immunity [2–4] and in auto-immunity [5, 6].

This antibody-induced cytotoxicity of lymphoid cells has also been described in animal tumor cases. Certain animal serum has been reported to make specifically cytotoxic lymphocytes from non-sensitized donors [7] or to potentiate the cytotoxic effect of lymphocytes from sensitized donors [8]. Serum-induced cytotoxicity has been detected in certain cancer patient sera [9–12].

Antisera to HL-A antigens on lymphocytes have been shown to be capable of inducing the killing of these target cells by normal non-immune lymphoid cells. There was a general agreement between the HL-A specificities determined by the lymphocyte-dependent antibody activity and the specificities shown by a complement-mediated cytotoxic assay [13].

2–4 dinitrochlorobenzene (DNCB) has been used successfully for the treatment of basal and squamous cell carcinoma of the skin [14]. Complete regression of cutaneous metastases of melanoma has also been reported under DNCB non-specific immunotherapy [15].

During the immunological evaluation of forty melanoma patients by the chromium-51 microcytotoxic assay, we found that the sera

Accepted 17 September 1974.

*This work was supported by a grant, No. 20,300 from the Fonds de la Recherche Scientifique Médicale.

from five patients could arm and/or potentiate lymphocyte-mediated cytotoxicity.

In order to establish whether the serum-induced lymphocyte cytotoxicity was directed against tumour specific antigens rather than against HL-A antigens of the allogeneic melanoma cells, the presence of anti-HL-A antibodies in the serum of the melanoma patients was assessed by a complement-mediated cytotoxic microassay.

This paper presents the study of these five patients.

MATERIAL AND METHODS

Sera

The sera from forty melanoma patients (29 women and 11 men) were tested in this study. The serum from clotted blood samples was heat-inactivated and generally used fresh otherwise after storage at −20°C.

Control lymphocytes donors

Control lymphocytes and serum donors were healthy adults or patients with neoplasms other than melanoma.

Melanoma cell lines

The NKI2, NKI4, NKI8, NKI10 and Me26 cell lines were a gift from J. de Vries (The National Cancer Institute, Amsterdam, The Netherlands). The HM6 cell line was kindly provided by Dr. F. Lejeune. (Institut Jules Bordet, Brussels, Belgium). The HuMe3 cell line was obtained from Dr. G. Meyer (Centre de Recherches Anticancéreux de Marseille, France). These seven cell lines were grown in Eagle's medium (Dulbecco's modification) supplemented with 15% fetal calf serum, penicillin (200 i.u./ml), streptomycin (100 μg/ml), 2% L-glutamine and 1% 1 M Hepes. Contamination by mycoplasmas was prevented by the addition of gentamycin (200 μg/ml) and amphotericin B (5 μg/ml). All cell culture reagents were obtained from Biocult. Laboratories, Scotland. The study of the seven tumoural cell lines at the electron microscope revealed the presence of promelanosomes and melanosomes in their cytoplasm. Two human leukaemic cell lines (lymphoblastic and monocytic) maintained in tissue culture in the same way were used as control target cell lines.

Lymphocytes

Forty ml of heparinized venous blood (50U/ml, Liquemine Roche) were drawn from each donor. Red cells were removed by sedimentation through Plasmagel (Laboratoires Roger Bellon, Neuilly, France) for 30 min at 37°C. The supernatant suspension was then centrifuged and the pellet resuspended in PBS (Ca^{2+} and Mg^{2+} free + 5u./ml of Liquemine). The cell suspension was then carefully layered upon a Ficoll–Isopaque mixture with a specific gravity of 1076–1078. The tube was then centrifuged for 30 min at room temperature at 1500 rev/min. The lymphocytes sedimented to the interface were carefully pipetted off. The cell suspension was then washed three times in heparinized PBS and finally resuspended in tissue culture medium plus 15% fetal calf serum counted in trypan blue with a hemacytometer and diluted to the desired concentration of viable cells. The viability of these lymphoid cell suspensions was always higher than 90%.

Microcytotoxic assay

A. *Labelling of target cells.* The melanoma cells of the various tumour lines and the leukaemic cells from the monocytic line were removed from the tissue culture bottles by gentle scraping with a rubber "policeman" and counted. The lymphoblastic leukaemic cells were grown in suspension. Five million viable cells (determined by a trypan blue exclusion) were suspended in 0·5 ml of tris phosphate buffered saline (pH 7·4) in the presence of 0·1 ml Cr^{51} (100 μCi. sodium^{51} chromate; specific activity 200 or more mCi/mg of chromium. Commission Energie Atomique, France) and incubated 40 min at 37°C. The cells were then washed four times by centrifugation with Eagle's medium (Dulbecco's modification) containing 15% inactivated calf serum, recounted and diluted to a final concentration of 5×10^4/ml.

B. *Cellular cytotoxic assay.* Cell bound immunity was evaluated by adapting the chromium-51 cytotoxic assay described by Brunner [16]. Volumes of 0·1 ml of labelled target cells were incubated in upright, flat-bottomed disposable glass tubes (10 × 60 mm) with the lymphocytes in a final volume of 1 ml. A lymphocyte to target cell ratio of 100:1 was generally chosen to minimize the non-specific effect of lymphocytes. Each lymphocyte-target cell combination was set up at least in triplicate. Control tubes containing target cells alone were included in each experiment. The mixtures were then incubated at 37°C for 20 hr. Following this incubation, the content of each tube was centrifuged at 2000 rev/min for 5 min, and 0·5 ml of the cell free supernatant was carefully removed and radioactivity measured in a well-type scintillation counter.

Each patient and each control lymphocytes suspension were tested on at least two occasions and on at least two melanoma cell lines.

The ability of serum of melanoma patients to potentiate or arm the cytotoxic effect of lymphocytes was assessed in tests in which melanoma cells were incubated with 0·1 ml serum (dilution 1:5) per tube, from the respective tumour patients or from controls. After 30 min preincubation at 37°C, the lymphocytes were added as described above. Control tubes containing target cells plus serum without lymphocytes were also included. Percentage of cytotoxicity by immune lymphocytes as well as potentiating and arming serum activity were calculated as follows:

Percentage of cytotoxicity = Cr^{51} release with lymphoid cells-Cr^{51} release of tumour cells alone × 100/Maximal Cr^{51} release-Cr^{51} release of tumoural cells alone.

Maximal Cr^{51} release (100%) was determined by incubation of target cells with distilled water.

Percentage of arming = (Max. Cr^{51} release-Cr^{51} release with control serum) − (Max. Cr^{51} release-Cr^{51} release with test serum) × 100/ Max. Cr^{51} release-Cr^{51} release with control serum.

Percentage of potentiation = % Cr^{51} release with test serum-% Cr^{51} release with control serum × 100/ % Cr^{51} release with control serum.

Anti HL-A antigens immunization

The presence of anti HL-A antibodies in the serum of the melanoma patients was assessed by a complement-mediated cytotoxic microassay [17] and by a microleukoagglutination assay [18]. The sera of the melanoma patients were tested on the leucocytes of 25 healthy individuals who had been HL-A typed for the following HL-A specificities: HL-A 1, 2, 3, 9, 10, 11, W28, W19; HL-A5, 7, 8, 12, 13, W5, W10, W14, W15, W17, W18, W27, with a panel of well-defined antisera obtained from the serum bank of the National Institutes of Health. The leucocytes were selected from the tissue typed panel in order to have most of HL-A specificities displayed at least once.

RESULTS

The clinical history of each patient will be summarized briefly before the description of the immunological data.

A. *Patients free of disease*

Table 1 summarizes the data from three melanoma patients free of disease whose serum has shown an arming and/or potentiating effect.

Patient 1. L.S., had surgery for a primary melanoma of the leg followed by a groin dissection in April 1971. The histological examination of the inguinal lymph nodes revealed a metastatic invasion. The patient has remained clinically tumour-free since the time of surgery.

L.S.'s serum had a potentiating as well as arming effect detectable respectively as an increased cytotoxicity with autologous and control's lymphocytes. Both effects were detected with HuMe3 and NKI4 tumor lines but not with leukaemic cells. The lack of cytotoxic activity of both sensitized lymphocytes on the Me26 and NKI2 cells incubated with L.S.'s serum suggests that melanoma cells have more than one tumour associated antigen.

Patient 2. L.M. had a wide surgical excision of primary melanoma of the leg followed by a groin dissection which was free of tumoural invasion. The patient has remained clinically tumour-free since surgery performed in April 1969.

Both potentiation and arming effects were seen with HuMe3, Me26 and NKI10 cell lines but not with leukaemic cells.

Patient 3. P.J., was operated upon for a primary melanoma of the eyelid which invaded the orbit. The primary treatment was followed by a parotidectomy and a submandibular lymph node dissection. The parotid and submandibular lymph nodes were free of metastases. She has been free of disease since surgery was performed in October, 1972.

Her serum had arming and potentiating effects on HuMe3, HM6 and leukaemic cells, potentiating and non-arming activities on NKI8 tumor line and arming and non potentiating effects on Me26 cells. In this case, a synergistic cytotoxicity was not detected when the coated melanoma cells were incubated with highly reactive control or autologous lymphocytes. As shown in Table 2, multispecific anti-HL-A antibodies were detected in her serum by lymphocytotoxic and leukoagglutination microtechniques. The existence of an anti-HL-A immunisation as well as the presence of potentiating and arming activities against leukaemic cells suggest that in this case, the serum lymphocyte dependent activity is directed mainly against the HL-A specificities of the various target cells.

Table 1. Arming and potentiating activities of the sera from three melanoma patients free of disease

Patient	Date of test	Target cells	Lymphocytes donor	Mean Cr^{51} release after incubation with: Lymphocytes alone	Lymphocytes plus Control serum	Lymphocytes plus Patient's serum	% arming	% potentiation
L. S. (*)	9/2/73	Hu Me 3	Control	8·1 ± 2·3	12·4 ± 2·6	61·2 ± 3·2	55·7 ± 3·4	
			Patient	25·0 ± 3·2	23·1 ± 1·0	55·6 ± 0·1		140·6 ± 0·6
		NKI4	Control	3·0 ± 1·0	6·1 ± 2·4	28·2 ± 2·7	23·5 ± 0·9	
			Patient	13·2 ± 1·3	14·2 ± 1·0	30·1 ± 2·2		111·9 ± 13·9
		Me26	Control	5·1 ± 0·1	7·5 ± 2·3	6·0 ± 2·1		
			Patient	19·2 ± 1·1	13·6 ± 1·0	14·3 ± 2·7		
	4/16/74	NKI2	Control	12·7 ± 1·5	13·6 ± 1·2	10·3 ± 1·4		
			Patient	10·4 ± 3·4	10·2 ± 3·4	7·6 ± 2·1		
		Leukaemia	Control	3·9 ± 1·4	6·6 ± 0·3	1·8 ± 1·5		
			Patient	6·1 ± 0·3	−1·5 ± 1·4	1·6 ± 1·3		
L. M. (†)	11/30/73	HuMe3	Control	4·2 ± 1·0	8·4 ± 1·7	45·6 ± 1·0	40·6 ± 1·1	
			Patient	24·7 ± 1·7	21·2 ± 3·5	51·7 ± 2·6		143·8 ± 24·0
		Me26	Control	2·4 ± 1·0	7·2 ± 2·1	37·4 ± 0·2	32·6 ± 0·6	
			Patient	14·2 ± 2·1	13·6 ± 3·0	49·2 ± 1·2		261·7 ± 13·1
	4/18/74	NKI10	Control	15·2 ± 1·6	14·1 ± 2·7	32·1 ± 1·1	20·9 ± 1·2	
			Patient	10·2 ± 2·7	16·2 ± 4·6	46·4 ± 0·4		186·4 ± 61·0
		Leukaemia	Control	7·1 ± 0·4	10·3 ± 1·2	13·6 ± 3·7		
			Patient	4·2 ± 3·2	−2·1 ± 0·4	0·3 ± 1·3		
P. J. (‡)	10/23/73	HuMe3	Control	1·1 ± 1·2	2·1 ± 1·9	43·1 ± 0·1	41·8 ± 1	
			Patient	7·2 ± 3·6	2·0 ± 0·4	16·2 ± 3·0		> 500
		NKI8	Control	36·1 ± 3·6	38·0 ± 2·7	40·4 ± 4·2		
			Patient	30·2 ± 1·7	23·3 ± 3·4	62·7 ± 5·6		169·0 ± 13·2
		Me26	Control	1·8 ± 2·1	0·5 ± 0·3	33·7 ± 3·9	33·3 ± 3·5	
			Patient	45·7 ± 0·7	39·5 ± 1·2	41·4 ± 2·1		
	4/23/74	HM6	Control	8·9 ± 0·7	12·9 ± 2·4	43·1 ± 2·8	34·6 ± 0·5	
			Patient	10·3 ± 3·8	11·6 ± 1·4	37·0 ± 1·6		218·9 ± 22·0
		Leukaemia	Control	0·1 ± 1·4	3·1 ± 2·1	24·2 ± 1·7	21·7 ± 0·1	
			Patient	2·7 ± 0·3	6·1 ± 2·1	37·3 ± 2·6		> 500

*Free of disease since April 1971.
†Free of disease since April 1969.
‡Free of disease since October 1972.

Table 2. HL-A specific of P.J.'s serum determined by lymphocytotoxic and leucoagglutination microtechniques*

HL-A specificities	2 7 5	2 11 7 W5	1 9 8 W10	2 9 W15	2 3 7 W10	2 12	1 W5 W17	2 11 12 5	2 7 W5	3 W28 W5
Leuco-Agglutination†	++	++	++	+++	++	++++	+	++	++	++
Lympho-Cytotoxicity‡	+++	++++	++	++++	+	++++	++++	++++	++	+++

*Two pregnancies
†+ : Weakly positive.
++ : Positive
+++ : Very positive.
++++ : Highly positive.
‡+ : 15–20% cell lysis.
++ : 50–60% cell lysis.
+++ : 75–90% cell lysis
++++ : 100% cell lysis.

HL-A specificities	W28 9 12	2 W15	2 12	2 5	2 9 W27	1 9 W27	3 8 W5	3 10 W5	W28 2 8 12	2 10 W5 W15
Leuco-Agglutination†	++	++++	++++	++	++	++	++	+++	++	++
Lympho-Cytotoxicity‡	++++	+++	++++	++++	++++	++++	—	—	—	++++

*Two pregnancies
†+ : Weakly positive
++ : Positive.
+++ : Very positive.
++++ : Highly positive.
‡+ : 15–20% cell lysis.
++ : 50–60% cell lysis.
+++ : 75–90% cell lysis.
++++ : 100% cell lysis.

B. *Patients under DNCB immunotherapy*

Patient 1. C.M., had a primary melanoma of the leg treated by application of 2 mg of DNCB on the lesion every week for three weeks followed by the wide excision of the treated melanoma. The histological analysis of the surgical specimen revealed the presence of an important lymphoplasmocytic infiltration which disrupted the tumour.

As shown in Table 3, it has been possible to assay the evolution of both cell mediated immunity and serum activity on autologous and allogeneic lymphocytes. No serum lymphocyte-dependent cytotoxicity could be detected before the DNCB pretreatment. Synergistic cytotoxicity appeared after completion of the pretreatment and was still present five months after.

Patient 2. P.G., was admitted to the hospital with a malignant melanoma stage III (distant metastases). A subcutaneous metastasis was surgically removed to reduce the tumoural mass but the primary lesion was left intact and treated by weekly application of 3% of DNCB ointment.

This patient was tested three different times during the non-specific immunotherapy. The data are collected in Table 4. Three months after his admission (10/16/73), the tumoural progression was controlled by the immunotherapy which was the only treatment applied. At that time, his serum had the ability to increase lymphocyte cytotoxicity of his lymphocytes only on the NKI8 melanoma cells. Three months later (1/7/74), this patient had inguinal metastases growing progressively in spite of a potentiating effect of his serum on the Me26 tumor line. On the last occasion (3/21/74), the synergistic cytotoxicity was still detectable despite a rapid clinical progression. As P.G.'s serum had not been tested before immunotherapy, it is impossible to establish if the presence in his serum of potentiating and arming factors is dependent on the non-specific immunotherapy. He died in June, 1974.

Data are presented in Table 5 on the cytotoxic effect of lymphocytes suspensions from four healthy donors on Me26 cell line incubated with C.M.'s serum on two different occasions. Important variations in the arming effect are observed with the various lymphocyte populations suggesting individual response in displaying a serum dependent lymphocyte cytotoxicity.

The effect of serial dilutions of the sera from the five patients on their ability to arm control lymphocytes is shown in Fig. 1. The four sera

Table 3. *Sequential analysis of cell-mediated immunity and serum activities of C.M.*

Date of test	Target cells	Lymphocytes donor	Mean Cr^{51} release after incubation with: Lymphocytes alone	Lymphocytes plus Control serum	Lymphocytes plus Patient's serum	% arming	% potentiation
9/19/73 (*)	HuMe3	Control	10·1 ± 1·4	13·2 ± 0·8	6·1 ± 4·2		
		Patient	44·1 ± 3·6	44·0 ± 4·1	39·1 ± 0·3		
	Me26	Control	8·1 ± 3·2	8·8 ± 6·1	10·3 ± 4·1		
		Patient	27·1 ± 4·3	33·1 ± 5·6	30·6 ± 3·2		
	NKI4	Control	15·1 ± 6·2	17·3 ± 3·1	20·4 ± 3·1		
		Patient	34·1 ± 0·1	34·1 ± 0·2	16·1 ± 2·3		
10/9/73 †	HuMe3	Control	10·1 ± 3·1	2·1 ± 0·1	43·1 ± 4·3	41·9 ± 5·2	
		Patient	12·3 ± 4·1	7·2 ± 3·1	50·1 ± 3·1		500
	NKI8	Control	14·2 ± 0·8	24·0 ± 1·3	40·1 ± 0·8	21·1 ± 0·2	
		Patient	16·4 ± 3·1	15·1 ± 3·6	40·3 ± 2·3		166·7 ± 38·9
11/27/73	HuMe3	Control	7·1 ± 3·2	12·6 ± 2·7	70·2 ± 8·1	65·9 ± 14·4	
		Patient	42·1 ± 4·2	36·5 ± 2·4	75·2 ± 1·5		106·9 ± 8·9
	Me26	Control	5·3 ± 1·2	1·2 ± 0·4	49·1 ± 10·2	48·4 ± 8·7	
		Patient	45·8 ± 1·6	37·8 ± 1·6	99·0 ± 0·7		161·9 ± 7·1
3/11/74	NKI4	Control	11·1 ± 2·0	20·1 ± 0·8	22·1 ± 0·4		
		Patient	28·1 ± 4·2	30·8 ± 1·8	100·0 ± 0·1		224·6 ± 17·6
	Leukaemia	Control	6·7 ± 2·4	10·1 ± 3·2	12·7 ± 1·6		
		Patient	8·1 ± 0·4	16·1 ± 2·9	13·2 ± 4·1		

*Before DNCB pretreatment.
†After DNCB pretreatment.

Table 4. Sequential analysis of cell-mediated immunity and serum activities of a melanoma patient (stage III) under DNCB immunotherapy

Date of test	Target cells	Lymphocytes donor	Mean Cr^{51} release after incubation with: Lymphocytes alone	Lymphocytes plus Control serum	Lymphocytes plus Patient's serum	% arming	% potentiation
10/16/73 (*)	HuMe3	Control	1·4 ± 0·2	3·6 ± 1·7	4·2 ± 2·4		
		Patient	10·8 ± 1·2	14·3 ± 3·1	12·8 ± 0·7		
	NKI8	Control	6·2 ± 3·6	4·2 ± 1·7	8·1 ± 2·7		
		Patient	38·2 ± 1·7	35·6 ± 5·6	90·1 ± 1·5		153·0 ± 30·7
1/7/74 (†)	Me26	Control	4·5 ± 3·1	8·2 ± 1·6	4·1 ± 2·7		
		Patient	36·1 ± 2·1	32·3 ± 0·6	63·2 ± 4·1		95·6 ± 8·9
3/21/74 (‡)	NKI4	Control	0·5 ± 2·9	2·4 ± 2·1	2·4 ± 3·2		
		Patient	14·5 ± 4·2	35·0 ± 3·7	62·4 ± 4·9		106·8 ± 32·9
	NKI2	Control	2·7 ± 0·5	−1·7 ± 0·7	10·6 ± 0·9	13·7 ± 0·4	
		Patient	10·4 ± 1·1	10·0 ± 3·4	7·6 ± 1·7		
	Leukaemia	Control	3·9 ± 1·4	6·6 ± 0·3	1·8 ± 1·5		
		Patient	6·1 ± 0·3	16·0 ± 1·3	−1·5 ± 1·4		

*clinical progression: very slow
†clinical progression: rapid
‡clinical progression: rapid

Table 5. Influence of four different lymphocyte populations on the arming activity of C.M.'s serum with the ME 26 tumour line

Date of test	Melanoma cell line	Lymphocytes donor	Mean Cr^{51} release after incubation with: Lymphocytes alone	Lymphocytes plus Control serum	Lymphocytes plus C.M.'s serum	% arming
12.18.73	Me 26	V.R.	9·7 ± 0·2	2·6 ± 0·7	30·1 ± 2·4	28·2 ± 2·9
		S.E.	0·4 ± 0·6	7·5 ± 4·8	10·0 ± 0·6	2·7 ± 0·9
		A.I.	3·0 ± 1·8	3·9 ± 1·4	59·7 ± 0·2	58·0 ± 0·4
		P.J.	9·2 ± 0·7	14·5 ± 6·2	96·4 ± 0·4	95·7 ± 0·2
1.8.74	Me 26	V.R.	6·1 ± 1·4	1·3 ± 0·5	27·1 ± 4·1	26·1 ± 3·8
		S.E.	3·1 ± 1·2	5·1 ± 2·0	13·1 ± 0·4	8·4 ± 1·5
		A.I.	9·2 ± 1·4	18·1 ± 3·2	64·2 ± 3·1	56·3 ± 2·1
		P.J.	1·1 ± 0·2	4·2 ± 1·0	89·1 ± 1·4	88·6 ± 1·3

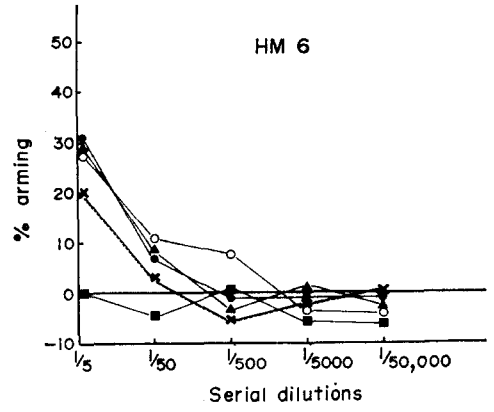
which are HM6 cell line specific show a rapid drop in their activity with the serial dilutions. The only serum which reacts with the leukaemic cells shows a similar drop in its activity with the dilution.

Figure 2 shows the effect of decreasing the number of effector cells on their ability to be involved in an arming activity when incubated with HM6 or leukaemic cells sensitized with the various sera. A progressive decrease in the percentage of arming activity is observed, but ten lymphocytes are still able to induce target cell lysis when incubated with the optimal dilution of the appropriate sera.

DISCUSSION

The data presented in this study indicate that *in vitro* killing of melanoma target cells can occur in the presence of non-sensitized as well as sensitized lymphoid cells and heat inactived sera from five out of forty melanoma patients. The simultaneous presence of lymphoid cells and serum is necessary to induce target cell destruction.

During sequential immunological studies on a large number of patients with various types of tumours, Hellström *et al.*, [9, 10] found that certain patient sera have arming and



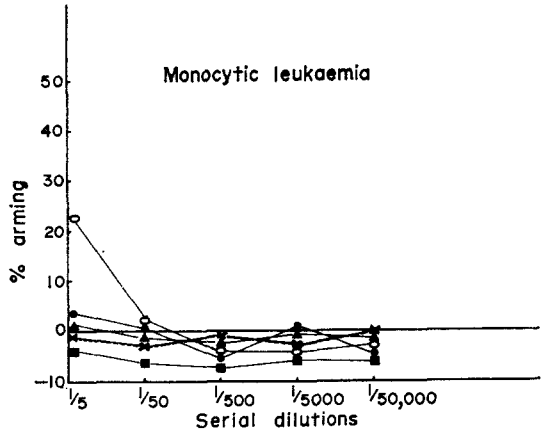
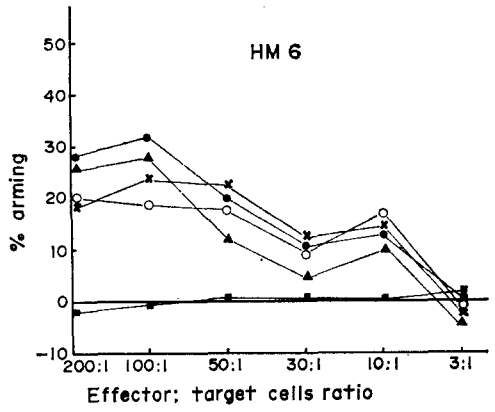


Fig. 1. Influence of serial dilutions on the arming activity of L.S.'s (×——×), P.J.'s (○——○), C.M.'s (●——●), L.M.'s (▲——▲) and P.G.'s (■——■) sera on control lymphocytes to HM6 melanoma cells and leukaemic cells. Lymphocytes: target cell ratio 100:1, incubation time: 20 hr.



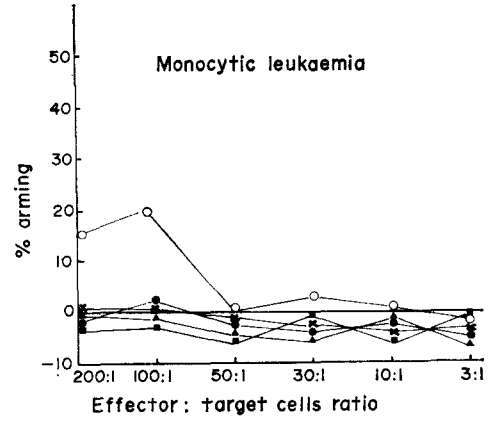


Fig. 2. Influence of decreasing the effector: target cells ratio on the arming activity of L.S.'s (×——×), P.J.'s (○——○), C.M.'s (●——●), L.M.'s (▲——▲) and P.G.'s (■——■) sera on control lymphocytes to HM6 melanoma cells and leukaemic cells. Serum dilution 1/5; incubation time: 20 hr.

potentiating activities. In their study, no correlation could be made between a synergistic cytotoxic and favorable clinical evolution. A similar potentiating effect was found by other authors in the serum from melanoma patients who had inactive disease at the time of the test [11]. An arming activity which is to induce the killing of allogeneic myeloblasts by normal lymphoid cells has been found in the serum from a high proportion of patients with acute myeloid leukaemia [12]. Preliminary results suggest that lymphocyte dependent serum activity might be useful in predicting the clinical course of leukaemic patients under immunochemotherapy. In our study, potentiating and/or arming activity is relatively infrequent (five out of forty patients). Such activities were detected in the serum from three patients free of disease and two under immunotherapy. No overall correlation could be made between the presence of such serum activities and a favourable clinical evolution since they were found in the serum of a patient with active metastatic melanoma.

To what extent are the serum factors melanoma specific?

Lymphocyte-dependent antibody activity has been demonstrated against the common transplantation antigens using HL-A antisera [13]. There was a general agreement between the specificities determined by the lymphocyte dependent antibody assay and by a complement-mediated cytotoxicity test in routine use for tissue typing. The main difference between the two assays was an increased number of serologically detectable antigens apparent from assay of lymphocyte dependent activity of antisera due to the higher sensitivity of this last assay. In our study, a multispecific anti-HL-A immunization was detected in the serum of one out of the five patients. This serum also reacts with allogeneic leukaemic cells suggesting that the lymphocyte activity is directed against HL-A specificities.

Arming or potentiating activities directed against melanoma specifics were never found in the serum from 45 healthy donors (unpublished data).

As shown by the sequential analysis of the serum of one patient (C.M.) whose primary melanoma had been pretreated with DNCB, a potentiating activity appeared in her serum after completion of the pretreatment and remained stable for several weeks. Although unique, this observation nevertheless suggests that assay of lymphocyte dependent serum activity might have importance in following the clinical evolution of melanoma patients. Indeed, the loss of arming activity against myeloblasts has been reported to announce subsequent clinical relapse in acute lyeloid leukaemia [12].

The fact that some patients' sera (L.S. and P.G.) have arming and potentiating activities on some lines of cultivated melanoma cells and not on others suggests that melanoma cells have more than one tumour-associated antigen. Our data confirm those published by other authors who demonstrated with other microassays of the immune response, the existence of more than one melanoma antigen [19–21]. Besides, as for the HL-A antigens, additional melanoma specificities might be revealed by serum-induced lymphocyte cytotoxicity.

Arming and potentiating activities are usually, but not always associated. Dissociation of the two activities seems to be related mainly to an unresponsiveness of the lymphoid cell populations of certain healthy individuals to display a serum-induced lymphocyte cytotoxicity when tested on the same target cell line incubated with the same serum. Although variations due to technical reasons could not be ruled out, the individual variability is probably due to different percentages of the blood effector cells which are responsible for the serum-dependent cytotoxicity. This important observation needs further investigation.

Contrary to the described models of this killing system [1, 12, 13, 22, 23], the killing of human melanoma cells is shown to decrease rapidly with the serial dilution of the sera. No blocking effect of the immune effector lysis was observed by diluting the serum as it was described in other systems [8, 24]. When incubated with the optimal dilution of the serum, the target cell can be lysed by a very low number of effector cells, although there exists an optimal number of attacking cells. This observation is similar to that made by others with the sera from multiply transfused patients who have anti-HL-A antibodies which can also mediate an arming effect [4].

The demonstration of serum-dependent lymphocyte activity against melanoma cells provides additional insight into this disease. The relative importance *in vivo* of this mechanism compared to other defined cytotoxic mechanisms has yet to be determined.

Addendum—Just as the manuscript was reviewed (August 1974), the patient P. J. was readmitted in the hospital with multiple distant metastases (stage III).

Acknowledgements—The excellent technical assistance of Mrs. M. C. Moor, A. Ooms and P. Leclerc is highly acknowledged.

REFERENCES

1. P. Perlmann and G. Holm, Cytotoxic effects of lymphoid cells *in vitro*. *Adv. Immunol.* **11,** 117 (1969).
2. E. Möller, Contact induced cytotoxicity by lymphoid cells containing foreign isoantigens. *Science.* **147,** 873 (1965).
3. J. Bubenik, P. Perlmann and M. Hasek, Induction of cytotoxicity of lymphocytes from tolerant donors by antibodies to target cell alloantigens. *Transplantation* **10,** 290 (1970).
4. I. Yust, J. R. Wunderlich, D. L. Mann and D. N. Buell, Cytotoxicity mediated by human lymphocyte dependent antibody in a rapid assay with adherent target cells. *J. Immunol.* **110,** 1672 (1973).
5. B. Ringertz, J. Wasserman, T. L. Packalen and P. Perlmann, Cellular and humoral responses in experimental autoimmune thyroiditis. *Int. J. Arch. Allergy* **40,** 917 (1971).
6. E. A. Calder, W. J. Penhale, D. McLennan, E. W. Barnes and W. J. Irvine, Lymphocyte-dependent-antibody-mediated cytotoxicity in Hashimoto thyroiditis. *Clin. exp. Immunol.* **14,** 153 (1973).
7. S. Pollack, G. Heppner, R. J. Brawn and K. Nelson, Specific killing of tumor cells *in vitro* in the presence of normal lymphoid cells and sera from hosts immune to the tumor antigens. *Int. J. Cancer* **9,** 316 (1972).

8. H. M. Skurzak, E. Klein, T. O. Yoshida and E. W. Lamon, Synergistic and antagonistic effect of different antibody concentration on *in vitro* lymphocyte cytotoxicity in the Moloney sarcoma virus system. *J. exp. Med.* **135,** 997 (1972).
9. I. Hellström, K. E. Hellström, H. O. Sjögren and G. A. Warner, Serum factors in tumor free patients cancelling the blocking of cell mediated tumor immunity. *Int. J. Cancer* **8,** 185 (1971).
10. I. Hellström, K. E. Hellström and G. A. Warner, Increase of lymphocyte-mediated tumor-cell destruction by certain patient sera. *Int. J. Cancer* **12,** 348 (1973a).
11. G. H. Heppner, L. Stoblach, M. Byrne, F. J. Cummings, E. McDonough, and P. Calabresi, Cell-mediated and serum blocking reactivity to tumor antigens in patients with malignant melanoma. *Int. J. Cancer* **11,** 245 (1973).
12. P. Hersey, I. C. M. McLennan, A. C. Campbell, R. Harris and C. B. Freeman, Cytotoxicity against human leukaemic cells. I. Demonstration of antibody-dependent lymphocyte killing of human allogeneic myeloblasts. *Clin. exp. Immunol.* **14,** 159 (1973a).
13. P. Hersey, P. Cullen and I. C. M. McLennan, Lymphocyte-dependent cytotoxic antibody against human transplantation antigens. *Transplantation* **16,** 9 (1973b).
14. E. Klein, Hypersensitivity reactions at tumor site. *Cancer Res.* **29,** 2351 (1969).
15. S. Malek-Mansour, S. Castermans-Elias and C. M. Lapiere, Regression de métastases de mélanome aprés thérapeutique immunologique. *Dermatologica* (Basel) **146,** 156 (1973).
16. K. T. Brunner, J. Mauel, J. C. Cerottini and B. Chapuis, Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells *in vitro*: inhibition by isoantibody and by drugs. *Immunology* **14,** 181 (1968).
17. F. Kissmeyer-Nielsen and E. Thorsby, Current methods in histocompatibility testing. In *Transplantation Reviews.* Edited by G. Möller, Vol. 4, p. 121. Munskgaard, Copenhagen (1970).
18. J. J. van Rood, A. van Leeuwen and R. Zweerus, The 4a or 4b do they or do not they? In *Histocompatibility Testing.* Edited by P. J. Tereseki, **93,** Munksgaard, Copenhagen (1970).
19. G. Fossati, M. I. Colnaghi, G. Della Porta, N. Cascinelli and U. Veronesi, Cellular and humoral immunity against human malignant melanoma. *Int. J. Cancer* **8,** 344 (1971).
20. J. E. Devries, P. Rumke and J. L. Bernheim, Cytotoxic lymphocytes in melanoma patients. *Int. J. Cancer* **9,** 567 (1972).
21. K. E. Hellström and K. I. Hellström, Some recent studies on malignant melanoma. *Fed. Proc.* **32,** 156 (1973b).
22. J. R. Wunderlich, E. B. Rosenberg and J. M. Conolly, Human lymphocyte-dependent cytotoxic and mechanisms of target cell destruction *in vitro*. *Prog. Immunol.* **1,** 473 (1972).
23. G. Möller and S. E. Svehag, Specificity of lymphocyte-mediated cytotoxicity induced by *in vitro* antibody-coated target cells. *Cell. Immunol.* **4,** 1 (1972).
24. R. Kiessling and E. Klein, Cytotoxic potential of mouse spleen cells on H-2 antibody-treated target cells. *J. exp. Med.* **137,** 527 (1973).

Europ. J. Cancer Vol. 11, pp. 277–279. Pergamon Press 1975. Printed in Great Britain.

Fibrinolysis in Human Bronchogenic Carcinoma*

H.-I. PETERSON, S. LARSSON and L. ZETTERGREN

Department of Sorgery I, Thoracic Surgery and Pathology II, University of Göteborg, Sahlgrenska sjukhuset, Göteborg, Sweden.

Abstract—*The fibrinolytic activity in tumour cells from 52 human bronchogenic carcinomas was studied by the histochemical technique described by Todd. A comparatively high fibrinolytic activity was found in tumour cells from 40–50% of bronchogenic carcinomas classified as epidermoid carcinoma and adenocarcinoma, while the majority of small cell anaplastic carcinomas had a comparatively low fibrinolytic activity. No correlation was found between fibrinolytic activity and local lymph node involvement. However, in epidermoid carcinomas, the frequency of intravascular tumour growth was increased significantly in tumours with high fibrinolytic activity compared to tumours with low fibrinolytic activity. Three bronchial adenomas separately studied showed high fibrinolytic activity.*

INTRODUCTION

SPONTANEOUS fibrinolytic activity has been found in carcinoma of the prostate and in other human malignant tumours [1–4].

This activity, which is based on a tissue plasminogen activator, was localized to the tumour cells from both transplantable experimental and human malignant tumours [5, 6]. The fibrinolytic activity of different tumours varied and for this reason the activity of separate tumour specimens was quantitated by a histochemical method. The reliability of such a quantitation was confirmed in a separate study [7].

Extravascular deposition of fibrin was found in human malignant tumours and it was suggested that this favoured the infiltrative growth of tumours [8]. On the other hand, the results obtained in experimental studies in mice on the effect of induced fibrinolysis and antifibrinolysis on tumour growth and spread suggested that fibrin deposition could be part of a mechanism against the growing tumour [5].

This was supported by a clinical study in which a high fibrinolytic activity in human breast carcinomas was correlated to an increased frequency of local lymph node metastases and an increased tendency to invasive growth [6].

Increased blood fibrinolysis during and after operation in patients with malignant disease was usually associated with circulating malignant cells, but the interpretation of this remains obscure [9]. Fibrin split products in blood was found to be a bad prognostic sign in patients with malignant disease [10]. Furthermore, malignant transformation in cell culture of various virus-infected animal fibroblasts initiated fibrinolytic activity of these cells. A cell factor which was found to be a plasminogen activator could be isolated from the transformed cells [11].

The aim of the present study was to investigate the fibrinolytic activity of various bronchogenic carcinomas and to correlate this activity to the histological type of carcinoma, to local lymph node involvement and to the tendency to intravascular tumour infiltration.

MATERIAL AND METHODS

Specimens from 52 bronchogenic carcinomas and 3 bronchial adenomas were studied. The specimens were taken from primary tumours at the time of operation.

The *fibrinolytic activity* of various carcinomas

Accepted 12 December, 1974.

*The original research reported in this communication was supported by a grant from the Swedish Cancer Society (grant No. 73:131).

was estimated on mechanically-produced cell suspensions by a histochemical method modified from Todd (Fig. 1) and described previously in detail [5, 7]. By this method a reliable semiquantitation of the fibrinolytic activity of tumour cells could be made. Due to the semiquantitative character of the estimation, the fibrinolytic activity is described only as high or low.

The *histological examination* of the various tumours was made by one of the authors (Zettergren) independent of the semiquantitation of fibrinolytic activity. The classification of tumours was made in accordance with recommendations from the WHO International Reference Centre for the Histological Definition and Classification of Lung Tumour. Thus, the bronchogenic carcinomas in the present material could be divided into four groups: epidermoid carcinoma, small cell anaplastic carcinoma, adenocarcinoma and large cell carcinoma. The majority of epidermoid carcinomas and adenocarcinomas in the present material were slightly differentiated, while only two tumours in each of these groups were highly differentiated.

Mediastinal lymph node metastases in the lung preparations removed at operation were verified histologically and intravascular invasion of the tumour was noted in sections from the operative preparations.

RESULTS

The results are presented in Table 1. Twenty-nine tumours were classified as epidermoid carcinoma, 12 as small cell anaplastic carcinoma, 8 as adenocarcinoma and 3 as large cell carcinoma.

High fibrinolytic activity was found in 40–50% of the epidermoid carcinomas and adenocarcinomas, while the majority of small cell anaplastic carcinomas showed a low fibrinolytic activity.

Local lymph node metastases were distributed equally in tumours with high or low fibrinolytic activity. The frequency of intravascular invasive growth observed was increased significantly in epidermoid carcinomas with high fibrinolytic activity compared to epidermoid carcinomas with low fibrinolytic activity ($P < 0{\cdot}05$).

Most tumours were anaplastic or slightly differentiated. One highly differentiated epidermoid carcinoma showed high fibrinolytic activity, local lymph node involvement and intravascular tumour growth, while another showed low fibrinolytic activity and only lymph node involvement. Two highly differentiated adenocarcinomas showed low fibrinolytic activity, no lymph node involvement and no observable intravascular tumour growth. The three bronchial adenomas included in the study had high fibrinolytic activity.

DISCUSSION

In the present study a comparatively high fibrinolytic activity was found in tumour cells from 40–50% of bronchogenic carcinomas classified as epidermoid carcinoma and adenocarcinoma, while the majority of small cell

Table 1. *Fibrinolytic activity of* 52 *bronchogenic carcinomas correlated to histological type, occurrence of regional lymph node metastases and observed intravascular tumour growth. s = significant difference compared with tumours with low fibrinolytic activity* ($P < 0{\cdot}05$)

Histological type	Epidermoid carcinoma		Small cell anaplastic carcinoma		Adenocarcinoma		Large cell carcinoma	
Fibrinolytic activity	high	low	high	low	high	low	high	low
Regional lymph node metastases	4/12	9/17	2/2	8/10	1/4	1/4	1/1	0/2
Intravascular tumour growth	7/12[s]	3/17	1/2	4/10	0/4	1/4	0/1	0/2
Number of tumours	12	17	2	10	4	4	1	2

anaplastic carcinomas had a comparatively low fibrinolytic activity. No correlation was found between fibrinolytic activity and local lymph node involvement. However, in epidermoid carcinomas a high fibrinolytic activity was associated with a significantly increased frequency of intravascular tumour infiltration.

With the exception of an increased occurrence of epidermoid carcinomas in the present material, the distribution of histological tumour type is very similar to a recently-presented large material of bronchogenic carcinomas [12]. In the present material 45% of the epidermoid carcinomas showed local lymph node metastases, while in the larger material the frequency was only 17·3%. The latter figure, however, includes only the mediastinal lymph node metastases.

In a previous study on breast carcinomas [6] a high fibrinolytic activity of tumour cells was correlated to an increased frequency of local lymph node metastases. Such a correlation was not found in the present study on bronchogenic carcinoma. However, the increased frequency of intravascular growth in epidermoid carcinomas with high fibrinolytic activity might suggest a promoting effect of fibrinolysis on tumour spread. In this respect the histological type of bronchogenic carcinoma must be considered.

The estimation of intravascular tumour growth in the present study was based on a limited number of sections from the tumours. This probably means a high tendency to intravascular growth in tumours in which this was observed, but intravascular growth might be seen in all bronchogenic carcinomas with a sufficiently comprehensive examination of the slides.

The increased tendency to intravascular growth of tumours with high fibrinolytic activity might be interpreted as a promoting effect of tumour cell fibrinolytic activity on tumour spread. This is in accordance with previous experimental and clinical studies. The limited material in the present study makes definite conclusions difficult. A subsequent follow-up of the survival of the patients, including criteria of peripheral metastasis formation, might add valuable information. Increased fibrinolytic activity in tumour cells, giving rise to an increased tendency to tumour spread, might also be a mechanism which could be open for treatment with antifibrinolytic agents.

REFERENCES

1. H. J. Tagnon, W. F. Whitmore Jr. and N. R. Shulman, Fibrinolysis in metastatic cancer of the prostate. *Cancer* **5,** 9 (1952).
2. H. J. Tagnon, W. F. Whitmore Jr., P. Schulman and S. C. Kravitz, The significance of fibrinolysis occurring in patients with metastatic cancer of the prostate. *Cancer* **6,** 63 (1953).
3. H. J. Tagnon, P. Schulman, W. F. Whitmore Jr. and L. A. Leone, Prostatic fibrinolysin. *Amer. J. Med.* **15,** 875 (1953).
4. E. E. Cliffton and C. E. Grossi, Fibrinolytic activity of human tumours as measured by the fibrin-plate method. *Cancer* **8,** 1146 (1955).
5. H.-I. Peterson, Experimental studies on fibrinolysis in growth and spread of tumour. *Acta chir. scand.* **134,** Suppl. 394 (1968).
6. H.-I. Peterson, I. Kjartansson, K. Korsan-Bengtsen, C.-M. Rudenstam and L Zettergren, Fibrinolysis in human malignant tumours. *Acta chir scand.* **139,** 219 (1973).
7. H.-I. Peterson, B. Petrusson and K. Korsan-Bengtsen, Fibrinolytic activity of human carcinomas—A comparative methodological study. *Thrombos. Diathes. haemorrh.* (Stuttg.) **30,** 133 (1973).
8. R. A. Q. O'Meara, Fibrin formation and tumour growth. *Thrombos. Diathes. haemorrh.* (Stuttg.) Suppl. **28,** 137 (1968).
9. A. J. Salsbury, C. White, P. Tsolakidis, J. A. McKinna and J. D. Griffiths, Fibrinolysis and circulating malignant cells. *Surg. Gynec. Obstet.* **136,** 733 (1973).
10. S. Carlsson, Fibrinogen degradation products in serum from patients with cancer. *Acta chir. scand.* **139,** 499 (1973).
11. E. Reich, Tumour-associated fibrinolysis. *Fed. Proc.* **12,** 2174 (1973).
12. S. Larsson, Pretreatment classification and staging of bronchogenic carcinoma. *Scand. J. thorac. cardiovasc. Surg.* **4,** Suppl. 10 (1973).

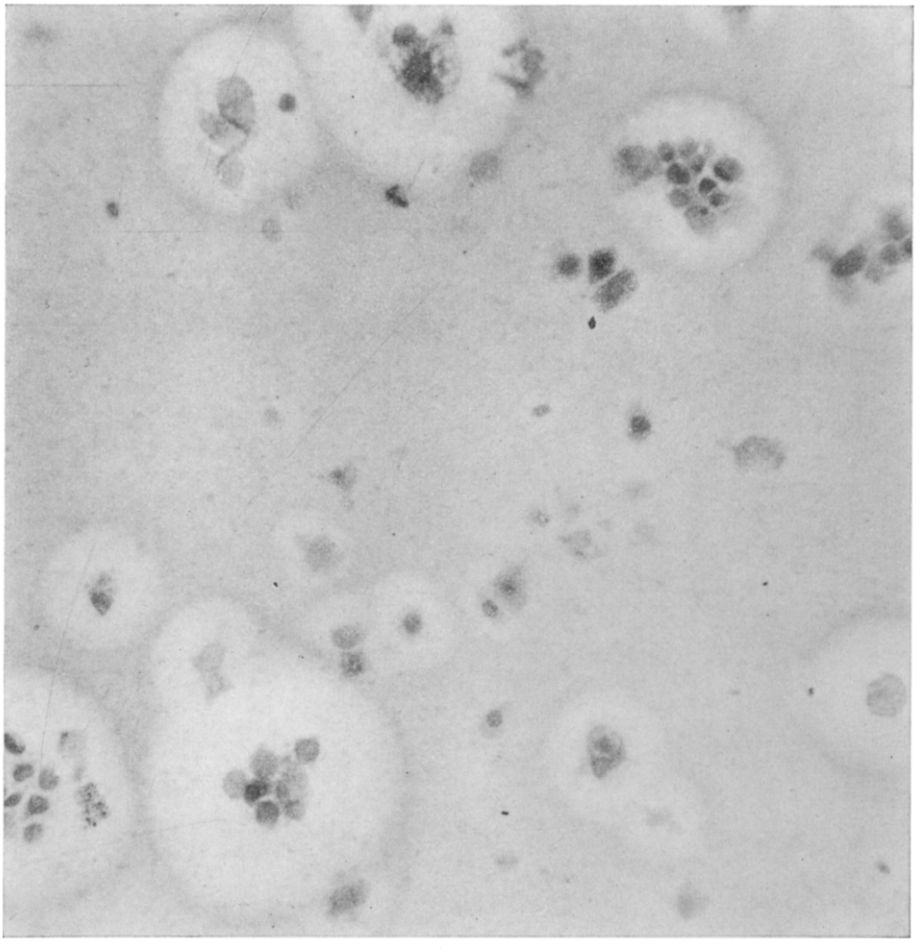


Fig. 1. *Zones of fibrinolysis around individual and aggregated cells from a slightly differentiated epidermoid bronchogenic carcinoma. Original magnification* × *75.*

(to face p. 278)

Europ. J. Cancer Vol. 11, pp. 281–289. Pergamon Press 1975. Printed in Great Britain.

The Dose-related Effect of Tumour Extract on the *In Vitro* Migration of Leucocytes from Patients with Renal Carcinoma*

MOGENS KJAER

Division of Clinical Immunology, Medical Department TA, and Surgical Department C, Rigshospitalet, University Hospital of Copenhagen, Denmark

Abstract—*Systematic titration experiments with allogeneic hypernephroma and normal renal tissue extracts in the capillary tube leucocyte migration technique (LMT) were carried out using peripheral blood leucocytes from 43 patients with renal carcinoma and 83 control patients. The investigation revealed a dose-related specifically-altered reactivity towards allogeneic hypernephroma tissue extract of leucocytes from renal carcinoma patients without distant metastases.*

INTRODUCTION

RECENTLY, a number of reports have described detection of tumour-directed, cellular hypersensitivity by means of the capillary tube leucocyte migration technique, (LMT). Such studies include patients with mammary carcinoma [1–6] oral leukoplakia [7], malignant melanoma [5, 6, 8, 9], colonic carcinoma [10], squamous cell carcinoma of the cervix [11], lymphomas [12], different types of malignant tumours [13–15], and renal carcinoma [16].

Also, difficulties with the technique have been reported [17, 18]. As antigen in the test, most studies have used soluble extracts of tumour material obtained at operation, but also fresh intact cells [19], cells frozen in dimethyl sulphoxide [20], cryostat sections from tumour biopsies [3, 4], and formalin-fixed tumour cells [21] have been used.

As concerns soluble tumour extracts, one of the main problems in the LMT is the standardisation of the concentrations employed, especially determination of the maximal concentration of extract not causing unspecific (toxic) stimulation or inhibition of normal leucocyte migration (maximal non-toxic dose).

It is characteristic for the LMT that: (1) Almost any compound may cause leucocyte migration inhibition if introduced into the tissue culture chamber in sufficiently high concentration, and (2) that high concentrations of antigen are often required to induce specific leucocyte migration inhibition in hypersentitive individuals [22]. To demonstrate a specific cellular hypersensitivity, it is necessary therefore to show a dose-related effect of tissue extracts on leucocyte migration [23]. In a previous report [16], tumour-directed, cellular hypersensitivity towards soluble extracts of autologous and allogeneic renal tumour tissue was described in patients with renal carcinoma. The present study reports on systematic tumour extract titration experiments in the LMT carried out on leucocytes from patients with renal carcinoma and different types of control patients.

Accepted 9 December 1974.

*This study was supported by the Danish Cancer Society, by the Danish Hospital Foundation for Medical Research, Region on Copenhagen Faeroe Island, and Greenland (J. No. 71/72, 32), and by The Danish Medical Research Council.

MATERIAL AND METHODS

A total of 126 persons was investigated, divided into the following groups:

1. Forty-three patients with renal carcinoma, (age 17–75, mean 57·8). Seventeen of these patients had demonstrable distant metastases at the time of initial treatment. Five patients had no operation performed. The histological diagnoses of the 38 patients were: Hypernephroma (25), transitional cell carcinoma of the renal pelvis (7), nephroblastoma (3), renal sarcoma (2), and adenopapillifery carcinoma (1 patient).
2. Four patients (age 24–70, mean 49·0), with various forms of benign renal disease including solitary renal cyst (2 patients), polycystic renal disease (1 patient), and renal adenoma (1 patient).
3. Seven patients (age 42–75, mean 61·0) with carcinoma of non-kidney origin including: colonic adenocarcinoma (3 patients), and gastric adenocarcinoma (4 patients). All patients in this group were without signs of disseminated disease at the time of test.
4. Forty-five patients (age 18–87, mean 47·3) with various forms of benign disease. These patients had no signs of malignant and benign tumours, hypertension or autoimmmune diseases, and had not received steroids or cytostatic drugs within the last 6 months.

   This group included: Duodenal ulcer (7), cerebral thrombosis (2), cholelithiasis (4), acute appendicitis (1), diverticulosis (6), presacral cyst (1), sequelae after gastric resection (1), haemorrhoids (1), stricture of the oesophagus (1), pilonidal cyst (3), oophoretic cyst (1), achalasia of the oesophagus (1), inguinal hernia (2), hydrocele testis (1), fracture (7), chronic pancreatitis (1), hemicrania (1), prostatic hypertrophia (1), and ureterolithiasis (2 patients).
5. Twenty-seven healthy blood donors from the blood donor group of Rigshospitalet Copenhagen, (age 20–65, mean 42·9). Persons above the age of 65 are not permitted to act as blood donors. Therefore, the mean age of this control group was lower than that of the renal carcinoma group.

The leucocyte migration technique

The preparation of tumour extracts has been described earlier in detail [16]. In essence, specimens of tumour and normal renal tissue were cut into small pieces, suspended in Hanks' solution, and homogenized. The homogenates were kept at 4°C overnight and then centrifuged at 1000 ***g*** for 20 min. The protein concentration of the supernatant was adjusted to 1 mg per ml by dilution with Hanks' solution and lyophilized. The LMT was performed as described by Bendixen and Søborg [24], but micromodified according to Maini *et al.* [23] in the following way:

Forty ml peripheral blood was collected from a cubital vein into 10 ml polysterol tubes containing 250 i.u. of heparin without preservatives, and 2·0 ml of 5 % Dextran 250. The tubes were carefully inverted 10 times, and placed for 1 hr at 37°C. After sedimentation of the erythrocytes, the leucocyte-rich supernatant was transferred to polyethylene tubes, and centrifuged at $1000 \times g$ for 5 min. The leucocytes were washed three times in Hanks' solution, and resuspended in TC 199 with 10 % horse serum to a concentration of $2{\cdot}2 \times 10^8$ cells per ml. The leucocytes were then collected in a suitable number of 20 μl glass capillary tubes (Drummond microcaps, Drummond Scientific Supplies, internal diameter, 0·6 mm). Each capillary tube contained 3×10^6 leucocytes. After sealing one end in a flame and centrifuging at 2000 rev/min for 10 min, the capillary tubes were cut just below the cell-fluid interphase, and placed in a 0·5 ml culture chamber (disposable polystyrene chamber for cell migration, Sterilin Ltd., England) containing TC 199 and 10 % horse serum. The adequate volume of tissue extract was added, and the chamber was sealed with a microscope coverslip. Four culture chambers were set up for each concentration of tissue extract employed. Within a set of identical cultures the variation from one migration to another did not exceed 5 % [25].

After 24 hr at 37°C, the migration area of the leucocytes around the opening of each capillary tube was measured in a projection microscope. The average migration area of the antigen-containing (M_x) and control cultures (M_0) from the same blood sample determine the migration index MI:

$$MI - M_x/M_0.$$

A *MI* of less than 1·0 indicates an inhibition, and *MI* greater than 1·0 stimulation of leucocyte migration.

Test protocol

Three to eight tests (group of initial tests) were performed in each patient in the pre- and immediate postoperative period, employing non-pooled extracts from 2–6 different allo-

geneic hypernephromas and corresponding normal renal tissues. In each test, at least two tumour- and normal renal tissue extracts were used at 10, 50, 100, 300, 400, and sometimes 600 μg of protein, per ml.

The previously described groups of control patients (Groups 2, 3, and 4) were tested according to the same protocol. However, blood donors (Group 5) were tested only once.

Statistical evaluation

A computerized combined Wilcoxon Mann–Whitney and Lepage test [26, 27] was used to compare the different distributions of *MI* at fixed tissue extract concentrations. This was done in order to analyse distributions for differences in position as well as dispersion.

When significant differences in position were found, these were corrected, and differences in dispersion were analysed by means of the Ansari–Bradley test [26]. Level of significance: 5 %.

Calculations of normal ranges, *i.e.*, $\bar{x} \pm 2$ s.d. of the MI's, were performed in the control groups (Groups 4 and 5) at allogeneic hypernephroma extract concentrations of 10, 50, 100, 300, 400 and 600 μg of protein per ml. The normal ranges found were as follows:

Group 4

10 μg per ml MI, 0·81–1·17.
50 μg „ MI, 0·80–1·16,
100 μg „ MI, 0·82–1·14,
300 μg „ MI, 0·79–1·19,
400 μg „ MI, 0·79–1·19,
600 μg „ MI, 0·45–1·25,

Group 5

10 μg per ml MI, 0·83–1·15,
50 μg „ MI, 0·81–1·13,
100 μg „ MI, 0·80–1·12,
300 μg „ MI, 0·78–1·18,
400 μg „ MI, 0·79–1·19,

Thus, the normal range at 400 μg of protein per ml (0·79–1·19) was used as a cut-off limit of normal leucocyte reactivity.

RESULTS

The results are presented in Fig. 1 – 7. Figure 1 diagramatically shows the reactivity in the LMT of a patient with hypernephroma without distant metastases and a control patient when leucocytes were tested towards allogeneic hypernephroma extract at concentrations ranging from 10–600 μg of protein per ml.

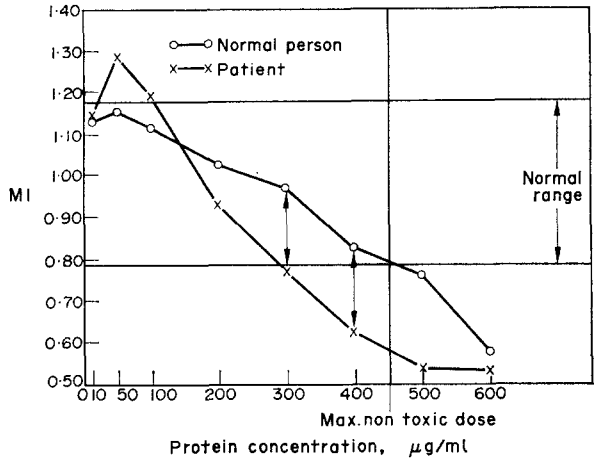


Fig. 1. The dose-related effect of tumour extract on the in vitro *migration of leucocytes from a patient with hypernephroma without detectable distant metastases and a control patient.*

In the hypernephroma patient at low tumour extract concentrations, a slight stimulation of migration was seen. At 300 and 400 μg of protein per ml significant migration inhibition could be demonstrated in the patient. At these concentrations control leucocytes were still unaffected. At 600 μg of protein per ml, inhibition of both patient and control leucocytes became apparent. The present study does not allow the conclusion that this is a general finding. However, titration experiments with other tumour extracts indicates that 600 μg of protein per ml regularly does inhibit both patient and control leucocytes [28].

Out of 26 renal carcinoma patients without distant metastases, 18 patients exhibited reactivity compatible with Fig. 1. Thus, at 300 and 400 μg of protein per ml, an altered reactivity of hypernephroma leucocytes towards components in the tumour extract could be demonstrated compared to that of control leucocytes.

In Fig. 2–7, the reactivity of leucocytes from hospitalized control patients, blood donors, patients with various types of solid carcinoma, patients with benign renal disease and renal carcinoma patients with and without detectable distant metastases towards allogeneic hypernephroma and corresponding normal renal tissue extracts is shown.

The abscissa indicates MI, the ordinate the percentage of observations in different MI-intervals out of the total number of observations in the group.

When leucocyte reactivity at fixed tissue extract concentrations was compared in hospitalized control patients, blood donors, patients with various types of carcinoma and benign renal disease, no significant differences could

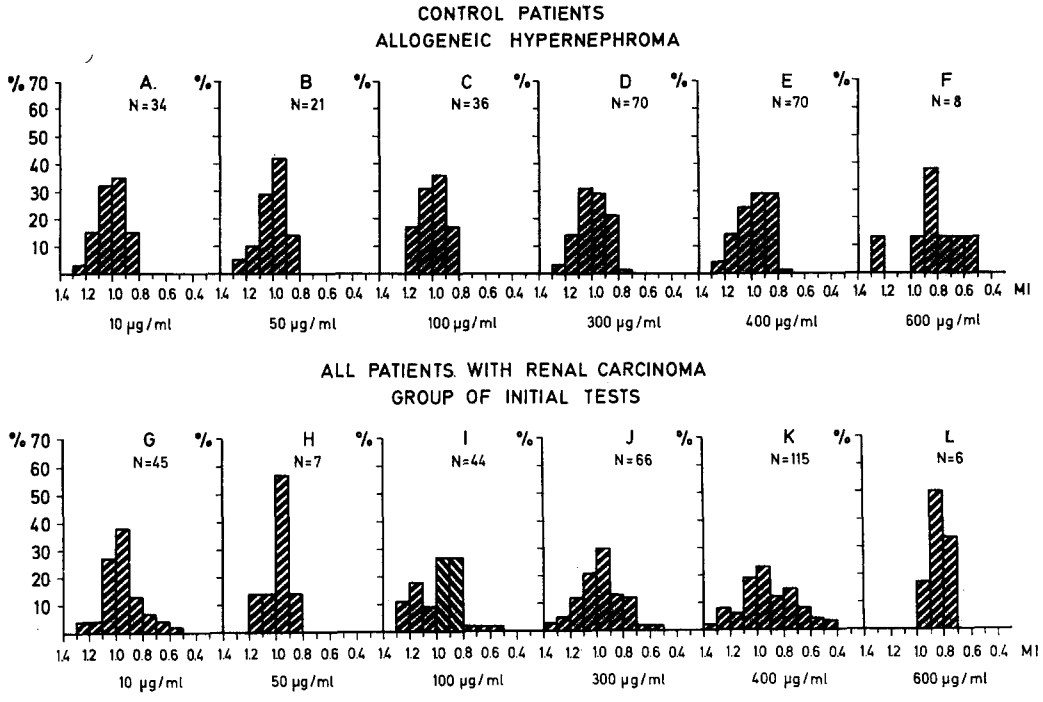


Fig. 2. *The dose-related effect of tumour extract on the* in vitro *migration of leucocytes from hospitalized control patients, (Group* 4*) and all patients with renal carcinoma.*

Wilcoxon Mann–Whitney test and Lepage test:

A–G :	$P > 0{\cdot}05$	$P = 0{\cdot}306$	D–J :	$P > 0{\cdot}05$	$P = 0{\cdot}003$
B–H :	$P > 0{\cdot}05$	$P = 0{\cdot}245$	E–K :	$P > 0{\cdot}05$	$P = 0{\cdot}001$
C–I :	$P > 0{\cdot}05$	$P = 0{\cdot}003$	F–L :	$P > 0{\cdot}05$	$P = 0{\cdot}001$

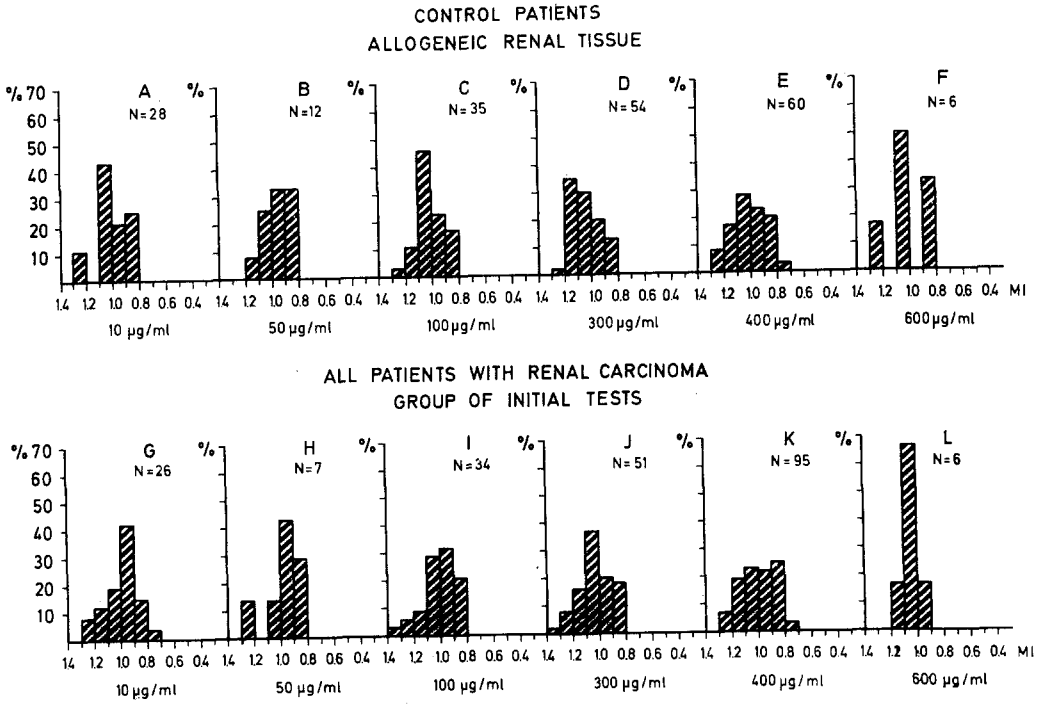


Fig. 3. *The dose-related effect of normal renal tissue extract on the* in vitro *migration of leucocytes from hospitalized control patients, (Group* 4*) and all patients with renal carcinoma.*

Wilcoxon Mann–Whitney test and Lepage test:

A–G :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	D–J :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
B–H :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	E–K :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
C–I: :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	F–L :	$P > 0{\cdot}05$	$P > 0{\cdot}05$

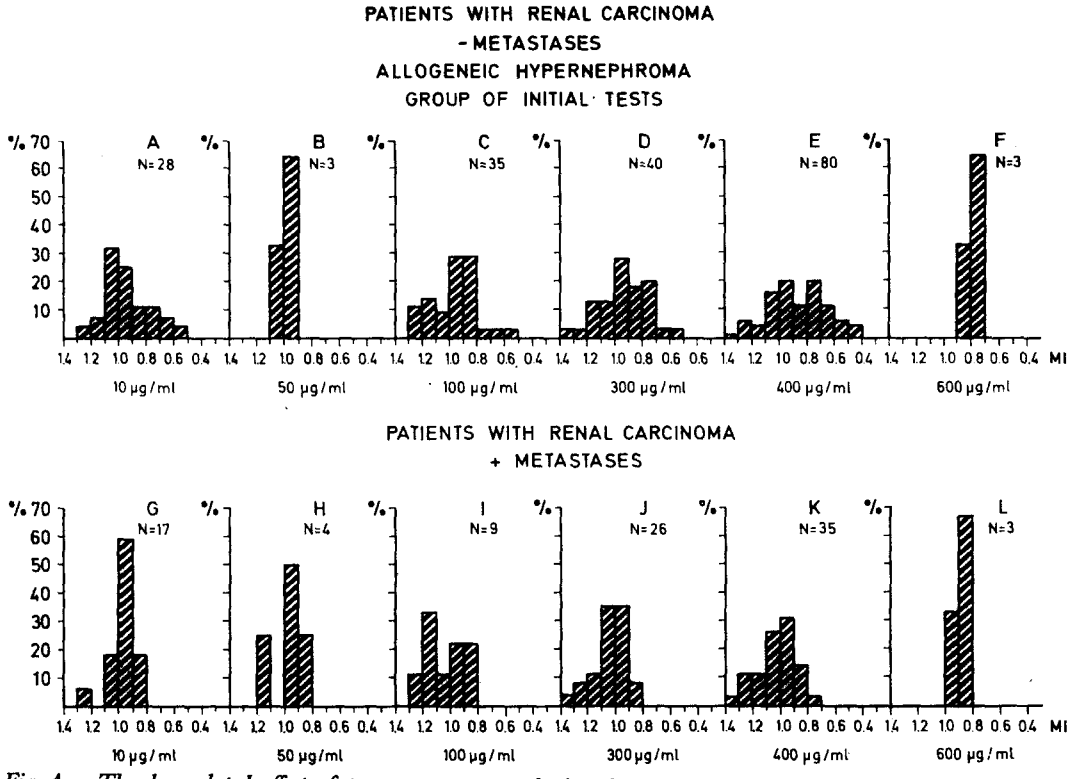


Fig. 4. *The dose-related effect of tumour extract on the* in vitro *migration of leucocytes from patients with renal carcinoma with and without clinically evident distant metastases at the time of initial treatment.*
Wilcoxon Mann–Whitney test and Lepage test:

A–G :	$P > 0{\cdot}05$	$P = 0{\cdot}311$	D–J :	$P < 0{\cdot}01$	$P = 0{\cdot}713$
B–H :	$P > 0{\cdot}05$	$P = 0{\cdot}510$	E–K :	$P < 0{\cdot}001$	$P = 0{\cdot}001$
C–I :	$P > 0{\cdot}05$	$P = 0{\cdot}596$	F–L :	$P > 0{\cdot}05$	$P = 0{\cdot}600$

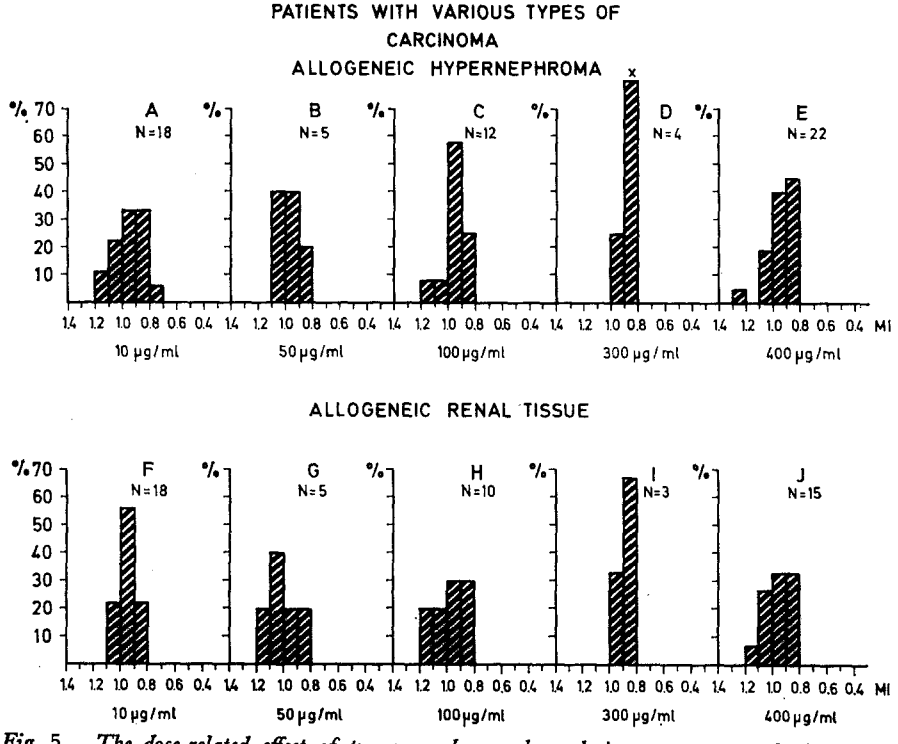


Fig. 5. *The dose-related effect of tumour and normal renal tissue extract on the* in vitro *migration of leucocytes from patients with various types of carcinoma, (Group* 3).
(×) 75%
Wilcoxon Mann–Whitney test and Lepage test:

A–F :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	D–I :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
B–G :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	E–J :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
C–H :	$P > 0{\cdot}05$	$P > 0{\cdot}05$			

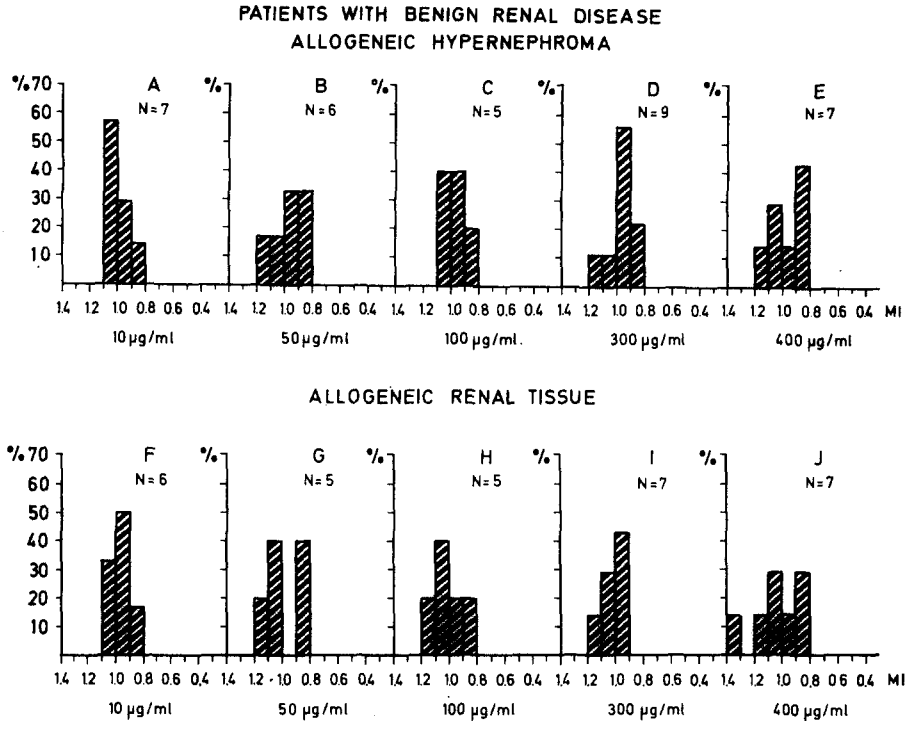


Fig. 6. The dose-related effect of tumour and normal renal tissue extract on the in vitro *migration of leucocytes from patients with benign renal disease, (Group 2).*
Wilcoxon Mann–Whitney test and Lepage test:

A–F :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	D–I :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
B–G :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	E–J :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
C–H :	$P > 0{\cdot}05$	$P > 0{\cdot}05$			

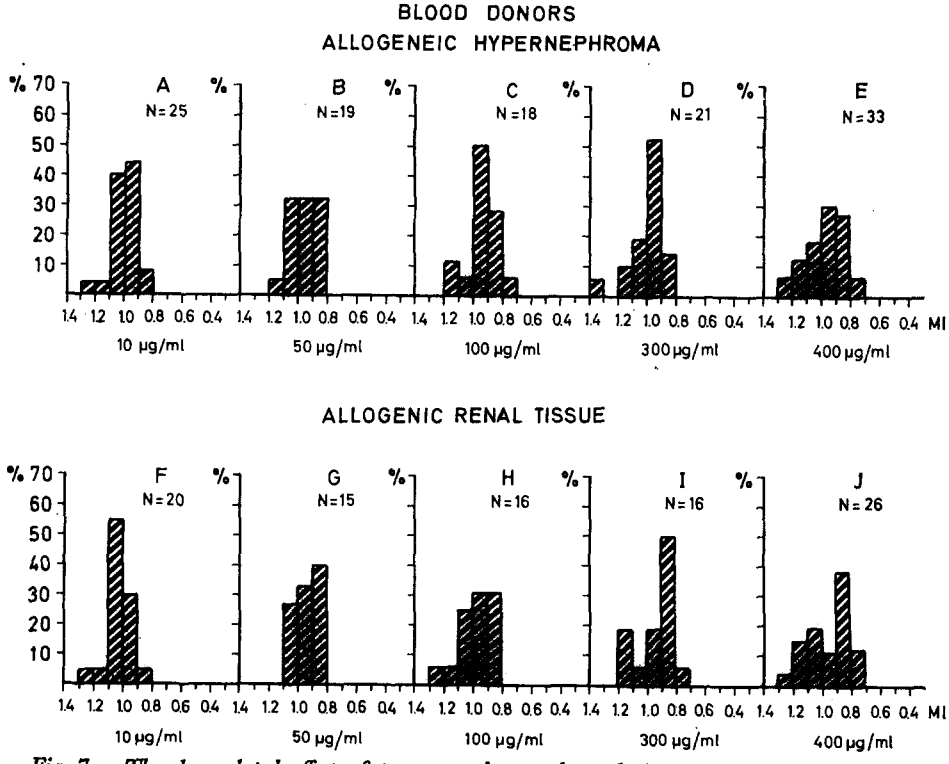


Fig. 7. The dose-related effect of tumour and normal renal tissue extract on the in vitro *migration of leucocytes from healthy blood donors, (Group 5).*
Wilcoxon Mann–Whitney test and Lepage test:

A–F :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	D–I :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
B–G :	$P > 0{\cdot}05$	$P > 0{\cdot}05$	E–J :	$P > 0{\cdot}05$	$P > 0{\cdot}05$
C–H :	$P > 0{\cdot}05$	$P > 0{\cdot}05$			

be found at any tissue extract concentration tested regardless of the type of tissue extract used. Likewise, the reactivity of renal carcinoma patients towards allogeneic normal renal tissue extract did not differ significantly from any other group tested.

However, when the total number of renal carcinoma patients was compared to control patients, significant differences in dispersion were found at 100, 300, and 400 μg of protein per ml when leucocytes were tested towards allogeneic hypernephroma tissue extract (Fig. 2). When reactivity of leucocytes from patients with renal carcinoma with and without distant metastases towards allogeneic hypernephroma tissue extract was compared (Fig. 4) significant differences in position were found at 300 and 400 μg of protein per ml.

When reactivity of leucocytes from renal carcinoma patients with distant metastases was compared to control leucocyte reactivity (Fig. 2, column A–F, Fig. 4, column G–L) towards allogeneic hypernephroma tissue extract, no significant differences could be found. However, when reactivity of leucocytes from renal carcinoma patients without distant metastases was compared to control leucocyte reactivity (Fig. 2, column A–F, Fig. 4, column A–F) both significant differences in position and dispersion of the distributions could be demonstrated at 50, 100, 300, and 400 μg of protein per ml. The values being: A–A, $P > 0{\cdot}05$ $P = 0{\cdot}204$, B–B, $P > 0{\cdot}05$ $P = 0{\cdot}05$, C–C, $P > 0{\cdot}05$ $P = 0{\cdot}005$, D–D, $P = 0{\cdot}01$ $P = 0{\cdot}045$, E–E, $P < 0{\cdot}001$ $P = 0{\cdot}001$, F–F, $P > 0{\cdot}05$ $P = 0{\cdot}01$. The results thus indicate a specifically altered, dose-related *in vitro* leucocyte reactivity in renal carcinoma patients without distant metastases. The reactivity is directed towards components of allogeneic hypernephroma extract and manifest at concentrations of 50, 100, 300, and 400 μg of protein per ml culture medium.

DISCUSSION

At least three factors seem to be critical for the outcome of the interaction between leucocytes and antigen *in vitro* employing the LMT:

(*a*) the sensitivity of the cell donor,

(*b*) the antigen concentration in the culture chamber,

(*c*) the structural identity between the antigens on tumour cell *in vivo* and in the tissue preparation used *in vitro*.

Systematic tumour-extract titration experiments in the LMT, to our knowledge, have not been published earlier. However, in brucella hypersensitivity, using brucella bacteria as antigen, Søborg [29] demonstrated a correlation between the degree of hypersensitivity as judged by the i.c. test and the LMT. Patients with a low to moderate degree of hypersensitivity tended to exhibit stimulation of leucocyte migration at low antigen concentrations and inhibition at high antigen concentrations. Leucocytes from patients with a high degree of hypersensitivity were inhibited at low and at high antigen concentrations.

In a study of tuberculin hypersensitivity, Federlin *et al.* [30] demonstrated that at low PPD concentrations a continuum of reactions from pronounced stimulation, over the normal range, to inhibition of leucocyte migration was found in the Mantoux-positive group. At high antigen concentrations, all the Mantoux-positive patients exhibited migration inhibition. At all antigen concentrations employed, the Mantoux-negative patients showed reactivity within the normal range. The antigens employed in these studies were microbial antigens. The outcome of the LMT was determined by the degree of hypersensitivity of the patients, and by the concentration of antigen in the culture chamber.

In the present investigation, at low antigen concentrations (10 μg per ml), no difference in leucocyte migration could be demonstrated in the renal carcinoma group compared to various types of control groups. At higher antigen concentrations (300–400 μg per ml), a continuum of leucocyte reactivity from pronounced stimulation to inhibition was found. At the same antigen concentrations, the reactivity of control leucocytes was unaffected (maximal non-toxic dose). At very high antigen concentrations (600 μg per ml), significant leucocyte migration inhibition was detected also in the control groups.

A similar distribution of leucocyte reactivity at the maximal non-toxic dose has been demonstrated in patients with cirrhosis of the liver using soluble extracts of foetal liver tissue as antigens [31].

As a tentative explanation of the leucocyte reactivity found in the present study, at least two possibilities exist:

(1) patients with renal carcinoma generally have a low degree of hypersensitivity towards the tissue extracts employed. Therefore, in this test system, the concentration of extract discriminating the renal carcinoma patients with a specific cellular hypersensitivity will not be very far from

the concentration with a toxic effect on normal leucocytes.

(2) the structural identity between antigens on tumour cells *in vivo* and in the tissue extracts employed in the present study was poor. Thus, unspecific effects might account for the wide distribution of reactions.

Isolation and characterization of the antigen(s) may clarify this point. Such studies are in progress.

However, using the same type of crude extraction of foetal kidney tissue, at protein concentration of 100 μg per ml (maximal non-toxic dose), Bendixen [32] examined patients with active glomerulonephritis. Thirteen out of fifteen patients showed inhibition of leucocyte migration, but stimulation was not detected in any of the patients.

In the present study, it is shown that the reactivity of renal carcinoma patients with distant metastases does not differ from that of different types of control patients. It is generally agreed that patients with distant metastases have low or absent tumour-directed, cellular hypersensitivity [16]. These observations might indicate that the outcome of the LMT as regards crude tissue extracts depends rather on the degree of hypersensitivity of the cell donor than on the purity of the extract used. It seems possible that stimulation as well as inhibition of leucocyte migration on contact with the specific antigen could indicate a state of cellular hypersensitivity in the patient studied. At different concentrations of tissue extract, a specific reactivity of the same patient may show either stimulation or inhibition of leucocyte migration.

Acknowledgements—I thank Mrs. Lidy Mayland, Mrs. Aase Bendixen, and Mrs. Anne Kjerulff for competent technical assistance. Likewise I want to thank Dr. G. Bendixen and Dr. V. Andersen for helpful discussions during the initial period of the investigations and while preparing the manuscript.

REFERENCES

1. V. Andersen, G. Bendixen and T. Schiødt, An *in vitro* demonstration of cellular immunity against autologous mammary carcinoma in man. *Acta. med. scand.* **186,** 101 (1969).
2. V. Andersen, O. Bjerrum, G. Bendixen, T. Schiødt and I. Dissing, Effect of autologous mammary tumour extracts on human leucocyte migration *in vitro*. *Int. J. Cancer* **5,** 357 (1970).
3. M. M. Black, D. H. Moore, B. Shore, R. E. Zachrau and H. P. Leis, Jr., Effect of murine milk samples and human breast tissues on human leucocyte migration indices. *Cancer Res.* **34,** 1054 (1974).
4. M. M. Black, H. P. Leis, Jr., B. Shore and R. E. Zachrau, Cellular hypersensitivity to breast cancer. Assessment by a leukocyte migration procedure. *Cancer* **33,** 952 (1974).
5. A. J. Cochran, W. G. S. Spilg, R. M. Macie and C. E. Thomas, Post-operative depression of tumour-directed cell-mediated immunity in patients with malignant disease. *Brit. Med. J.* **4,** 67 (1972).
6. A. J. Cochran, R. M. Mackie, C. E. Thomas, R. M. Grant, D. E. Cameron-Mowat and W. G. S. Spilg, Cellular immunity to breast carcinoma and malignant melanoma. *Brit. J. Cancer* **28,** suppl. 1, 77 (1973).
7. B. Roed-Petersen, J. Roed-Petersen and G. Bendixen, Cell-mediated immunity to human oral leukoplakia demonstrated by the leukocyte migration test. *Int. J. Cancer* **12,** 66 (1973).
8. R. M. Mackie, W. G. S. Spilg, C. E. Thomas and A. J. Cochran, Cell-mediated immunity in patients with malignant melanoma. *Brit. J. Derm.* **87,** 523 (1972).
9. A. J. Cochran, U. W. Jehn and B. P. Gothoskar, Cell-mediated immunity in malignant melanoma. *Lancet* **1,** 1340 (1972).
10. D. M. Bull, J. R. Leibach, M. A. Williams and R. A. Helms, Immunity to colon cancer assessed by antigen-induced inhibition of mixed mononuclear cell migration. *Science* **181,** 957 (1973).
11. M. S. Goldstein, B. Shore and S. B. Gusberg, Cellular immunity as a host response to squamous carcinoma of the cervix, *Amer. J. Ogstet. Gynec.* **111,** 751 (1971).
12. M. Braun, L. Sen, A. E. Bachmann and A. Pavlovsky, Cell migration inhibition in human lymphomas using lymph node and cell line antigens. *Blood* **39,** 368 (1972).

13. A. SEGALL, O. WEILER, J. GENIN, J. LACOUR and F. LACOUR, *In vitro* study of cellular immunity against autochthonous human cancer. *Int. J. Cancer* **9,** 417 (1972).
14. W. H. WOLBERG, Inhibition of migration of human autogenous and allogenic leukocytes by extracts of patients' cancers. *Cancer Res.* **31,** 798 (1971).
15. W. H. WOLBERG and M. L. GOELZER, *In vitro* assay of cell mediated immunity in human cancer: Definition of leukocyte migration inhibition factor. *Nature (Lond.)* **229,** 632 (1971).
16. M. KJAER, *In vitro* demonstration of cellular hypersensitivity to tumour antigens by means of the leucocyte migration technique in patients with renal carcinoma. *Europ. J. Cancer* **10,** 523 (1974).
17. M. D. LOCKSHIN, J. WAXMAN and M. W. JENKINS, Mechanism of buffy coat migration inhibition test. *Experienta (Basel)* **29,** 340 (1973).
18. S. A. ROSENBERG, Problems with the leukocyte migration inhibition technique in the study of human tumour immunity. Conf. and Workshop on Cellular Immune Reactions to Human Tumour Associated Antigen. June 8–9, 1972. *Natl. Cancer Inst.. Monogr.* **37,** 139 (1973).
19. A. J. COCHRAN, E. KLEIN and G. PETRANYI, Migration of lymph node cells during the primary immune response. *Transplantation* **12,** 523 (1971).
20. J. M. ANDERSON, F. KELLY, S. E. WOOD, K. E. RODGER and I. FRESHNEY, Evaluation of leucocyte functions six years after tumour autograft in human mammary cancer. *Brit. J. Cancer* **28,** suppl. 1, 83 (1973).
21. C. E. ROSS, A. J. COCHRAN, D. E. HOYLE, R. M. GRANT and R. M. MACKIE, Formalin-fixed tumour cells in the leucocyte migration test. *Lancet* **1,** 1087 (1973).
22. V. ANDERSEN, M. KJAER and G. BENDIXEN, *In vitro*-demonstration of cellular hypersensitivity to tumour antigens in man. *Ser. Haemat.* **5,** 3 (1972).
23. R. N. MAINI, L. M. ROFFE, J. T. MAGRATH and D. C. DUMONDE, Standardization of the leucocyte migration test. *Int. Arch. Alllergy* **45,** 308 (1973).
24. G. BENDIXEN and M. SØBORG, A leucocyte migration technique for *in vitro* detection of cellular (delayed type) hypersensitivity in man. *Dan. med. Bull.* **16,** 1 (1969).
25. M. KJAER and G. BENDIXEN In preparation.
26. J. W. BRADLEY, *Distribution-Free Statistical Tests.* Prentice-Hall, Englewood Cliffs. N. J. Jersey, (1968).
27. Y. LEPAGE, A combination of Wilcoxon's and Ansari-Bradley's statistics. *Biometrica* **58,** 213 (1971).
28. M. KJAER, Unpublished observations.
29. M. SØBORG, *In vitro* migration of peripheral human leucocytes in cellular hypersensitivity. *Acta med. scand.* **184,** 135 (1968).
30. K. FEDERLIN, R. N. MAINI, A. S. RUSSELL and D. C. DUMONDE, A micromethod for peripheral leucocyte migration in tuberculin sensitivity. *J. clin. Path.* **24,** 533 (1971).
31. M. G. M. SMITH, A. L. W. F. EDDLESTON and R. WILLIAMS, Leucocyte migration in active chronic hepatitis and primary biliary cirrhosis. In *Immunology of the Liver* (Edited by M. SMITH and R. WILLIAMS), Proc. Int. Meet. at King's College Hosp. Med. School. July 6–7 (1970).
32. G. BENDIXEN, Organ-specific inhibition of the *in vitro* of leucocytes *migration* in human glomerulonephritis. *Acta med. scand.* **184,** 99 (1968).

Europ. J. Cancer Vol. 11, pp. 291–299. Pergamon Press 1975. Printed in Great Britain

The Response of Mouse Skin to Fractionated X Rays

S. B. FIELD,* C. MORRIS,* J. DENEKAMP† and J. F. FOWLER†
*M.R.C. Cyclotron Unit, Hammersmith Hospital, London W12 0HS,
†Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex HA6 2RN, Great Britain

Abstract—*The feet of mice were irradiated with single doses or with 4, 8, 16 or 32 fractions of X rays, all in the same overall time of 43 days. Skin reactions and late deformities were scored and the results are discussed in terms of cell survival curve models. It is concluded that at low doses per fraction there is a significant component of irreparable damage, but it is not clear whether this is due to a high LET component of the radiation or to a variable sensitivity of the cells of the skin.*

The applicability of the Ellis formula is discussed and is found to be a reasonable approximation over a limited range of dose per fraction.

INTRODUCTION

IN RADIOTHERAPY, it is usual to give a course of treatment in many small fractions. A large number of radiobiological experiments have been designed to explain the processes occurring between fractions, and the majority of these have employed two doses only. Such experiments on cells *in vitro* and *in vivo* have yielded detailed information regarding accumulation and repair of sublethal damage, sensitivity of cells in different phases of their cycle, their reassortment after irradiation, and repopulation [1–4]. However, the doses used in such investigations on tissues are generally much larger than those employed by the clinician, and recently more attention has been focussed on experiments employing smaller doses per fraction [4–6]. Since the range of total dose over which radiation damage in tissues can be assessed is rather limited, it is necessary to employ many fractions in order to use small doses, and such experiments are therefore similar to radiotherapy practice.

This paper will compare the results of irradiating mouse skin with up to 32 doses of X rays all given in the same overall time of 43 days, so that it can be assumed that the total repopulation throughout each treatment is the same. This may not be strictly correct, but the repopulation is likely to be similar for various treatments given in the same overall time. Skin reactions and late deformity of the feet were measured and also in some cases, the effects of an additional "topping up" dose at the end of the fractionation regime.

The results will be discussed in terms of two theoretical approaches: (1) the consideration of the dose in N fractions in comparison to the *single* dose required to produce the same level of damage, and the employment of a correction for repopulation during the N fractions; (2) that of Dutreix, Wambersie and Bounik [5] who considered the additional dose required when N fractions are increased to $2N$ subfractions in the same overall time, with an adequate time allowed for repair of sublethal damage between each of the subfractions.

An interpretation of the present results in terms of survival curves has been attempted and this approach focusses attention on the sensitivity of cells at low doses, *i.e.*, on the shape of the cell survival curve in the "shoulder" region. Previous work has been influenced mainly by the sensitivity of the cells in the exponential region of the survival curve (characterised by D_0) because large doses per fraction have been used.

Accepted 28 November 1974.

MATERIAL AND METHODS

Male SAS/TO albino mice aged 10–12 weeks were used. For each dose point, 8 mice were irradiated simultaneously; they were anaesthetised with 60 mg/kg pentobarbitone sodium and were irradiated in an atmosphere of oxygen warmed to 25 ± 1°C flowing through the jig enclosure at 6 l/min. The left leg was secured in position by lightly cellotaping the toes. The whole foot and ankle were irradiated. The X rays were generated by a 250 kVp Maximar X-ray generator with 0·25 mm of copper and 1 mm of aluminium added filtration, giving a HVT of 1·3 mm copper. The dose rate was 180 rads/min.

Four, 8, 16 or 32 fractions were given, at equal intervals in the same overall time of 43 days. For the 32 fraction experiment, 48 mice were anaesthetised for each irradiation, and only one died. Only eight mice died altogether out of 210, with a total of 2,748 administrations of anaesthetic. This was achieved by noting the effect of each anaesthetic dose on each mouse, modifying the subsequent dose accordingly, and allowing all mice to recover from the anaesthetic in a box maintained at 30°C.

Table 1. *Skin reaction scoring system*

Score	Observation
0·5	Doubt as to whether there is any abnormality
1·0	Definite abnormality with reddening or foot slightly swollen
1·5	Moist desquamation in one small area (usually between the toes)
2·0	Breakdown of a large area and/or toes stuck together
2·5	About 1/2 total foot broken down

In practice the system has been developed so that differences of 1/6th of a unit can be recorded.

Table 2. *Deformity scoring system*

Score	Observation
1	Slight change in shape
2	Definite change in shape and/or toes stuck together
3	Considerable change in shape and/or toes missing
4	Almost shapeless with only proximal parts of toes remaining
5	Foot shapeless with no toes

In practice intermediate scores of 0·5 are used.

RESULTS

Skin reactions occurring after irradiation of the feet of mice and the methods of scoring them have been well documented. The skin reaction and deformity scores used in the present experiments are tabulated in Tables 1 and 2.

After single doses, the feet became slightly swollen and erythematous at about 7 days, reaching a peak reaction of moist desquamation (if the dose was high enough) at about day 20. Healing was usually complete by day 30, except for the highest dose groups. In the multi-fraction treatments given over 43 days, the initial appearance of the reaction was at about 15 days after the first irradiation, *i.e.*, a delay of about 8 days in comparison to a single dose. The peak reaction was reached at about day 30 in the 32 fraction experiment, and day 35 in the 16 and 8 fraction experiments, *i.e.*, before the treatment was completed. In the 4 fraction experiment in which the spacing of the doses was about 2 weeks, the reaction showed 3 separate peaks, at about days 25, 40 and 55. The shape of the curve relating skin reaction to time after irradiation was similar for single doses, 8, 16 and 32 fractions but rather different for 4 fractions.

The lower dose groups in all fractionation experiments developed only very small skin reactions, not exceeding an erythema, and since this was difficult to score accurately, these groups all received an additional 1000 rads on day 43, in order to raise the level of damage into the range where it could be scored. This additional dose resulted in a further wave of reaction starting almost immediately, having a maximum about 12 days later and healing very rapidly.

The feet became progressively deformed over a period of months, although unlike rat feet, they did not develop a further wave of moist desquamation [7]. The damage seen at 30 weeks was used as a measure of late deformity (Table 2). Many mice were kept for one year after irradiation, and in no case did the late damage progress beyond 6 months, despite the large doses used, up to 10,000 rads in 30 fractions in 43 days.

Figure 1 shows the early skin reaction averaged over a 28 day period after treatment, plotted as a function of dose for the various treatment regimes. The single dose data were averaged from day 7 to day 35 and the fractionated data from day 16 to day 44. In this way, the timing of the overall rise and fall of the reactions was roughly matched. (This was not so for 4 fractions). Figure 2 shows

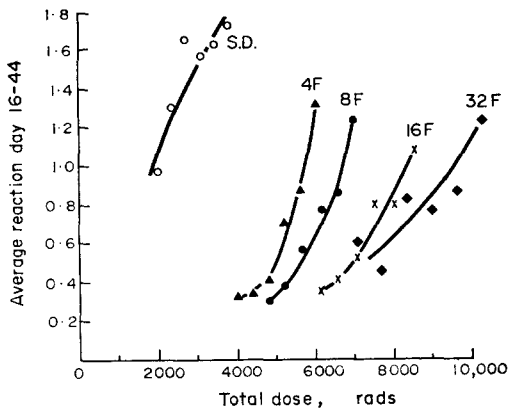


Fig. 1. *Average early skin reactions plotted as a function of total dose for single doses (S.D.) and various numbers of fractions all given in the total time of* 43 *days.*

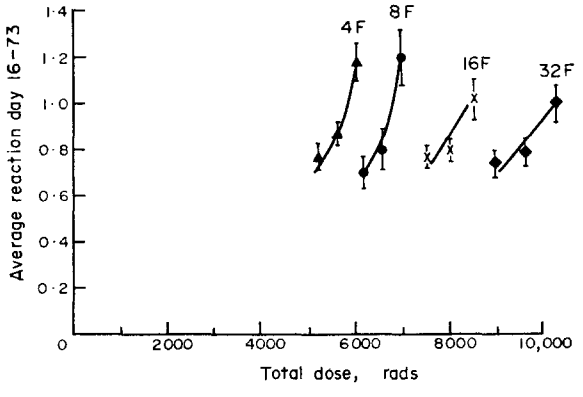


Fig. 2. *Skin reactions averaged from* 16–73 *days after the first dose plotted as a function of total dose, for irradiations given in various numbers of fractions, all given in a total time of* 43 *days.*

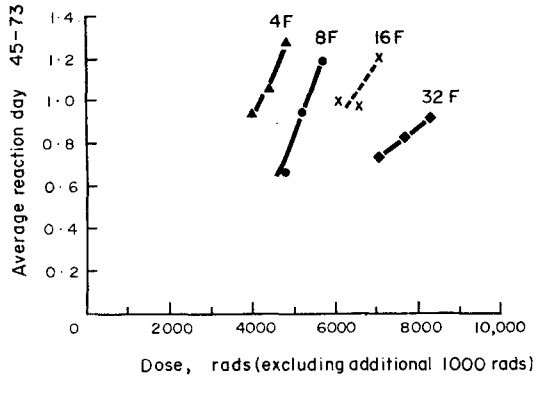


Fig. 3. *Skin reactions averaged over a* 19 *day period after the final dose of various fractionation regimes, all given in the total time of* 43 *days. On day* 43 *an additional* 1000 *rads was given to all mice.*

similar data, but averaged from day 16 to 73; the single dose data are omitted. In this case, only the 3 highest dose groups are included: the lower dose groups received an additional 1000 rads on day 43. Figure 3 shows the data for these lower dose groups, analysed from day 45 to 73 *i.e.*, from 3 days after the "topping up" dose. They are consistent with the pattern of results obtained using the other methods of analysis. Figure 4 shows the dose response curves for foot deformity at 30 weeks.

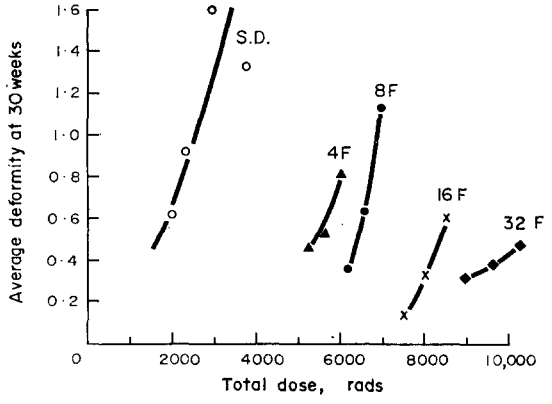


Fig. 4. *Average deformity plotted as a function of total dose for single doses (S.D.) and various numbers of fractions, all given in the total time of* 43 *days.*

In all four methods of analysis, as the number of fractions was increased, the total dose to produce a given reaction also had to be increased. These results are discussed below in relation to the results of other recent experiments in which many small fractions have been employed. In order to make these comparisons, some theoretical concepts must first be clarified, as is done in the following section.

DISCUSSION

General theory

It is assumed throughout the following discussion that the gross radiation response of skin is the result of damage to its proliferative cells and can be interpreted in terms of cell survival curves. This approach is supported by the similarity between results obtained using skin reactions and survival of epidermal cells [3, 8].

Most mammalian cells yield survival curves with a "shoulder" characterized by D_Q [9] and with a final exponential region with slope $-1/D_0$. With small doses of low LET radiation, the shoulder region represents a large proportion of the radiation being used to inflict sublethal damage. This can usually be repaired providing further radiation is not given immediately to convert the damage to the lethal form. Two theoretical survival curves are shown in Fig. 5(*a*). Curve (A) represents the multi-target model, *i.e.*, 1 hit in each of m targets, given by:

$$S = 1-(1-e^{-D/D_0})^m.$$

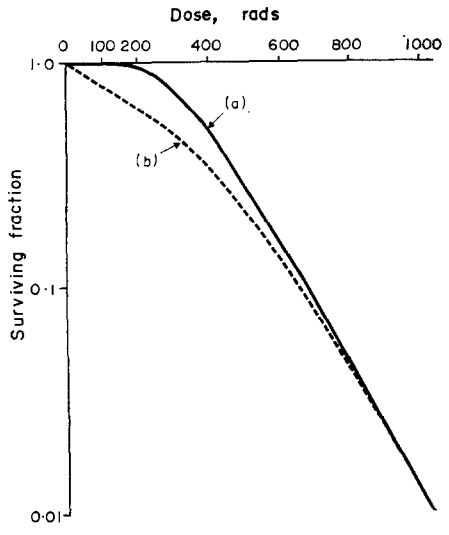


Fig. 5a. (*A*) *multi-target survival curve* $D_0 = 150$ *rad and* $m = 10$.
(*B*) *Two component survival curve* $D_0/D' = 0{\cdot}33$, $m = 10$.

This curve has zero initial slope in contrast with curve (B), which represents the two component model, *i.e.*, single hit, multi-target, given by:

$$S = e^{-D}/D' \,(1-(1-e^{-D}/D'')^m).$$

In this case, the initial region is exponential with slope $-(1/D')$, the final slope of the multi-target component is given by $-(1/D'')$ and the final slope of the combination is given by: $-(1/D^0)$where

$$\frac{1}{D_0} = \frac{1}{D'} + \frac{1}{D''}.$$

If the radiation is given in two fractions, and if repair of the sublethal damage from the first dose is complete, the shoulder is repeated in the second dose curve. This can be measured as $D_2 - D_1$, where D_2 is the dose in two fractions and D_1 the single dose to produce the same level of damage. $D_2 - D_1$ is the additional dose used in accumulating sublethal damage in the second dose, *i.e.*, is a measure of the "shoulder" of the second dose survival curve. For large doses per fraction, $D_2 - D_1 = D_Q$, providing there are no complicating factors. For small doses $D_2 - D_1$ is the same as the parameter D_r defined by Dutreix *et al.* [5]. These authors developed an ingenious experimental technique for measuring the "additional dose" D_r for small doses per fraction by amplifying the difference using many small fractions. They compared the effect of N fractions with $2N$ subfractions given in the same overall time, and assuming repopulation is the same in both cases, then

$$D_r = \frac{\text{total dose in } 2N \text{ fractions} - \text{total dose in } N \text{ fractions}}{N}$$

Figure 5(*b*) shows curves of D_r as a function of the larger dose per fraction D_N/N, designated D_s by Dutreix *et al* [5]. Curve (A) corresponds to the multi-target model (A) in Fig. 5(*a*). According to this model, as the dose is decreased, an increasing fraction of the dose is "wasted" on inflicting sublethal damage. If, on the other hand, D_r reaches zero at a positive value of dose per fraction, the implication is that the critical region of the survival

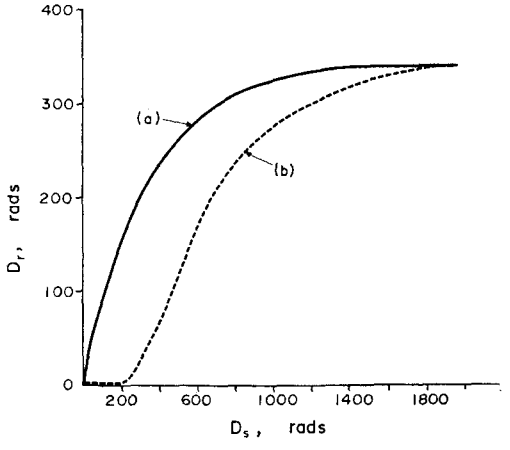


Fig. 5b. D_r *plotted as a function of* D_s *for two theoretical models*: (*A*) *Multi-target model with* $D_0 = 150$ *rad*, $m = 10$ *and* (*B*) *Two component model with* $D_0 = 150$ *rad*, $D_0/D' = 0{\cdot}33$, $m = 10$.

curve is exponential and that no "addition dose" is required to inflict sublethal damage, *i.e.*, in this situation, as the dose is decreased, the *proportion* of dose inflicted as sublethal damage remains constant. This is shown in curve (B) in Figs. 5(*a*) and 5(*b*). As the dose per fraction is increased, D_r in both models tends to D_Q.

The ratio of the initial slope relative to the final slope of the survival curve is D_0/D' and is a parameter of considerable interest. We term it the "slope ratio" and it can be derived from the comparison of N fractions D_N with a single dose D_1 to produce the same level of damage, *i.e.*,

$$\text{slope ratio} = \frac{D_0}{D'} \simeq \frac{D_1 - D_Q}{D_N}$$

(Fig. 6).

The initial "slope" derived in this way is actually a cord, but is likely to be close to a slope if N is large. A correction must be applied for repopulation and D_Q must be determined separately from $D_2 - D_1$.

Figure 5(*c*) shows the plots of slope ratio for the multi-target model (A) and the two component model (B), consistent with the D_0 curves shown in Fig. 5(*b*) and the survival curves shown in Fig. 5(*a*). For the multi-target model, (A), the slope ratio tends to zero as the dose per fraction tends to zero, *i.e.*, the initial slope of the survival curve is zero. For the two component model, (B), the slope ratio becomes constant with decreasing dose, *i.e.*, the initial region is exponential. In the example given, the slope ratio becomes 0·33.

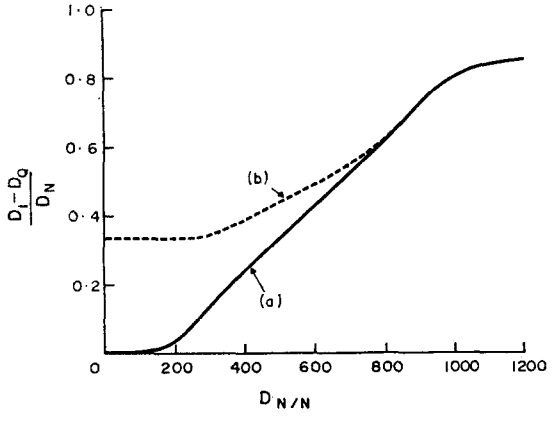


Fig. 5c. Slope ratio, $(D_1 - D_Q)/D_N$, *plotted as a function of dose per fraction* (D_N/N) *for two theoretical models. (A) Multi-target model with* $D_0 = 150$ *rad,* $m = 10$ *and (B) Two component model with* $D_0 = 150$ *rad* $D_0/D' = 0{\cdot}33$, $m = 10$.

There are, of course, other models of cell survival which could be considered. The two chosen were used mainly for illustration, although one possible implication of the two component model is that the beam contains a mixture of low and high LET radiation. It must be pointed out, however, that such a survival curve could be obtained for entirely different reasons. For example, cells in different stages of their cycle have different survival curves, so that by assuming a distribution of cells in different stages of their cycle, each with its own survival curve, a wide range of composite survival curves can be constructed, including curves similar to those derived from the two component model described above in B. Whereas for the two component model described above, the surviving fractions for each component are multiplied together, in this case the surviving fractions are *added* in proportion to the fraction of cells in each stage of the cycle.

Consideration of experimental data

The data obtained in the present experiments (shown in Figs. 1–4), are plotted in Fig. 7 as D_r against D_s. The lowest doses were from a comparison of 32 and 16 fractions, with D_s (the dose per fraction in the 16 fraction experiment) having a minimum value of about 400 rads. Because a finite dose is required to produce any reaction at all with very small doses, it becomes difficult to resolve the dose effect curves at very low doses, which may have introduced an artefact in deriving the lowest data point shown in

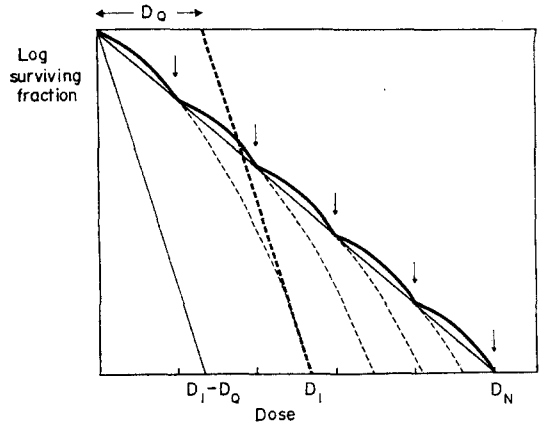


Fig. 6. Hypothetical survival curves for single doses, reaching a given level of damage with dose D_1 *and for* N *fractions reaching the same level of damage with dose* D_N. *The ratio of the final slope of the survival curve to the intercept slope to dose* D_N/N *is given by* $(D_1 - D_Q)/D_N$. *This is the "slope ratio".*

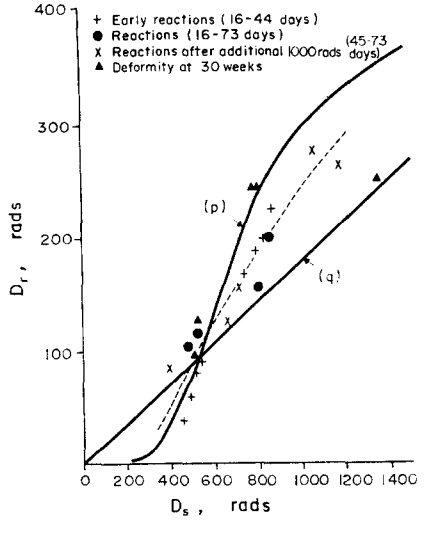


Fig. 7. D_r *plotted as a function of* D_s *for the various skin reactions and deformity shown in Figs. 1–4. Curve (P) is the best fit which may be obtained with the two component model, with* $D_0 = 150$ *rad,* $D_0/D' = 0{\cdot}33$, $m = 12$. *Curve Q is from the Ellis formula* [10].

Fig. 7. As was found by Dutreix *et al.* [5] no reasonable fit to the data could be obtained using the multi-target model, no matter how the parameters were chosen. A better fit was obtained with the two component model, but again no choice of parameters would enable the theoretical curve to fit the data exactly. The best fit with a two component model was obtained with D_0 = 150 rads m = 12 and a slope ratio of 1:3. If the exponential component was replaced by a two target component (as might be the case if the high LET component of the X-ray beam was not sufficiently high to produce an exponential survival curve) more repair at low doses would be predicted. However, there is very little difference between the best fit obtained in this way and curve P in Fig. 7. Curve Q is derived from the Ellis formula [10], the slope depending on the exponent for the number of fractions. This will be discussed later.

The present data (broken line in Fig. 7) are compared in Fig. 8 with data from similar experiments by other authors. The human skin reaction data are taken from Dutreix *et al.* [5]. The intestinal damage is from Wambersie, Dutreix, Guelette and Lellouche [11] using mouse LD_{50}. The lung results are from Field, Kutsutani and Hornsey [12]. Most of the data from Dutreix *et al.* [5] and Wambersie *et al.* [11] were obtained using time intervals of 2–6 hr between subfractions, but they have evidence showing that the results are not different for subfractions given 24 hr apart. The time intervals between fractions in the present skin experiments were a minimum of 24 hr and a maximum of 15 days, since the overall time was kept constant at 43 days. The lung data [12] were similarly obtained, except that the overall time was 39 days. It is assumed in our interpretation that repopulation is independent of the number of fractions used, and depends only on the overall time. Although this may not be true [13], no satisfactory alternative assumption is presently available.

In view both of the differences in experimental techniques and the different tissues used, it is hardly surprising that the present results are not in exact agreement with those of Dutreix *et al.* [5] and Wambersie *et al.* [11] (Fig. 8), but the differences are not great. The present data from mouse skin are similar to those for human skin [5].

The data shown in Figs. 1–4 have also been used to plot slope ratio as a function of dose per fraction, (Fig. 9). D_Q was taken to be 500 rads [3, 6, 8]. A correction was applied for repopulation during the 43 days taken for each of the various fractionation regimes by assuming that a certain dose was required to counteract it. This will depend strictly upon the size of the dose fractions used, because the killing efficiency of the radiation is less at low doses per fraction and more dose is required to counteract the same increase in cell number [14]. However, there is inadequate information available for making an accurate correction, and 3 rough approximations are used in calculating the slope ratio in Fig. 9. It appears that the slope ratio extrapolates to a value of about 0·1 to 0·2 as the dose per fraction tends to zero, so that the results cannot be fitted at all using the multi-target model, or by using the same slope ratio of 0·33 in the two compo-

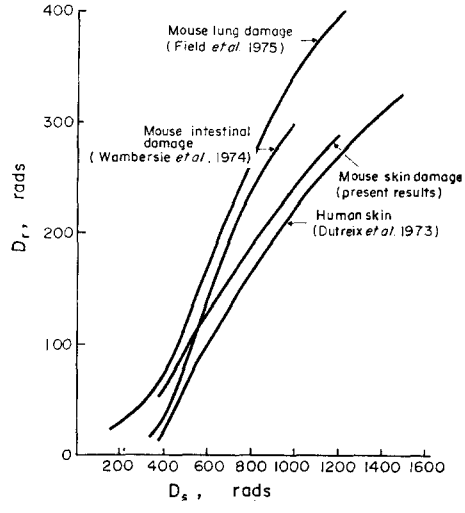


Fig. 8. D_r *plotted as a function of* D_s *for the best fit, drawn by eye through the present results, together with data for human skin* [5], *mouse intestine* [11] *and mouse lung damage* [12].

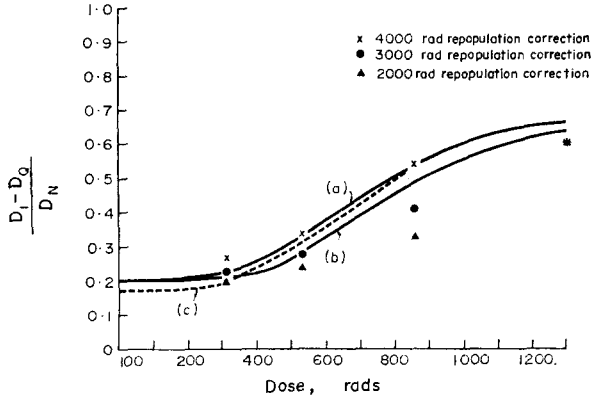


Fig. 9. $(D_1 - D_Q)/D_N$ *plotted as a function of the dose per fraction* D_N/N *for the present experimental results, with repopulation corrections taken as* 2000, 3000 *or* 4000 *rad in* 43 *days. The lines are derived from the* 2 *component model, all with* D_0 *of* 150 *rad. A is for* m = 15 *and* D_0/D' = 0·2, *B for* m = 20 *and* D_0/D' = 0·2 *and C for* m = 15 *and* D_0/D' = 0·17.

nent model as for curve P in Fig. 7. A moderate fit can be obtained with this model if the ratio of initial to final slope is 0·17–0·20. However, with this ratio it is not possible to obtain a reasonable fit to the results expressed in terms of D_r (Fig. 7). Since the slope ratio is independent of any cell survival model, the implication of these results is that the two component model, although a better approximation than the multi-target model, is still not a true representation of a survival curve for cells of the skin.

The Ellis formula

Ellis [10] suggested an empirical relationship between the total dose (TD) required in radiotherapy to produce a full tolerance reaction in skin and subcutaneous tissue, the number of fractions (N) and the overall treatment time (T), *i.e.*,

$$TD = NSD.\ N^{\cdot 24}\ T^{\cdot 11}$$

where NSD is the nominal standard dose. Whilst this simple formula cannot be expected to be exact, it is thought by many to serve as a reasonable approximation, which can be tested when new data such as those presented in this paper become available.

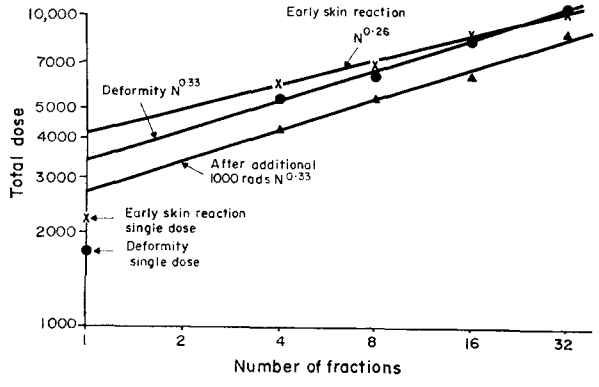


Fig. 10. *Log. total dose plotted as a function of the log. number of fractions (all given in the same overall time of* 43 *days). The slope denotes the exponent for N in the Ellis formula.*

We have used the data shown in Fig. 1, 3 and 4 to plot log. total dose against log. number of fractions to produce the same level of injury (Fig. 10). It is seen that the data points are reasonably well fitted by straight lines. Since all irradiations were performed in the same time, the slope of the lines in Fig. 10 give the exponent for N in the Ellis formula. This is $N^{\cdot 26}$ for the early skin reaction and $N^{\cdot 33}$ for skin reactions after an additional 1000 rads and also for the later deformity. It is unlikely that these exponents for early and late damage are significantly different. However, it does appear that the value proposed by Ellis [10] *i.e.*, $N^{\cdot 24}$, is not grossly wrong for mouse skin. Some other experimental data have been shown previously to support this view [15, 16].

We have also calculated, from the Ellis formula, the relationship between D_r, and the dose per fraction as was shown in Fig. 7. This method of analysis provides a more severe test of the formula than the fit on a log. log. plot. It is seen that for very low doses per

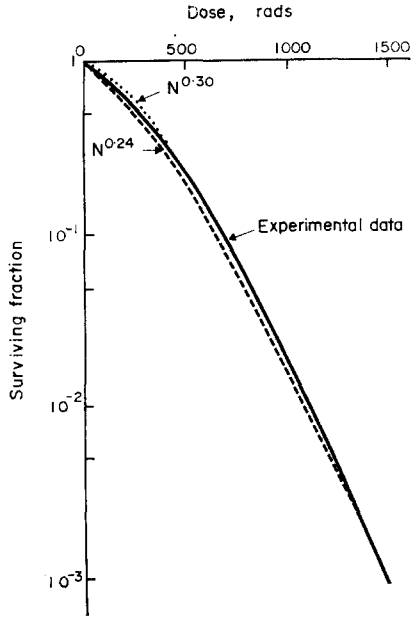


Fig. 11. *Survival curve derived from the experimental data as described in the text. Curves derived from the Ellis formula* ($N^{\cdot 24}$) *and also for* $N^{\cdot 30}$ *are also shown.*

fraction and for large doses it deviates from the experimental data (Fig. 7), but it was not claimed by Ellis that the formula was valid for less than 4 fractions, *i.e.*, in practice, for doses greater than about 700 rads per fraction. Up to this dose level, the formula provides a reasonable fit to the data.

The time factor $T^{\cdot 11}$ in the Ellis formula seems unlikely to be correct for the skin of mice. Denekamp [4] showed that the rate of repopulation in mouse skin increases with increasing damage during a course of fractionation. The present data also show this because by day 30 to 35 in the 8, 16 or 32 fraction exposures the reactions were already healing despite continued irradiation, implying very rapid repopulation indeed. In contrast, $T^{\cdot 11}$ predicts a slowing in repopulation with increasing time, as did Cohen [17]. Since the homeostatic control mechanism of proliferation in

skin is likely to be similar in mouse and man, the T factor is probably also wrong for human skin.

Construction of a cell survival curve for mouse skin

The results of the present experiments may be used to construct a dose response curve from the plot of D_r versus D_s in Fig. 7. If a given survival level, s, is reached with a dose D_s, when the number of fractions is doubled, the dose D_s+D_r is required to produce the same level of damage. Thus the dose $(D_s+D_r/2)$ will reduce the survival level to a linear half on a log. scale, *i.e.*, to $\sqrt{s}$. By repeating this process with smaller and smaller dose fractions the curve in Fig. 11 was obtained. A starting point was obtained by assuming a D_Q of 500 rads [3, 6, 8] and a D_0 of 135 rads [8] *i.e.*, 1440 rads gave a surviving fraction of 10^{-3}. The solid curve was thus obtained from the dotted curve shown in Fig. 7. If the Ellis formula is used to construct a survival curve, it is seen to be similar. For $N^{0.3}$ (instead of $N^{0.24}$) the fit is even better, except in the "shoulder" region, below 400 rads. The curve shows that no exponential region is reached up to a dose of at least 1,500 rads. From this "experimental" survival curve, 30 fractions of 200 rads, typical of the regime used in radiotherapy, would reduce the survival level to about 10^{-6}.

General discussion

The data shown in Figs. 1–4 are in agreement with the suggestion that the survival curve for the cells of the skin does not fit a multi-target model. A two component model provides a better fit with a slope ratio of about 1:3. However, with the constraint that the slope ratio is about 1:5 or 1:6, as shown in Fig. 9, no reasonable fit to the data is possible.

There are two obvious possible mechanisms to explain this result. (1) The cells are a mixed population in terms of their position in the cell cycle. It is well known that the survival curves obtained from irradiation of synchronised cells in culture depend on their phase in the cycle. For example, cells in mitosis generally yield an exponential survival curve and those in late S generally show a large shoulder. The resultant curve for a mixed population would have a finite initial slope and therefore could well fit the model described above. (2) The secondary electrons produced by X or gamma rays have a range of LET values, reaching a maximum of about 30 KeV/μ in the Bragg Peak. Thus, there is a component of the total LET spectrum which is of moderately high LET which would modify the initial region of the cell survival curve [18, 19]. It is probable that both factors contribute to the presence of an irreparable component of damage.

CONCLUSIONS

(1) When the feet of mice are irradiated with many fractions over a period of several weeks, the peak skin reaction is reached during the treatment, and healing takes place despite continued treatment. This is explained by a rapid increase in proliferation rate, consistent with the findings of Denekamp [4].

(2) The relationship between the early skin reaction and subsequent late deformity is similar for all the fractionation regimes used and for single doses.

(3) The relationship found between D_r (the extra dose required per fraction if the fraction number is doubled) and D_s (the dose per fraction) is similar to that found by other workers.

(4) We agree with the conclusions of Dutreix *et al.* [5] that the data obtained from fractionation experiments on skin are not consistent with a simple multi-target model of cell survival. A more reasonable fit may be obtained either if the component for single hit damage is included, or by an inhomogeneous population of cells having different sensitivities (as would be the case in different stages of the cell cycle). Probably both mechanisms are effective.

(5) The ratio of initial to final slope of the survival curve for skin was found to be about 1:5 or 1:6. This is significantly less than values suggested elsewhere [4–6, 11, 20].

(6) When the Ellis formula is examined in detail by these methods of analysis it is found to be in reasonable agreement with the experimental data over a limited range of dose per fraction, but there is a deviation at both high and low doses per fraction in the direction suggested by Liversage [21]. It also seems likely that for many small fractions, given over a long time period, the moderate agreement may be fortuitous, in that the exponent for N should be smaller and the effect of repopulation greater.

(7) With certain assumptions, the data may be used to construct a cell survival curve. For such a curve the shoulder is continually bending so that no exponential region is reached at least for doses up to 1,500 rads.

Acknowledgements—It is with pleasure that we thank Professor R. Oliver for the use of an X-ray machine and for providing laboratory space, Mr. K. Butler for excellent technical assistance, Mr. R. Pengilley for computing assistance, Dr. D. K. Bewley, Professor J. Dutreix, Dr. W. Liversage and Professor A. Wambersie for many valuable discussions.

REFERENCES

1. M. M. Elkind and H. Sutton, Radiation response of mammalian cells grown in culture. I. Repair of X-ray damage in surviving Chinese hamster cells. *Radiation Res.* **13,** 556 (1960).
2. T. Terasima and L. J. Tolmach, Changes in X-ray sensitivity of HeLa cells during the division cycle. *Nature (Lond.)* **190,** 1210 (1961).
3. J. Denekamp, M. M. Ball and J. F. Fowler, Recovery and repopulation in mouse skin as a function of time after X-irradiation. *Radiation Res.* **37,** 361 (1969).
4. J. Denekamp, Changes in the rate of repopulation during multi fraction irradiation of mouse skin. *Brit. J. Radiol.* **46,** 381 (1973).
5. J. Dutreix, A. Wambersie and C. Bounik, Cellular recovery in human skin reactions: Application to dose fraction number overall time relationship in radiotherapy. *Europ. J. Cancer* **9,** 159 (1973).
6. J. F. Fowler, J. Denekamp, C. Delapeyre, S. R. Harris and P. W. Sheldon, Skin reactions in mice after multi-fraction X-irradiation. *Int. J. Radiat. Biol.* **25,** 213 (1974).
7. S. B. Field, Early and late reactions in skin of rats following irradiation with X-rays or fast neutrons. *Radiology* **92,** 381 (1969).
8. E. W. Emery, J. Denekamp, M. M. Ball and S. B. Field, Survival of mouse skin epithelial cells following single and divided doses of X-rays. *Radiation Res.* **41,** 450 (1970).
9. T. Alper, J. F. Fowler, R. L. Morgan, D. D. Vonberg, F. Ellis and R. Oliver, The characterisation of the "Type C" survival curve. *Brit. J. Radiol.* **35,** 722 (1962).
10. F. Ellis, Dose time and fractionation: a clinical hypothesis. *Clin. Radiol.* **20,** 1 (1969).
11. A. Wambersie, J. Dutreix, J. Gueulette and J. Lellough, Early recovery for intestinal stem cells, as a function of dose per fraction evaluated by survival rate after fractionated irradiation of the abdomen of mice. *Radiation Res.* **58,** 498 (1974).
12. S. B. Field, Y. Kutsutani and S. Hornsey, In preparation (1974).
13. W. E. Liversage, Ph.D. Thesis, University of London (1973).
14. S. B. Field, The overall treatment time in radiotherapy. *Brit. J. Radiol.* In press (1974).
15. J. F. Fowler, Experimental animal results relating to time dose relationships in radiotherapy and the "ret" concept. *Brit. J. Radiol.* **44,** 81 (1971).
16. S. B. Field, The Ellis formulae for X-rays and fast neutrons. *Brit. J. Radiol.* **45,** 315 (1972).
17. L. Cohen, Theoretical "iso-survival" formulae for fractionated radiation therapy. *Brit. J. Radiol.* **41,** 522 (1968).
18. H. H. Rossi, Biophysical considerations concerning the existence of a threshold dose for radiation effects. *Amer. J. Roentgenol.* **93,** 196 (1965).
19. G. W. Barendsen, H. M. D. Walter, J. F. Fowler and D. K. Bewley, Effects of different ionizing radiations on human cells in tissue culture. III. Experiments with cyclotron accelerated alpha particles and deuterons. *Radiation Res.* **18,** 106 (1963).
20. G. W. Barendsen, T. L. J. Beusker, A. J. Vergroesen and L. Budke, Effects of different ionizing radiations on human cells in tissue culture. II. Biological experiments. *Radiation Res.* **13,** 841 (1960).
21. W. E. Liversage, A critical look at the ret. *Brit. J. Radiol.* **44,** 91 (1971).

Europ. J. Cancer Vol. 11, pp. 301–302. Pergamon Press 1975. Printed in Great Britain

Announcements

BREAST: NON-CANCEROUS DISEASES

June 29–July 3, 1976, Strasbourg, France.

FOURTH INTERNATIONAL SYMPOSIUM ON SENOLOGY

under the auspices of the European Association of Radiology (E.A.R.)

Round Tables – Invited Lectures – Free Communications
Scientific and Technical Exhibitions
Simultaneous Translation: English, French, German

Congress Center

Program and Information: Professeur Charles Gros, Symposium 1976
Laboratoire d'Electroradiologie, Faculté de Médecine
11 rue Humann, 67085 Strasbourg Cedex
France.

CANCER WEEK

Concerning Recent Progress in Basic Immunology

23 June–28 June, 1975, Villejuif, France.

For information contact: Secretarial Office,
Professor G. MATHÉ,
Institut de Cancérologie et d'Immunogénétique,
Hôpital Paul-Brousse,
14 avenue Paul-Vaillant-Couturier,
948000 VILLEJUIF (France).

SCIENTIFIC PLENARY SESSION OF THE EORTC

Concerning BCG and Cancer

27 June, 1975, Villejuif, France

For information contact: Secretarial Office,
Institut de Cancérologie et d'Immunogénétique,
Hôpital Paul-Brousse,
14, avenue Paul-Vaillant-Couturier,
94800 VILLEJUIF (France).

MEETING OF THE CLINICAL COOPERATIVE GROUPS OF THE EORTC

28 June, 1975, Paris, France

For information contact: EORTC Secretarial Office,
Institut Jules Bordet.
1, rue Héger-Bordet,
BRUXELLES (Belgium).

Europ. J. Cancer Vol. 11, pp. 303–306. Pergamon Press 1975. Printed in Great Britain

Acknowledgement

The Editor gratefully acknowledges the generous assistance of the following reviewers who served between November 1970 and June 1974.

S. Aaronson
G. Abelev
M. Abercrombie
R. Adamson
A. Aisenberg
P. Alexander
A. Allison
J. Althoff
J. Ambrus
J. Amiel
R. Anderson
F. Ansfield
C. Appfel
M. Apple
D. Armstrong
G. Asherson
G. Atassi
N. Atkin
D. Atkins
M. Attia
N. Bachur
D. Bainbridge
C. Baglay
K. Bagshawe
E. Baker
R. Baldwin
G. Barendsen
J. Barnes
A. Bartolucci
M. Basombrio
P. Bassleer
A. Basten
S. Bauminger
A. Beale
D. Beard
J. Bekesi
Q. Bell
A. Bendich
W. Benedict
P. Bentvelzen
C. Berdjis
I. Berenblum
L. Berman
W. Bernhard
F. Bertalanffy
J. Bertino
M. Bessis
I. Betel
C. Biancifiori
P. Biberfield
P. Bichel
I. Bickiss
A. Billiau
R. Billingham
S. Biran
J. Blakeslee
H. Bleiberg
K. Bloch
J. Block
H. Blomgren
H. Bloom
O. Bodansky
G. Bodey
B. Boeryd
M. Boiron
W. Bollag
R. Bomford
G. Bonadonna
V. Bono
C. Boone
M. Boranic
A. Borkowski
J. Borsa
C. Bourgaux
J. Bourgoin
R. Boutwell
E. Boyland
J. Brachet
J. Bradfield
W. Bradner
R. Bradshaw
K. Breur
H. Brevick
J. Brewen
M. Brin
L. Broder
J. Broerse
K. Broerse
P. Brookes
R. Brooks
W. Brooks
M. Broome
G. Brown
J. Brown
T. Bucher
R. Bulbrook
W. Bullough
M. Burger
D. Burk
E. Burns
A. Burny
R. Burr
J. Burton
P. Calabresi
A. Cantarow
P. Carbone
C. Carruthers
A. Carsten
S. Carter
T. Casperson
J. Cerf
C. Chae
T. Chalmers
H. Chantrenne
M. Chirigos
G. Chisholm
B. Christine
W. Churchill
E. Ciaranfi
N. Clapp
B. Clarkson
D. Clayson
K. Clifton
M. Cline
F. Clowes
J. D. Cobb
J. P. Cobb
A. Cochran
J. Cohen
M. Cohen
M. Cohn
T. Connors
E. Cooper
C. Cori
A. Coune
P. Cox
W. Creasy
P. Cresswell
J. Crocker
E. Cronkite
E. Daniels
K. Dano
P. Darcy Hart
C. Davidson

D. Davies
C. Dawe
K. Dawson
L. De Busschere
J. De Grouchy
E. De Harven
G. Della Porta
L. Delmonte
U. Del Monte
P. Dendy
J. Denekamp
S. Denham
S. Deodhar
E. Desombre
P. De Somer
G. De The
L. Dethlefsen
V. De Vita
J. De Vries
J. De Waard
G. Deyson
F. Dianzani
H. Diggleman
J. Di Paols
E. Dirksen
A. Dodge
R. Doll
P. Dombernowski
M. Donelli
R. Dougherty
A. Drochmans
R. Duff
J. Dumont
C. Dunn
T. Dunn
J. Duplan
P. Dustin
J. Dutreix
M. Eaton
J. Edsall
A. Edwards
M. Elkind
L. Elson
C. Elston
P. Emmelot
E. Englesman
S. Epstein
P. Ernst
M. Errera
L. Espey
J. Evans
S. Farber
P. Farnes
J. Ferrer
P. Ferris
G. Fetterman
E. Field
M. Finland
B. Fisher
E. Fisher
J. Fisher
G. Fletcher
R. Flickinger
T. Fliedner
E. Fluckiger
J. Forman
I. Forsyth
J. Fowler
W. Fox
G. Franchi
S. Freedman
M. Freid
E. Freireich
C. Friend
J. Friend
E. Frindel
J. Frueling
L. Frye
T. Gallagher
R. Gallo
S. Garattini
W. Gardner
F. Gavosto
H. Gelboim
W. Gepts
R. Gershon
J. Gillette
L. Golberg
P. Gold
A. Goldberg
S. Goldblatt
D. Goldenberg
S. Goldfarb
A. Goldin
T. Goodfriend
C. Goodhall
A. Goussev
G. Graff
C. Graham
F. Graham
S. Granick
B. Greenberg
W. Greene
I. Gresser
D. Griffiths
D. Griswold
C. Grobstein
P. Gross
C. Grosvenor
P. Guillou
K. Gumaa
H. Gutmann
J. Hadden
G. Haemmerli
B. Hagmar
F. Hagueneau
G. Hahn
T. Hall
M. Hamilton
G. Hamilton-Fairley
H. Hansen
I. Hansteen
N. Haran-Ghersa
K. Harrap
H. Harris
M. Harris
J. Hartley
J. Haskill
E. Hawkins
L. Hayflick
C. Heath
R. Heath
C. Heidelberger
R. Heiman
A. Heimens
E. Heise
I. Hellstrom
H. Helminen
I. Henderson
M. Henderson
H. Hennings
H. Hers
A. Herzfield
J. Heuson
H. Hewitt
J. Higginson
J. Hildebrand
R. Hilf
C. Hill
M. Hill
J. Hobbs
G. Hodges
K. Hofer
J. Hoffman
D. Hollis
D. Holland
J. Holland
H. Holm
B. Holmberg
J. Holtzman
F. Homburger
B. Horton
C. Huang
P. Hubinot
H. Isliker
J. Iversen
O. Iversen
F. Izsak
D. James
L. Jamieson
E. Jensen
H. Johanson
G. Johnson
R. E. Johnson
R. K. Johnson
W. Johnson
P. Jungblutt
M. Kadin
R. Kahn
T. Kakefuda
H. Kalant
J. Kalbfleisch
H. Kaplan
M. Karim
M. Karon
L. Kasza
Y. Kenis
B. Kennedy
G. Kenney

S. Killmann
C. King
R. King
K. Kiossoglov
H. Kirkman
A. Klainer
J. Klastersky
G. Klein
H. Klein
A. Kleinzeller
D. Knook
G. Kolodney
R. Kramm
M. Krant
L. Kronenberg
J. Krupey
T. Kuipers
S. Kumaoka
T. Kuroki
L. Kuznezova
A. Laird
L. Lajtha
M. Landy
A. Latner
L. Law
G. Lawford
G. Leclercq
D. Legler
E. Legrand
J. Lehmann
F. Lejeune
A. Leonard
H. Lettre
A. Levan
J. Levi
R. Levi-Montalcini
S. Levin
V. Levin
C. Levinson
H. Levy
P. Libby
F. Lilly
M. Linder
H. Lindsay
M. Lohmann-Matthes
M. Loring
R. Love
A. Loveless
W. Lowenstein
J. Luce
R. Lukes
P. Lurie
A. Lwoff
H. Maass
A. Macgillivray
E. Macintyre
B. Macmahon
I. Macpherson
P. Magee
R. Malmgren
N. Mantel
E. Manuelidis
J. Mark
T. Marquardt
R. Martin
W. Martin
G. Martz
F. Mauro
R McCarthy
I McConnel
M McEneny
C McGrath
W. McGuire
G. Melnykovych
M. Meltzer
J. Mester
D. Metcalf
T. Michaelis
S. Michaelson
R. Michell
T. Miettinen
C. Migeon
Z. Mikulska
C. Miles
E. Miller
J. Miller
S. Mirvish
W. Mitchison
N. Mohr
J. Moloney
R. Montesano
G. Moore
L. Morasca
K. Mori
H. Moroson
H. Morris
G. Muhlbock
R. Murray
K. Musshoff
C. Nadolney
G. Nagel
H. Narang
M. Nass
P. Neiman
B. Nelson
T. Nettesheim
K. Nilausen
A. Novikoff
R. Nowinski
C. Ockey
H. Oettgen
L. Ornstein
B. Ortwerth
C. O'Toole
H. Otsuka
K. Overgaard
C. Parish
J. Pasteels
N. Paweletz
O. Pearson
B. Peckham
P. Pederson
S. Peel
A. Percy
P. Perlmann
S. Perry
L. Perryman
H. Pertoft
L. Perves
M. Peters
R. Petersdorf
F. Philips
B. Phillips
L. Piessens
W. Piessens
M. Pike
M. Pimm
A. Pines
R. Polzer
V. Potter
R. Powers
R. Prehn
D. Pressman
T. Puck
M. Rajewsky
H. Ramseier
H. Rappaport
M. Rapport
R. Raven
E. Reerink-Broughers
B. Reid
H. Reinhold
F. Reyes
W. Richert
K. Rifi
V. Riley
A. Rios
D. Roberts
J. Roberts
E. Robinson
F. Roe
J. Rosai
S. Rosenberg
J. Roth
W. Rowe
G. Rudali
P. Rumke
R. Rutman
H. Ryser
T. Rytomaa
W. Sachsemaier
A. Sandberg
F. Sanders
K. Sanford
G. Santos
A. Saunders
F. Schapira
R. Scharff
M. Schneiderman
C. Schneyer
A. Schrecker
D. Schubert
D. Schwartz
O. Schweisguth
N. Seeds
J. Selkirk
H. Selye
R. Shamberger
S. Sherlock

M. Shimkin
R. Simmons
L. Simpson-Herren
M. Siperstein
H. Sjogren
L. Skinner
H. Skipper
N. Sladeck
T. Slaga
M. Slavik
M. Sluyser
W. Sly
E. Smith
I. Smith
P. Smith
K. Snell
M. Sonenberg
L. Sordhal
S. Sorof
C. Southam
A. Spiers
H. Stahelin
M. Staquet
M. Stearn
G. Steel
R. Stenge
J. Steward
F. Stewart
J. Stjernsward
C. Stock
P. Strauli
G. Stroobandt
P. Stryckmans
H. Suit
R. Sullivan
W. Sutow
J. Svoboda
M. Sweeney
J. Swift
J. Sykes
D. Szafarz
S. Tanneberger
L. Terenius
L. Thiry
R. Thomas
R. Thomlinson
G. Tisman
R. Tobey
H. Toolan
B. Toth
W. Troll
V. Trott
A. Trouet
M. Tubiana
R. Turkington
E. Unanue
S. Urbain
J. Urban
J. Vaage
G. Vamecq
W. Van Bekkum
H. Vandenberg
M. Vandeputte
K. Vanderveen
B. Van Der Werf-Messing
L. Van Putten
A. Verhest
M. Vogt
B. Volk
W. Waddel
L. Walter
J. Wanson
J. Warner
J. Watkins
R. Wattiaux
E. Weinbach
J. Weisberger
I. Weisman
D. Weiss
B. Weston
P. Wheeler
W. Whitmore, Jr.
F. Wiebel
L. Wiest
J. Williams
R. Williams
C. Wilson
M. Wilson
R. Wilson
W. Wingfield
A. Winsnes
M. Wintrobe
H. Withers
R. Withers
H. Witschi
N. Wivel
I. Wodinsky
J. Woessner
G. Wogan
S. Wood
M. Woodruff
J. Woods
M. Woods
J. Wybran
E. Wynder
D. Yesair
N. Zamcheck
B. Zbar
I. Zeidman
M. Zelen
G. Zubrod
N. Zuckerman
E. Zybler-Katz

Europ. J. Cancer Vol. 11, pp. 307–308. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer — *April 1975*

S. A. Chopra, C. A. Linsell, F. G. Peers and F. S. Chopra: Cancer of Asians in Kenya.

S. Stenman, J. Zeuthen and N. R. Ringertz: Expression of SV40 T antigen during the cell cycle of SV40-transformed cells.

A. Liabeuf, R. A. Nelson, Jr. and F. M. Kourilsky: The detection of the Epstein–Barr virus (EBV) nuclear antigen (EBNA) by anti-complement immunofluorescence. Immunoglobulin class of antibodies and role of complement.

M. M. Lieber, C. J. Sherr and G. J. Todaro: Transplantable murine tumors release mouse-tropic and xenotropic type C viruses.

E. Bjelke: Dietary vitamin A and human lung cancer.

B. M. Reedman, J. Hilgers, F. Hilgers and G. Klein: Immunofluorescence and anti-complement immunofluorescence absorption tests for quantitation of Epstein–Barr virus associated antigens.

E. Legrand, M. C. Kressmann and J. F. Duplan: Leucémogénèse et régénération du systeme lymphoide chez des hybrides F1 (C3H × AKR/T1ALD) restaurés par des cellules medullaires et thymiques parentales.

L. M. van Putten, L. K. J. Kram, H. H. C. van Dierendonck, T. Smink and M. Füzy: Enhancement by drugs of metastatic lung nodule formation after intravenous tumour cell injection.

M. Jondal and H. Pross: Surface markers on human B and T lymphocytes. VI. Cytotoxicity against cell lines as a functional marker for lymphocyte subpopulations.

T. D. K. Brown, I. Ernberg and G. Klein: Studies of Epstein–Barr virus (EBV)-associated nuclear antigen.

I. Assay in human lymphoblastoid cell lines by direct and indirect determination of ^{125}I-IgG binding.

B. Armstrong and R. Doll: Environmental factors and the incidence and mortality from cancer in different countries with special reference to dietary practices.

J. Yeh, M. Ahmed, J. Lyles, D. Larson and S. A. Mayyasi: Competition radioimmunoassay for Mason–Pfizer monkey virus: Comparison with recent isolates.

J. G. Bowen, R. A. Robins and R. W. Baldwin: Serum factors modifying cell mediated immunity to rat hepatoma D23 correlated with tumour growth.

V. Balakrishnan: An additional younger-age peak for cancer of the nasopharynx.

S. E. Martin and W. J. Martin: Anti-tumor antibodies in normal mouse sera.

A. A. Stavrovskaya, T. V. Potapova, V. A. Rosenblat and A. S. Serpinskaya: The effect of non-ionic detergent Tween 80 on colcemid-resistant transformed mouse cells *in vitro*.

G. Kimura, A. Itagaki and J. Summers: Rat cell line 3Y1 and its virogenic polyoma and SV40 transformed derivatives.

British Journal of Cancer *April 1975*

M. J. Frost, G. T. Rogers and K. D. Bagshawe: Extraction and preliminary characterisation of a human bronchogenic carcinoma antigen.

R. M. Gorczynski and R. A. Knight: Immunity to murine sarcoma virus induced tumours. IV. Direct cellular cytolysis of ^{51}Cr-labelled target cells *in vitro* and analysis of blocking factors which modulate cytotoxicity.

P. Dyson and A. G. Heppleston: Cell kinetics of urethane-induced murine pulmonary adenomas: I. The growth rate.

P. R. Twentyman and N. M. Bleehen: Changes in sensitivity to cytotoxic agents occuring during the life-history of monolayer cultures of a mouse tumour cell line.

J. L. Daehnfeldt and M. Schulein: High affinity oestradiol receptors and the activity of glucose-6-phosphate dehydrogenase and lactose synthetase in mammary carcinomas of post-menopausal women.

J. B. Adams and Karen Li: Biosynthesis of 17β-oestradiol in human breast carcinoma tissue and a novel method for its characterisation.

K. A. Karande, S. P. Taskar and K. J. Ranadive: Activation of murine leukaemogenic agent under different physiological conditions.

B. R. Davis, J. K. Whitehead, Margaret E. Gill, P. N. Lee, Ann D. Butterworth and F. J. C. Roe: Response of rat lung to 3,4-benzpyrene administered by intratracheal instillation in infusine with or without carbon black.

B. R. Davis, J. K. Whitehead, Margaret E. Gill, P. N. Lee, Ann D. Butterworth and F. J. C. Roe: Response of rat lung to tobacco smoke condensate or fractions derived from it administered repeatedly by intratracheal instillation.

B. R. Davis, J. K. Whitehead, Margaret E. Gill, P. N. Lee, Ann D. Butterworth and F. J. C. Roe: Response of rat lung to inhaled vapour phase constituents (VP) of tobacco smoke alone or in conjunction with smoke condensate or fractions of smoke condensate or fractions of smoke condensate given by intratracheal instillation.

B. R. Davis, J. K. Whitehead, Margaret E. Gill, P. N. Lee, Ann D. Butterworth and F. J. C. Roe: Response of rat lung to inhaled tobacco smoke with or without prior exposure to 3, 4-benzpyrene (BP) given by intratracheal instillation.

A. O. Williams, E. B. Chung, A. Agbata and M. A. Jackson: Intestinal polyps in American Negroes and Nigerian Africans.

Short Communication

H. Shimizu, D. Nagel and B. Toth: Tumor induction study with *n* amylhydrazine hydrochloride in Swiss mice.

Europ. J. Cancer Vol. 11, pp. 309-316. Pergamon Press 1975. Printed in Great Britain

Comparison of Adriamycin with DNA–adriamycin complex in Chemotherapy of Experimental Tumors and Metastases

G. ATASSI, M. DUARTE-KARIM and H. J. TAGNON*
Chimiothérapie Expérimentale, Service de Médecine Interne et Laboratoire d'Investigation Clinique, Institut Jules Bordet, Brussels, Belgium

Abstract—*The effects of adriamycin and of two different preparations of DNA–adriamycin complex were compared in mice on the following experimental tumors: leukemia L_{1210}, melanotic melanoma B16 and Lewis Lung carcinoma. Plasma levels were determined in different strains of mice: DBA/2, CDF_1 and BDF_1, bearing L_{1210T} leukemia (blood form). Increased therapeutic effectiveness was observed with the complex (prepared with Dr. Trouet's method) in DBA/2 mice bearing L_{1210T} leukemia (blood form) treated i.v. and in BDF_1 mice bearing melanoma B16 i.p. treated i.p.*

On the other hand, adriamycin alone, when given i.v., was more effective than the complex on primary solid tumors, including leukemia L_{1210T} in solid form. Therefore, on leukemia L_{1210T}, the Trouet complex preparation was more effective than adriamycin alone on the leukemic form, while adriamycin alone was more effective on the solid form. The therapeutic effect of the DNA–adriamycin (Trouet preparation) and adriamycin alone given i.v. was found to be similar on the formation of lymph node metastases in DBA/2 mice bearing L_{1210} s.c. inoculated i.v. and lung metastases in BDF_1 mice bearing Lewis Lung carcinoma i.m. treated i.v.

The disappearance of adriamycin from the plamsa of DBA/2 mice after treatment with the DNA–adriamycin complex is slower than the disappearance of the drug after a treatment with adriamycin alone.

The therapeutic action of DNA–adriamycin seems to require direct contact between the complex and the tumor cells. This is achieved by local injection in the case of solid tumors and by i.v. injection in the case of leukemia.

INTRODUCTION

In a previous study [1], the complex DNA–adriamycin tested on a line of L_{1210} leukemia in DBA/2 mice showed an enhanced therapeutic effectiveness as compared with adriamycin alone, and this was similar to the observation made by Trouet *et al.*, [2]. We attribute this difference to a preferential pynocytosis of the DNA–adriamycin complex by cancer cells followed by a release of active adriamycin within these cells by lysosome action [2, 3]. This explanation is still hypothetical.

The observations were not repeated by us on the standard line of leukemia which we use for testing in our Laboratory [1] in BDF_1 mice, but, we thought it interesting to study the effects of free and complexed adriamycin in solid tumors and in other strains of mice as well. The relationship between the therapeutic effectiveness and the drug levels in the plasma was also investigated. The results of these studies are given below.

Accepted 8 January 1975.

*With the technical assistance of Charles Schaus and Francine Bournonville.

MATERIAL AND METHODS

Drugs

Adriamycin. NSC 123127 was supplied by Farmitalia Benelux.

DNA. A solution from herring sperm was obtained from Dr. Trouet. The solution was prepared as described before [2].

DNA–adriamycin complex. This was prepared in two different laboratories, resulting in the production of two different DNA–adriamycin complexes, differing much from one another, especially in their molecular weights. The first preparation, corresponding to the original Trouet preparation, consisted of a solution made as described previously: 50 ml of DNA-solution containing 4·68 mg/ml were added aseptically to 1 ml of adriamycin solution containing 20 mg/ml. Under these conditions, the complex is formed spontaneously and the solution can be used immediately. The complex is called "complex T" hereunder.

The second preparation was prepared and obtained in lyophilized form (NSC 169534) by Farmitalia Benelux and the National Cancer Institute, Bethesda, Md. This complex is called "complex N" hereunder.

Experimental animals and tumors

BDF_1 mice (DBA/2 × C57B1), CDF_1 mice (BALB/C × DBA/2), C57B1 and DBA/2 mice (supplied by Charles River Laboratory) were used and maintained under conditions of uniform caging, at a temperature of 25°C, and at a relative humidity of 55 %; food and water were given *ad libitum.*

The two lines of lymphoid leukemia L_{1210} used for transplantation are maintained in this laboratory by serial ascitic inoculation in DBA/2 mice: the first line was originally obtained from Mr. I. Wodinsky (Arthur D. Little, Inc.). It is identified by the letter B (Bordet); the second line was obtained from Trouet's Laboratory (2) and is identified by the letter T (Trouet).

Melanotic melanoma B16 and the Lewis Lung carcinoma maintained in our Laboratory by serial transfer in C57B1 mice were also used.

Instrumentation

A Zeiss spectrophotofluorometer and a Beckman Spinco L265B ultracentrifuge were used for the determination of the drug plasma concentration.

Lymphoid leukemia L_{1210}

L_{1210} ascite was taken from DBA/2 mice and a suspension was made in Hank's balanced solution. 10^5 cells in 0·1 ml solution were injected i.v. into each male DBA/2, BDF_1 and CDF_1 mouse weighing 18–28 g.

In a first experiment, treatment was given 24 hr after implantation of the leukemic cells as a single dose of adriamycin or DNA–adriamycin administered i.v.

In a second experiment, treatment was started 24 hr after tumor inoculation and administered i.v. once a day for a total of 5 injections in DBA/2, CDF_1 and BDF_1 mice.

In a third method of treatment, in an attempt to reduce the cumulative toxicity, treatment was given i.v. on days 1, 4, 8, 12 and 17 after tumor implantation.

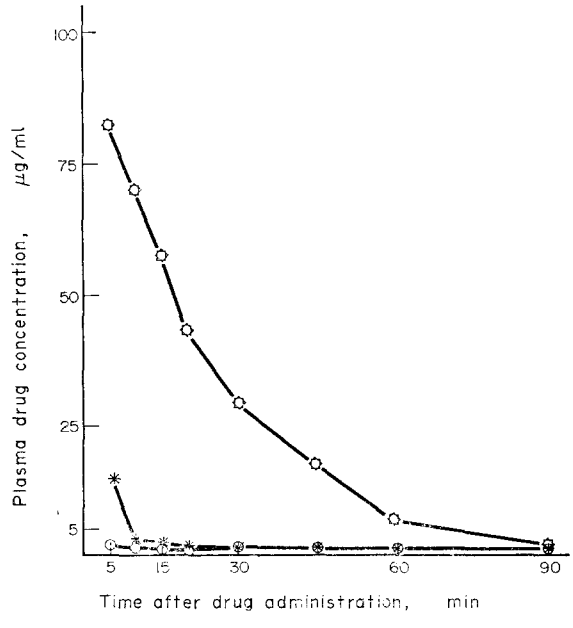


Fig. 1. Mean plasma concentration of adriamycin in DBA/2 male mice inoculated i.v. with 10^5 L_{1210T} leukemia cells (determination was made after dose of 6 mg/kg adriamycin or complexed adriamycin given i.v.). The fluorescence was compared with that of standard solution and expressed as total adriamycin (excitation: 465 nm; fluorescence: 595 nm).

✲ *DNA–adriamycin T*

✳ *DNA–adriamycin N*

○ *Free adriamycin*

Melanotic melanoma B16

Five-tenths ml of tumor suspension from a homogenate of 1 g tumor in 10 ml cold Hank's solution was implanted s.c. in one experiment and i.p. in a second experiment into BDF_1 mice weighing 18–28 g.

Treatment was started 24 hr after the inoculation of the melanoma B16 and continued once every four days for a total of 5 doses. The drugs were given i.v. in the case of s.c. transplantation and i.p. in the case of i.p. transplantation of the tumor.

L_{1210} solid form and Lewis Lung carcinoma

The L_{1210} leukemia inoculated s.c. at the inner side of the right thigh of DBA/2 mice was used, in order to compare the effectiveness of adriamycin and DNA–adriamycin against metastases. By this method, there is production of metastases, appearing mainly in the lymph nodes of the ipsolateral side of the animals [4]. End points considered included both animals' life spans and measurements of right axillary lymph nodes and tumor sizes on the 9th day after tumor inoculation. Treatment was started 24 hr after L_{1210} cells inoculation and continued every day for a total of 5 injections.

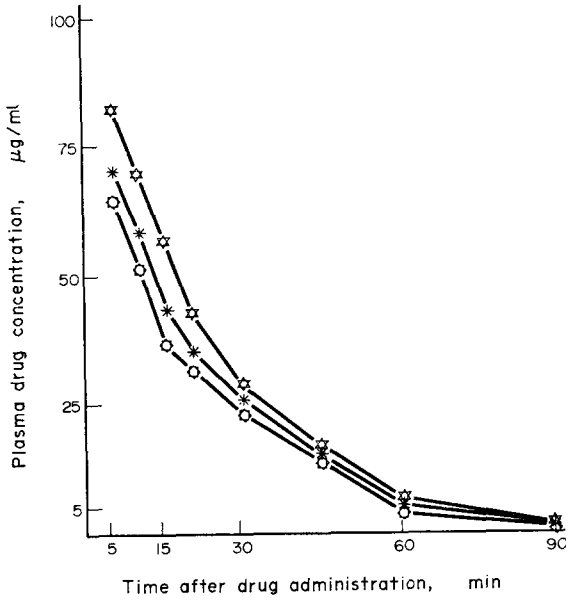


Fig. 2. Mean plasma concentration of adriamycin in DBA/2, CDF_1 and BDF_1 mice inoculated i.v. with 10^5 L_{1210T} leukemia cells. Determination was made after the administration of 6 mg/kg of complexed adriamycin solution. The fluorescence was compared with that of standard solution and expressed as total adriamycin.

✡ *Plasma concentration in DBA/2 mice*

✻ *Plasma concentration in CDF_1 mice*

✡ *Plasma concentration in BDF_1 mice*

Another method for the evaluation of the effect on metastases was based on the Lewis Lung carcinoma: male BDF_1 mice weighing 20–24 g were transplanted i.m. in the left posterior leg with $2{\cdot}10^6$ cells in cold Hank's balanced solution. This tumor produces lung metastases regularly. The end points are, in this case, inhibition of appearance of lung metastases and primary tumor regression. Treatment was started 24 hr after tumor implantation and continued on days 4, 9, 14 and 22 after tumor implantation.

Plasma/drug concentration

Mice bearing L_{1210T} tumor (10^5 cells/mouse) were injected i.v. with adriamycin and DNA–adriamycin as a single dose 24 hr after tumor inoculation. Venous blood samples were collected at intervals as shown in Figs. 1 and 2 from the inferior vena cava under open thoracotomy of the anaesthetized animals. The blood from 5 to 6 mice was pooled and the plasma was separated immediately, and stored at −20°C for a maximum of a month before drug determination.

Adriamycin fluorescence in plasma was determined by a modification of the method of Bachur *et al.* [5], as follows:

In the plasma of mice treated with DNA–adriamycin, adriamycin is generated, after extraction, from the two bonds present in complex T as well as complex N between the DNA base pairs and the chromophore on one hand, and a complex of external fixation on the other hand [7].

This sensitive fluorometric method for the determination of the concentration of adriamycin in plasma is based on the direct measurement of the fluorescence intensity extracted adriamycin with ethanol–HCl.

The frozen plasma was thawed and homogenized by sonication. Absolute ethanol (1·5 ml) and hydrochloric acid 2N (0·45 ml) were added to each plasma sample (1 ml). The mixture was allowed to stand at 4°C for 24 hr and centrifuged at 50,000 *g* for 25 min and the fluorescence of the plasma acid–alcohol extracts was determined at the optimum activating wave length of 465 nm and the optimum emission wave length of 595 nm. Standard solutions of adriamycin and DNA–adriamycin in plasma were used to draw a calibration curve. A corresponding blank containing no drug was also performed with the above-mentioned procedure and the relative fluorescence of the standard solutions of the drugs was corrected by subtracting the value obtained for the blank (endogenous fluorescing materials) from each standard.

All fluorescence intensity readings were referred to primary fluorescence standards.

RESULTS

The DNA–adriamycin complex used in all experiments was the complex T except when indicated otherwise. The doses in the tables are expressed in mg/kg adriamycin alone or in mg/kg adriamycin contained in the complex.

1. *Comparison of the effect of DNA–adriamycin and adriamycin on the life span of DBA/2, CDF_1 and BDF_1 mice bearing L_{1210} leukemia*

Tables 1 and 2 show that the DNA–adriamycin complex was therapeutically more active at the doses used here than adriamycin alone in leukemia L_{1210T} inoculated into DBA/2 and CDF_1 mice. However, when BDF_1 mice and L_{1210B} leukemia were used, no appreciable difference could be demonstrated between the therapeutic effectiveness of DNA–adriamycin and adriamycin alone.

tion of DNA followed by adriamycin resulted in as good an effect as DNA–adriamycin complex, while prior administration of adriamycin followed by DNA was less effective.

Table 4 shows the results obtained when drugs were administered at longer intervals for a longer period to DBA/2 mice bearing L_{1210T}. There remained a definite therapeutic superiority of the complex DNA–adriamycin over the free drug. However, when the DNA–adriamycin N was used in similar experiments, no superiority for the complex was apparent.

Table 1. Comparison of the effect of DNA–adriamycin and adriamycin on the life span of DBA/2 and BDF1 mice bearing L_{1210} leukemia

	DBA/2 mice bearing $L^{1210}{}_T$ (Trouet)				BDF1 mice bearing $L^{1210}{}_B$ (Bordet)			
	DNA–adriamycin T		Adriamycin		DNA–adriamycin T		Adriamycin	
Doses (mg/kg)	ILS*	No. of survivors after 30 days	ILS*	No. of survivors after 30 days	ILS*	No. of survivors after 30 days	ILS*	No. of survivors after 30 days
12	85	0	54	0	57	0	57	0

DBA/2 male mice (18–22 g) and BDF_1 male mice (21–26 g) were inoculated i.v. (Day 0) with 10^5 leukemic cells from stock DBA/2 mice bearing ascitic L^{1210}.
A single dose treatment was given on Day 1.
*Increase in Life Span in %.

Table 2. Comparison of the effect of DNA–adriamycin and adriamycin on the life span of DBA/2, CDF_1 and BDF_1 mice bearing L_{1210T} leukemia

	Results with DBA/2 mice				Results with CDF_1 mice				Results with BDF_1 mice			
	DNA–adr. T		Adriamyc.		DNA–adr. T		Adriamyc.		DNA–adr. T		Adriamyc.	
Dose (mg/kg)	ILS*	SD30†	ILS	SD30	ILS	SD30	ILS	SD30	ILS	SD30	ILS	SD30
6	216	1/13	83	0/13	120	0	82	0	6	0	0	0
5	216	0/13	141	0/13	120	0	54	0	12	0	0	0
3	125	0/13	92	0/13	43	0	33	0	100	0	50	0

DBA/2 male mice (18–22 g), CDF_1 male mice (21–25 g) and BDF_1 male mice (23–28 g) were inoculated i.v. on Day 0 with 10^5 leukemic cells from stock DBA/2 mice bearing ascite L_{1210}.
Treatment was given i.v. from Day 1 to Day 5.
*Increase in Life Span in %.
†Number of survivors after 30 days.

In order to verify whether the superior effect of the complex over the free drug on L_{1210T} given i.v. (Tables 1 and 2) was really due to the complex and not to separate actions of DNA and adriamycin, the following experiment was carried out: DNA and adriamycin were injected separately in DBA/2 mice bearing L_{1210T}, DNA being given first or adriamycin being given first and the results were compared. The data show (Table 3) that prior administra-

2. *Comparison of the effect of DNA–adriamycin and adriamycin administered chronically on BDF_1 mice bearing melanotic melanoma B16 subcutaneously*

Table 5 shows that at doses of 4 mg/kg, given 5 times at differing intervals, adriamycin alone administered i.v. was more effective than DNA–adriamycin. The observation was true when survival was used for evaluation. When tumor mass was used for evaluation, there was no statistical difference between the mean tumor

Table 3. Comparison of the effect of the association DNA and adriamycin given separately, adriamycin alone and DNA–adriamycin on the life span of DBA/2 mice bearing L_{1210T} leukemia

Dose (mg/kg)	DNA–adriamycin T		Adriamycin		DNA followed by by Adriamycin		Adriamycin followed by DNA	
	ILS*	SD30†	ILS	SD30	ILS	SD30	ILS	SD30
6	192	2/11	98	0/11	158	3/11	106	0/11

Leukemia bearing mice (20–22 g) were inoculated i.v. on Day 0 with 10^5 cells. 11 leukemic mice were used as a control and had a median survival time of 6 days.
Treatment was given i.v. from Day 1–5.
*Increase in Life Span in %.
†Number of survivors after 30 days/total number of mice.

mass in animals treated by either drug. Considering the very large variability of the tumor weights, it is not excluded that a difference in the inhibitory effect on tumor growth between both forms may exist and not be observed

However, when the same comparison was made on the same tumors inoculated by i.p. route, the animals being treated by the i.p. route also, an increased effectiveness of the complex was observed as evaluated by duration of survival. Tumor mass could not be measured in these experiments (Table 6).

Table 4. Comparason of the effect of two different forms of DNA–adriamycin complex and adriamycin administered chronically on the life span of DBA/2 mice bearing L_{1210T} leukemia

Dose (mg/kg)	DNA–adriamycin T		DNA–adriamycin N		Adriamycin	
	ILS*	SD30†	ILS	SD30	ILS	SD30
6	157	3/15	56	2/10	43	0/15
6	200	2/15	50	0/10	128	1/15

DBA/2 male mice (18–20 g) were inoculated i.v. on Day 0 with L^{1210} leukemic cells from stock DBA/2 mice bearing ascite L_{1210}. Thirty leukemic mice were used as a control.
Treatment was given i.v. on days 1, 4, 8, 12 and 17 after tumor inoculation.
*Increase in Life Span in %.
†Number of survivors after 30 days/total number of mice.

3. *Comparison of the effect of DNA–adriamycin and adriamycin on the life span and the lymph node metastases of DBA/2 mice bearing L_{1210T} leukemia (solid form)*

In these experiments (Tables 7 and 8), the free drug inoculated i.v. was more effective than the complex inoculated i.v. in preventing the development of the primary tumor, while both free drug and complex had an identical effect in preventing metastases. However, cures were obtained with adriamycin alone, presumably because of its better activity on the primary tumors, while such cures were not observed at the dosages used with the DNA–adriamycin complex. Here again, we observed in parallel experiments, that DNA–adriamycin N was less effective than the solution of DNA-adriamycin T prepared immediately before treatment (Table 8).

4. *Comparison of the effect of DNA–adriamycin and adriamycin against lung metastases of BDF_1 mice bearing Lewis Lung carcinoma intramuscularly*

On mice bearing Lewis Lung carcinoma, the therapeutic effectiveness of free adriamycin was slightly superior to the complex, both on

Table 5. Comparison of the effect of DNA–adriamycin and adriamycin on BDF_1 mice bearing melanotic melanoma B16 s.c.

	Dose (mg/kg)	Tumor weight Day 20 (g)	Increase in Life Span %	No. of survivors on Day 60 / Total number of mice
Free adriamycin	4	0·3679	52	2/12
DNA–adriamycin T	4	0·4302	12	1/12
Control		2·918		

BDF1 male mice (18–23 g) were inoculated s.c. on Day 0 with 0·5 ml of 1/10 tumor homogenate from a stock of C57B1 mice bearing B16 s.c.
Treatment was given i.v. on days 1, 4, 8, 13 and 19 after tumor implantation.

Table 6. Comparison of the effect of DNA–adriamycin and adriamycin on BDF_1 mice bearing melanotic melanoma B16 i.p.

Dose (mg/kg)	Adriamycin MST*	Adriamycin NS60†	DNA–adriamycin T MST*	DNA–adriamycin T NS60†	DNA then Adriamycin MST*	DNA then Adriamycin NS60†
6	14·5	4/10	> 60	9/10	> 60	6/10
4	> 60	6/09	> 60	5/07		
2	> 60	7/10	> 60	5/10		
Control	18·2					

BDF_1 male mice (± 25 g) were inoculated i.p. on Day 0, with 0·5 ml of 1/10 tumor homogenate from a stock of C57B1 mice bearing s.c. solid tumor. Nineteen mice were used as controls.
Treatment was given i.p. on Days 1, 5, 9, 13 and 17 after tumor implantation.
*MST: Median Survival Time.
†NS60: Number of survivors on Day 60/Total number of mice.

Table 7. Comparison of the effect of DNA–adriamycin and adriamycin on the life span and lymph node metastases of DBA/2 mice bearing L_{1210T} leukemia s.c.

	Tumor size on Day 9 (mm^3)	Axillary lymph node size on day 9 (mm^3)	Increase in life span %	No. of survivors after Day 30/Total number of mice
Adriamycin	17·22*	3·33	76	2/11
DNA-adriamycin T	167·25*	5·81	52	0/11
Control	599·4	29·71		
Intact mice		3·51		

10^6 L_{1210} cells/mouse were inoculated s.c. on Day 0.
Treated mice (18–22 g) received i.v. 6 mg/kg of adriamycin, free or complexed with DNA, from Day 1 to Day 5 after tumor implantation.
*Student test $p < 0{\cdot}01$.

Table 8. Comparison of the effect of DNA–adriamycin and adriamycin on the life span and lymph node metastases of DBA/2 mice bearing L_{1210T} leukemia s.c.

	Tumor size on Day 9 (mm^3)	Axillary lymph node size (mm^3)	Median survival time	Increase in life span %
Adriamycin	6·9	3·3	21	89
DNA–adriamycin T	34·6	6·8	18·0	62
DNA–adriamycin N	66·8	5·3	17·7	59
Control	597·3	16·95	11·1	
Intact mice		3·2		

10^5 L_{1210} cells/mouse were inoculated s.c. on Day 0.
Treated mice received i.v. 6 mg/kg adriamycin in a complexed form or in a free form, for 5 days, starting on Day 1.

Table 9. Comparison of the effect of DNA–adriamycin and adriamycin on lung metastases of BDF_1mice bearing Lewis Lung carcinoma i.m.

	Tumor weight (g)	No. of mice with metast. / No. of mice with tumor	Aver. No. of metast./mouse	Aver. wt. of metast./mouse (mg)
Adriamycin	7·59 ± 0·73	2/11	0·18	0·59
DNA–adriamycin T	9·54 ± 2·32	3/11	0·45	9·41
Control	10·47 ± 1·72	11/11	7	44·98

BDF1 male mice (20–24 g) were inoculated on Day 0 with $2{\cdot}10^6$ tumor cells/mouse. Animals received i.v. 6 mg/kg of free or complexed adriamycin on Days 1, 4, 9, 14, 18 and 22.

primary tumor and metastases, but this difference was not statistically significant (Table 9).

5. *Determination of plasma levels of adriamycin*

Figures 1 and 2 show the plasma–drug concentration at different intervals after injection. The drug concentration is expressed as adriamycin equivalents [6]. Each dot represents the results obtained on the plasma from 5–6 animals.

The plasma concentration of adriamycin was found to be higher in DBA/2 leukemic mice treated with the DNA–adriamycin complex form T than in mice treated with DNA–adriamycin form N or with free adriamycin (Fig. 1). It took 90 min to clear the plasma entirely of adriamycin after administration of DNA–adriamycin type T, while the clearance was complete about 10 min after treatment in mice given the DNA–adriamycin type N or free adriamycin.

Fig. 2 shows the fluctuations in the plasma concentrations of adriamycin in different strains of mice: DBA/2, CDF_1 and BDF_1 leukemic mice treated with 6 mg adriamycin contained in the DNA–adriamycin complex (type T). We noticed that the plasma drug concentration determined at different intervals was higher in DBA/2 mice than in CDF_1 and BDF_1 mice. After 90 min, the adriamycin–plasma concentration became equal in the 3 strains of mice.

DISCUSSION

We have shown previously that the complex DNA–adriamycin T was more active than adriamycin alone when tested on L_{1210T} in DBA/2 mice. This superior therapeutic activity could not be demonstrated in L_{1210} from this Laboratory (L_{1210B}) in DBA/2 mice. On L_{1210T} inoculated into BDF1 mice and on L_{1210B} inoculated into DBA/2 mice, the difference in activity between the DNA–adriamycin complex and adriamycin alone became insignificant [1].

In the study reported here, the data show that the increased therapeutic effectiveness of DNA–adriamycin complex T over adriamycin alone on L_{1210T} in DBA/2 or CDF_1 mice persisted with different schedules of administration. For instance one single dose 24 hr after tumor implantation or 5 daily doses or a schedule extending over a 17 day period. However, no difference was found between the complex T and the free drug when BDF_1 mice and L_{1210B} leukemia were used. The single dose and the prolonged schedule of administration were used in order to verify the hypothesis that the better therapeutic effectiveness of the complex might be due to a decreased toxicity associated with a prolonged action of adriamycin in complex form.

When the components of DNA–adriamycin were injected separately, one after the other, and the effect compared with adriamycin alone, there was a clear superiority of the association DNA plus adriamycin over adriamycin alone, provided DNA was administered first and adriamycin immediately thereafter. When adriamycin was administered first and DNA second, no increased therapeutic effectiveness as compared with adriamycin alone was noted. This observation suggests that DNA and adriamycin were able to recombine *in vivo* if administered in quick succession and if DNA was given first, thereby avoiding the fixation of free adriamycin within the plasma and vessel components. It also indicates that the specific effect of the DNA–adriamycin complex is not explained by a possible addition of separate effects of DNA and adriamycin, but that complex formation is necessary.

A few experiments are reported here (Tables 4 and 8) comparing the two forms of DNA–adriamycin complex available, one (form T) and the other (form N). They illustrate the fact that the N form is definitely less active than the T form. Actually, information is available that the N form of the DNA–adriamycin complex is not homogeneous, consisting of molecules of different sizes; the preparation of this form was attended by difficulties underlining the necessity of standardization and rigorous definition of the type of preparation used when experimenting with complexes.

The data on solid tumors show:

(*a*) there was no superiority of the DNA–adriamycin complex when administered i.v. in mice bearing s.c. melanotic melanoma B16 and Lewis Lung carcinoma in BDF_1 mice. However, when the same experiment was repeated on i.p. melanotic melanoma B16 by i.p. injections, the complex was found to be more effective than adriamycin alone. Direct contact of the DNA–adriamycin complex with the tumor cells thus appears to be the condition for the enhanced therapeutic activity of the complex over adriamycin alone.

(*b*) this interpretation receives confirmation from the experiments reported here on L_{1210T} as solid tumor and as a true leukemia. On the L_{1210T} solid tumor, the DNA–adriamycin complex was definitely inferior to adriamycin alone. Cures were obtained in this solid form of L_{1210T} with adriamycin alone and never with the DNA–adriamycin complex. In contrast, with the same L_{1210T} in leukemic form

in the same mice, the DNA–adriamycin complex was more effective than adriamycin alone.

Finally, the disappearance of free adriamycin from the plasma of DBA/2 leukemic mice was much quicker than the disappearance of the complex DNA–adriamycin T and this may explain the superior effectiveness of the complex upon the free drug, since the complex remains in contact longer with the tumor cells. Contrariwise, the heterogeneous complex N form showed essentially the same curve as the free adriamycin and one may assume that the form N of the complex will be as active as the free drug in the L_{1210T} solid tumor. It appears here also that the complex N behaves quite differently from complex T and from free adriamycin.

The difference in the disappearance of the DNA–adriamycin complex from the plasma in the different strains of mice explains the difference in the therapeutic effectiveness; actually, the complex was more active in DBA/2 mice than in CDF_1 mice and in CDF_1 mice than in BDF_1 inoculated i.v. with L_{1210} leukemia.

The therapeutic action of the DNA–adriamycin complex and its superiority over adriamycin alone appears to depend on the nature of tumor and in some cases on the strain of mice which are used. It also appears to require intimate and rather long contact between the complex and the tumor cells and may indicate a therapeutic limitation of the complex to leukemias and other tumors in which such a direct contact is possible. The importance of determining the drug plasma concentration during the treatment is evident.

Acknowledgements—Our thanks are due to Dr. Omar Karim from Farmitalia Benelux, for a generous supply of adriamycin and to Dr. André Trouet from the Laboratoire de Chimie Physiologique, Université de Louvain for providing the DNA used in this study.

REFERENCES

1. G. Atassi, H. J. Tagnon and A. Trouet, Comparison of adriamycin with the DNA–adriamycin complex in chemotherapy of L1210 leukemia. *Europ. J. Cancer* **10,** 399 (1974).
2. A. Trouet, D. Deprez-De Campanere and C. De Duve, Chemotherapy through lysosomes with a DNA–daunorubicin complex. *Nature New Biol.* **239,** 110 (1973).
3. A. Trouet, D. Deprez-De Campanere, M. Desmet and G. Atassi, Experimental leukemia chemotherapy with a "lysosomotropic" adriamycin–DNA complex. *Europ. J. Cancer* **9,** 405 (1974).
4. S. Tsukagoshi and Y. Sakurai, Cancer chemotherapy screening with experimental tumors which metastasize to lymph nodes. *Cancer Chemoth. Rep.* (*Part* 1) **54,** 311 (1970).
5. N. R. Bachur, A. L. Moore, J. G. Bernstein, A. Liu, Tissue distribution and disposition of Daunorubicin (NSC–82151) in mice: fluorometric and isotopic methods. *Cancer Chemoth. Rep.* (*Part* 1) **54,** 89 (1970).
6. A. S. Benjamin, C. E. Rigs and R. Bachur, Pharmakinetics and metabolism of adriamycin in mice. *Clinical Pharmacol. Ther. Part* 1, **14,** 592 (1973).
7. U. Hartel, M. Duarte-Karim, O. S. Karim, E. Arlandini, Some physiochemical properties of adriamycin and adriamycin–DNA complex. Second Int. Symp. on Adriamycin, Brussels, May 16–18, 1974 (Edited by M. Stafuet, H. Tagnon and Y. Kenis) European Press Mediton, pp. 75–83.

Europ. J. Cancer Vol. 11, pp. 317–320. Pergamon Press 1975. Printed in Great Britain

Methotrexate Effects on Enteric Mucosa Liver and Pancreas of Normal and Bled Rats*

G. CELLE, M. DODERO, I. PANNACCIULLI, G. FRESCO, C. MANSI, V. SAVARINO and G. BOGLIOLO

Istituto Scientifico di Medicina Interna, Università di Genova, Genova, Italy

Abstract—*The effects upon the enteric mucosa, liver and pancreas of normal and bled rats induced by methotrexate administered in one dose are reported.*

In normal rats, notwithstanding the severe enteric lesions, gross histological modifications of the liver and pancreas are absent. In rats made anemic by bleeding, the liver damage is marked with a diffuse fatty metamorphosis which reaches a peak at 48 hr after drug administration; in the pancreas there was only a pattern of mild cellular toxicity.

INTRODUCTION

TOXICITY studies on the antimetabolite methotrexate (MTX) have extensively covered the field of the experimental and clinical damage to enteric mucosa and to blood-forming organs [1–6].

A hepatotoxic effect of methotrexate is being reported with increasing frequency in patients treated for malignant and non-malignant disorders [7–22]. Available information leaves some doubt as to whether the underlying disease itself may be the cause of the observed abnormalities, in both malignant and non-malignant disorders (psoriasis, cyclitis, etc.). This is because few liver function tests have been carried out, and because virtually no liver biopsies have been performed before treatment. Moreover, reports on experimental research on liver damage induced by MTX are relatively few. MTX in human and mouse liver was detected for many months after administration [23, 24]. The drug mainly affects tissues with a high rate of proliferation, but it impairs, indirectly, the synthesis of proteins and, therefore, the whole hepatic function.

The theme of this paper is limited to a histological evaluation of the effects induced on the enteric mucosa, liver and pancreas of rats by MTX administered in one dose. This approach allows the use of relatively high dosage and follows the modern clinical trend to treat patients by intermittent administration of the drug. The toxic effects of the drug have been evaluated also in rats made anemic by bleeding.

MATERIAL AND METHODS

Male Wistar rats, weighing approx. 190 g, fed on standard diet, were observed for 10 days before starting the experiment.

At the beginning of the experiment, on day 0 and on day 1, 28 animals were bled by the usual technique. On each day an amount of blood approaching 18 % of the rat total blood volume was removed and in the two bleedings, more than a third of the total blood volume was removed, bringing the packed cell volume of the animals down to an average value of 28–33 %.

Accepted 2 January 1975.

*Supported by C.N.R. Grant No. 41:71.00862.

On the 6th day (5 days after the first bleeding) 18 rats of the bled group and 47 normal rats, not previously bled, were injected i.p. with MTX Lederle–Cyanamid Company (commercial brand for parenteral administration). The total dose was of the order of 10 mg/kg b.w., given in 4 portions, every 6 hr.

At intervals of 12, 24, 48 and 72 hr after the last drug administration, 5 non bled rats, 3 bled rats, and 2 bled, non-treated rats were killed after light ether anaesthesia.

Ninety-six hr after the last drug administration, 3 non-bled rats and 2 bled, non-treated, rats were sacrificed; no rat, bled and treated with MTX, survived to this time. All the bled, non-treated rats survived. Almost one half of the non-bled, treated, rats died, the peak of the mortality being some 65 hr after the last dose of the drug.

The liver, the pancreas and the whole digestive tube from the pyloroduodenal junction to the ileocaecal junction were removed from every animal sacrificed. These organs were prepared for histological examination, using hematoxylin eosin staining for all of them, a reticulum (Gomory's method) staining for liver and pancreas, and a lipid (Sudan III) staining for the liver only.

RESULTS

(a) *Normal rats which received 10 mg/kg of MTX*

Twelve hr after the last MTX dose, the enteric mucosa showed some slight changes, due mainly to a mild increase of cellular infiltration of the lamina propria.

Twenty-four hr, and more, (48 hr, 72 hr), after the last MTX dose, the changes became more striking, resulting in a pattern strongly resembling that of human sprue; the damage however was rather uneven, with differences between individual rats and in various tracts in the same animal. Intestinal villi became blunted and, in some tracts, disappeared. The lamina propria became heavily infiltrated with neutrophils, with lymphocytes and a few plasma-cells. The crypts showed the presence of some degenerating cells with alterations in size, staining properties and nuclei with a striking vacuolization of the cytoplasma. The same pattern, more and more pronounced, could be observed up to 96 hr after the last MTX dose. By this time the enteric mucosa appeared almost devoid of epithelia, consisting only of a heavily infiltrated lamina propria.

No significant changes could be observed in the liver or the pancreas.

(b) *Rats, made anaemic by bleeding, non-treated with MTX*

No changes were found in the organs examined.

(c) *Rats, made anaemic by bleeding, treated with MTX*

The damage to the enteric mucosa was the same as that found in normal rats treated with the same dose of MTX.

Twelve hr after the last dose of MTX, the hepatocytes showed cellular toxic changes with vacuolization of the cytoplasma, small areas of slight scattered necrosis, and a moderate degree of fatty metamorphosis.

After 24 hr, this damage was much more obvious. The reticulum appeared more evident, possibly because of approximation or collapse of the fibers (Fig. 1) and steatosis was particularly marked, although not uniform.

This fatty metamorphosis reached its peak at 48 hr when a true diffuse hepatic steatosis could be observed (Fig. 2). In rats sacrified later (72 hr) the degree of steatosis seemed to diminish.

No significant changes were observed in the pancreas of rats sacrified 12 and 24 hr after the last dose of MTX. At 48 and 72 hr, there was a pattern of mild cellular toxicity with changes in the size and staining properties of individual cells.

DISCUSSION

In rats, MTX at a dosage of 10 mg/kg administered at 24 hr, does not seem to cause hepatic or pancreatic damage as far as morphologic examination shows. At the same time the severe enteric lesions, observed by other authors also, testify to the overall toxicity of the given dose. In the absence of gross histological modifications, the cells, however, may show fine modifications which may be seen early by electromicroscopy, as in the liver of rats treated with L-asparaginase [25].

The possibility of functional alterations in hepatic or pancreatic cells has yet to be ascertained.

If MTX, in equal dosage, is administered to rats made anemic by bleeding, the liver damage is early, homogeneous, diffuse and marked. In addition to other modifications, the fatty metamorphosis of hepatocytes reaches a peak 48 hr after MTX administration. The livers of rats, made anemic by bleeding, but not treated with MTX, do not show any alteration.

A more prolonged assay of the steatogenic effect of MTX in anemic rats would require

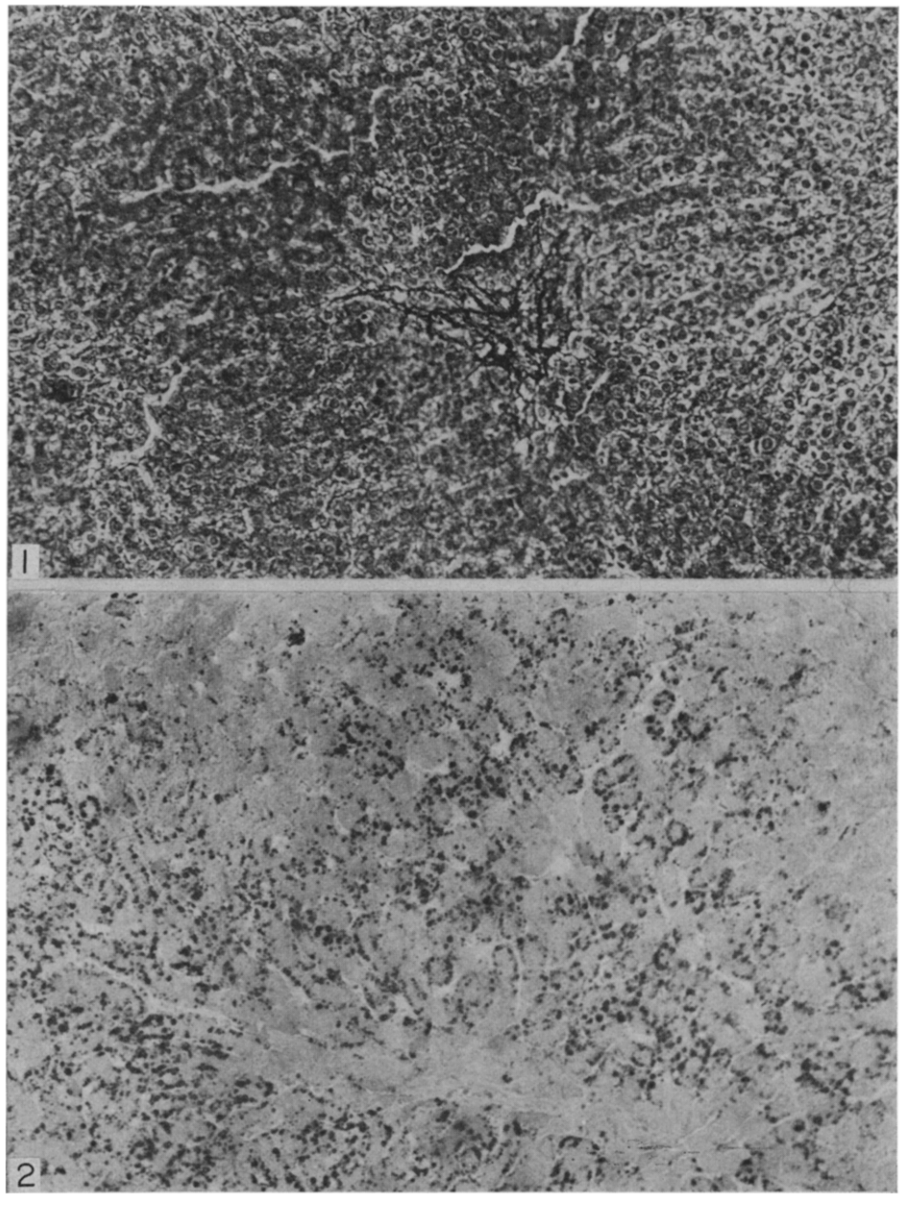


Fig. 1. *Bled and treated rat: small areas of the liver with collapse of the fibers of reticulum after* 24 *hr* (HE × 100).

Fig. 2. *Bled and treated rat: marked and diffuse fatty metamorphosis of the liver after* 48 *hr* (*Sudan III* × 100).

(to face p. 318)

further bleeding or a different method of inducing anemia. That would introduce new pathogenic factors (haemodynamic changes), which in turn would make less reliable the whole evaluation.

The similarity of enteric lesions in bled and non-bled animals demonstrates that, on the whole, the toxicity of MTX in the two groups was similar.

The mechanism of hepatotoxicity of MTX in rats made anemic by bleeding is not known. Anemic hypoxia could enhance the antimetabolic effect of MTX on the hepatic cell. However, at the levels of packed cell volume reached in this experiment, modifications of general circulation and/or of liver circulation are still possible to a degree sufficient to compensate for the anemic hypoxia.

After bleeding, the erythrogenic tissue was actively proliferating: at the time of MTX injection the average reticulocyte value was 13%. Thus, some competition could arise between the erythroid tissue and the liver cells, metabolically strained, for a common pool of anti-steatogenic factors which are, in part, also haemopoietic factors. However, the stores of these factors in the liver, at least for vit. B_{12} and tetrahydrofolate, are large and such a quick exhaustion of them seems unlikely. Moreover, the tetrahydrofolate may not be exhausted because of persistent folate reductase activity of the liver [17, 24]. This latter interpretation deserves comment. Equally unlikely is a quick effect of malabsorption from enteric damage.

If a similar situation takes place in man, then the administration of MTX to anemic patients with leukemia, solid tumors, psoriasis etc. would cause further severe damage to the liver. While it may be worth risking this in malignant conditions, the use of the drug is questionable in patients with non-malignant disorders, at least until after correction of the anemia.

REFERENCES

1. A. Baserga, La patologia arigenerativa del tubo digerente. *Minerva med.* **50,** 4186 (1959).
2. O. Schiraldi and R. Marano, Enteropatia acuta in ratti trattati con antimetaboliti (aminopterina). *Rass. Fisiopat. clin. terap.* **33,** 920 (1961).
3. B. J. Ryback, Electron microscopic studies of intestinal lesions. Aminopterin-induced lesions in mice. *Gastroenterology* **42,** 306 (1962).
4. J. S. Trier, Morfologic alteration induced by Methotrexate in the mucosa of human proximal intestine. Serial observations by light microscopy. *Gastroenterology* **42,** 295 (1962).
5. J. L. Achord, The effect of Methotrexate on rabbit intestinal mucosa enzymes and flora. *Amer. J. dig. Dis.* **14,** 315 (1969).
6. S. Margolis, F. S. Phillips and S. S. Sternberg, The cytotoxicity of Methotrexate in mouse small intestine in relation to inhibition of folic acid reductase and of DNA synthesis. *Cancer Res.* **31,** 2037 (1971).
7. G. Colsky, E. M. Greenspan and T. N. Warren, Hepatic fibrosis in children with acute leukemia after therapy with folic acid antagonists. *Arch. Path.* **59,** 198 (1955).
8. R. V. P. Hutter, F. H. Shipkey, C. T. C. Tan, M. L. Murphy and M. Chowdury, Hepatic fibrosis in children with acute leukemia: a complication of therapy. *Cancer* **13,** 288 (1960).
9. G. D. Amromin and R. D. Solomon, Necrotizing enteropathy: a complication of treated leukemia or lymphoma patients. *J. Amer. med. Ass.* **182,** 23 (1962).
10. R. A. O'Rourke and G. E. Eckert, Methotrexate-induced hepatic injury in an adult. A case report. *Arch. intern. Med.* **113,** 191 (1964).
11. E. M. Hersh, V. G. Wong, E. S. Menderson and E. Z. Freireich, Hepatotoxic effect of Methotrexate. *Cancer* **19,** 600 (1966).
12. J. Manaster, P. Barbier, J. Robert and E. Jacobs, Hepatoxicitè des antimitotiques. *Acta gastro-ent. belg.* **29,** 1058 (1966).
13. H. Sharp, J. White and W. Krivit, Methotrexate induced liver injury. *Proc. Amer. Ass. Cancer Res.* **8,** 60 (1967).
14. R. O. Coe and F. E. Bull, Cirrhosis associated with Methotrexate treatment of psoriasis. *J. Amer. med. Ass.* **206,** 1515 (1968).
15. H. Baker and M. G. C. Dahl, Methotrexate and the liver. *Brit. J. Derm.* **81,** 465 (1969).
16. G. A. Caron, Methotrexate: a calculated risk. *Arch. Derm.* **99,** 627 (1969).

17. E. H. Epstein and J. D. Croft, Cirrhosis following Methotrexate administration for psoriasis. *Arch. Derm.* **100,** 531 (1969).
18. S. A. Muller, G. M. Farrow, D. L. Martalock and M. Rochester, Cirrhosis caused by Methotrexate in the treatment of psoriasis. *Arch. Derm.* **100,** 523 (1969).
19. H. V. Dubin and F. C. Harrel, Liver disease associated with Methotrexate treatment of psoriatic patients. *Arch. Derm.* **102,** 498 (1970).
20. P. Amblard, P. Dugois, R. Imbert, S. P. Fillon and S. Martel, A propose de deux accidents survenus au cours de traitements generaux par le methotrexate. *Bull. Soc. franç. Derm. Syph.* **78,** 85 (1971).
21. M. G. C. Dahl and M. M. Gregory, Liver damage due to methotrexate in patients with psoriasis. *Brit. med. J.* **5750,** 625 (1971).
22. D. J. Philip, G. L. Logue, T. S. Harle and W. H. Farrar, Pulmonary and hepatic complication of Methotrexate therapy of psoriasis. *J. Amer. med. Ass.* **216,** 881 (1971).
23. S. Caharache, P. T. Condit and S. R. Humphreys, Studies on the folic acid vitamins: IV. The persistence of amethopterin in mammalian tissues. *Cancer* **13,** 236 (1960).
24. W. C. Werkheiser, The relation of folic acid reductase to aminopterin toxicity. *J. Pharmacol. exp. Ther.* **137,** 167 (1962).
25. G. Celle, M. Dodero and I. Pannacciulli, The liver damaging effect of L-Asparaginase. An experimental study of chronic toxicity. *Europ. J. Cancer* **55** (1973).

Europ. J. Cancer Vol. 11, pp. 321–326. Pergamon Press 1975. Printed in Great Britain

Immunochemotherapy of Human Malignant Melanoma with Chlorambucil-carrying Antibody*

T. GHOSE, S. T. NORVELL, A. GUCLU and A. S. MACDONALD
Departments of Pathology and Surgery, Faculty of Medicine, Dalhousie University and Victoria General Hospital, Halifax, Nova Scotia, Canada.

Abstract— *Heterologous anti-melanoma sera produced against any one patient's tumour cross-react, to varying degrees, with both the cytoplasmic and surface antigens of melanoma cells from other patients. Intravenous injections of cross-reacting anti-melanoma antibodies bound to chlorambucil were followed by the regression of a considerable number of skin metastases in a patient with disseminated malignant melanoma. When immunochemotherapy had to be discontinued because of anaphylaxis, many of the metastases which had almost completely regressed, reappeared and grew progressively in spite of chemotherapy. One metastatic nodule which had not regressed and another which appeared during immunochemotherapy contained a much larger proportion of tumour cells lacking the cross-reacting surface antigen, when compared to tumour cells in metastatic nodules excised before immunochemotherapy.*

INTRODUCTION

CHLORAMBUCIL can be non-covalently bound to anti-tumour antibodies without losing its alkylating activity or affecting the immunological reactivity of antibodies, and such chlorambucil-bound anti-tumour antibodies are more effective tumour inhibitors than chlorambucil or antibodies alone [1, 2]. We have reported the regression of metastatic nodules in a malignant melanoma patient after i.v. injections of chlorambucil-bound goat anti-melanoma globulin produced against the patient's own tumour [2]. We report here the regression of a number of metastatic nodules in the second melanoma patient treated with chlorambucil-bound anti-melanoma globulin.

Accepted 5 December, 1974.

*This work was supported by grants from the National Cancer Institute of Canada and Cancer Research Fund, Faculty of Medicine, Dalhousie University.

Our study of 52 heterologous anti-sera produced against 18 human malignant melanomas, reveals that human malignant melanoma cells, in contrast to other reports [3], have surface antigens which they share with other human malignant melanoma cells but neither with any normal adult human tissues nor with several other types of human malignant tumours. As tumour tissue adequate for animal immunization was not available from this patient, cross-reacting anti-melanoma sera were used for immunochemotherapy.

MATERIAL AND METHODS

Anti-human melanoma sera

Surgically excised, malignant melanoma specimens were received in sterile Hank's balanced salt solution at 4°C. After removal of skin, fibro-fatty and other non-neoplastic tissues, tumours were chopped into pieces about 5 mm^3,

washed several times with phosphate buffered saline (PBS, 0·1 M, pH 7·1) at 4°C, and then transferred to Ca and Mg free balanced salt solution containing EDTA [2]. As many dissociated tumour cells as possible were obtained from the tumour pieces by mechanical teasing. Tumour cells suspended in 2 ml PBS and mixed with 2 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) were injected i.m. into both flanks and both shoulders of adult goats or New Zealand white rabbits (4×10^8 viable tumour cells/animal). Identical injections were repeated after 1 week using tumour cells thawed and washed in PBS after storage in 10% dimethyl sulfoxide at −176°C. From one week after the second set of injections, the goats and rabbits were injected i.m. twice a week respectively with 100 mg and 5 mg of the tumour homogenate (prepared with a Waring blender and stored at −70°C) without any adjuvant. Any individual goat or rabbit received tumour materials from only one patient. Three days after the tenth injection of tumour homogenate, 5–10 ml of blood were obtained from all animals (test-bleeding). Test-bleeding sera were inactivated (56°C, 0·5 hr), then absorbed with AB red cells and then repeatedly with homogenates of pooled normal human tissues and tested, thereafter, by immunofluorescence, for specific anti-melanoma activity [2]. Immune sera were obtained from all animals showing titers of 1/64 or above of specific anti-melanoma activity. The resulting sera were inactivated and then successively absorbed with AB red cells and homogenates of normal human liver, lungs, kidney and spleen. Absorption with every tissue (tissue to serum ratio 1:3 by volume) was carried out at 37°C for 4 hr followed by another 8 hr absorption at 4°C. Absorbed sera were separated from the homogenates by centrifugation ($20{,}000 \times \mathbf{g}$) for 2 hr at 4°C. Absorptions were repeated (usually 2 absorptions each with human liver, spleen and lung homogenates and at least 4 absorptions with kidney homogenate) till the sera reacted only with the immunizing melanoma cells and not with the patient's own normal skin and peripheral blood lymphocytes or with other normal adult human tissues. Specific anti-melanoma sera (*i.e.* sera which after appropriate absorptions reacted only with malignant melanoma cells) against 18 malignant melanomas were produced in 14 goats and 38 rabbits. Immunofluorescence was performed either on 5 μm cryostat sections of melanoma and other human tissues snap-frozen at −176°C or on smears of unfixed snap-frozen and thawed tumour cells by the "sandwich method" using fluorescein isothiocyanate conjugated goat anti-rabbit, rabbit anti-goat or rabbit anti-human globulins with appropriate controls [1, 2]. For membrane immunofluorescence on viable melanoma cells, peripheral blood lymphocytes and cultured skin fibroblasts, the method of Phillips and Lewis [4] was used. Cytotoxicity of sera was assessed by the trypan blue exclusion method [2] using human AB blood group sera and a non-cytotoxic normal rabbit serum as complement sources [2]. Lymphocytes were isolated from sterile peripheral venous blood with carbonyl iron using a mechanical lymphocyte separator. Melanoma cells and normal skin fibroblasts were grown in Falcon flasks using medium 199 supplemented with human serum or 10% foetal calf serum [2].

Cross-reaction studies

All specific anti-melanoma sera were tested by immunofluorescence on smears from each of the 18 malignant melanomas. Five-tenths ml aliquots of four specific goat anti-melanoma sera were further absorbed with increasing amounts of homogenized melanoma cells or normal skin (2·5, and 5 and 10 mg/ml wet weight) from eight different patients. After absorption, increasing dilutions of sera were tested to find the titer of reactivity with cells from these 18 tumours.

Binding of globulins to chlorambucil

Varying amounts of chlorambucil were attached to anti-melanoma globulins at a pH of $3 \pm 0{\cdot}5$ when up to 90% of chlorambucil can bind to goat and rabbit globulins as observed by us [1, 2] and confirmed using similar procedures by others [5].

RESULTS

Cross reactivity of the melanoma cytoplasmic antigen

All specific anti-melanoma sera reacted by immunofluorescence with the cytoplasm of melanoma cells from most but not all malignant melanomas. With a few exceptions (*e.g.*, tumour from E.R., Table 1) melanoma cells usually reacted maximally with the specific sera produced against the test tumour and less with sera against other melanomas. When the reactivity of anti-melanoma sera with cells from the immunizing tumour were absorbed out with just enough homogenate of the

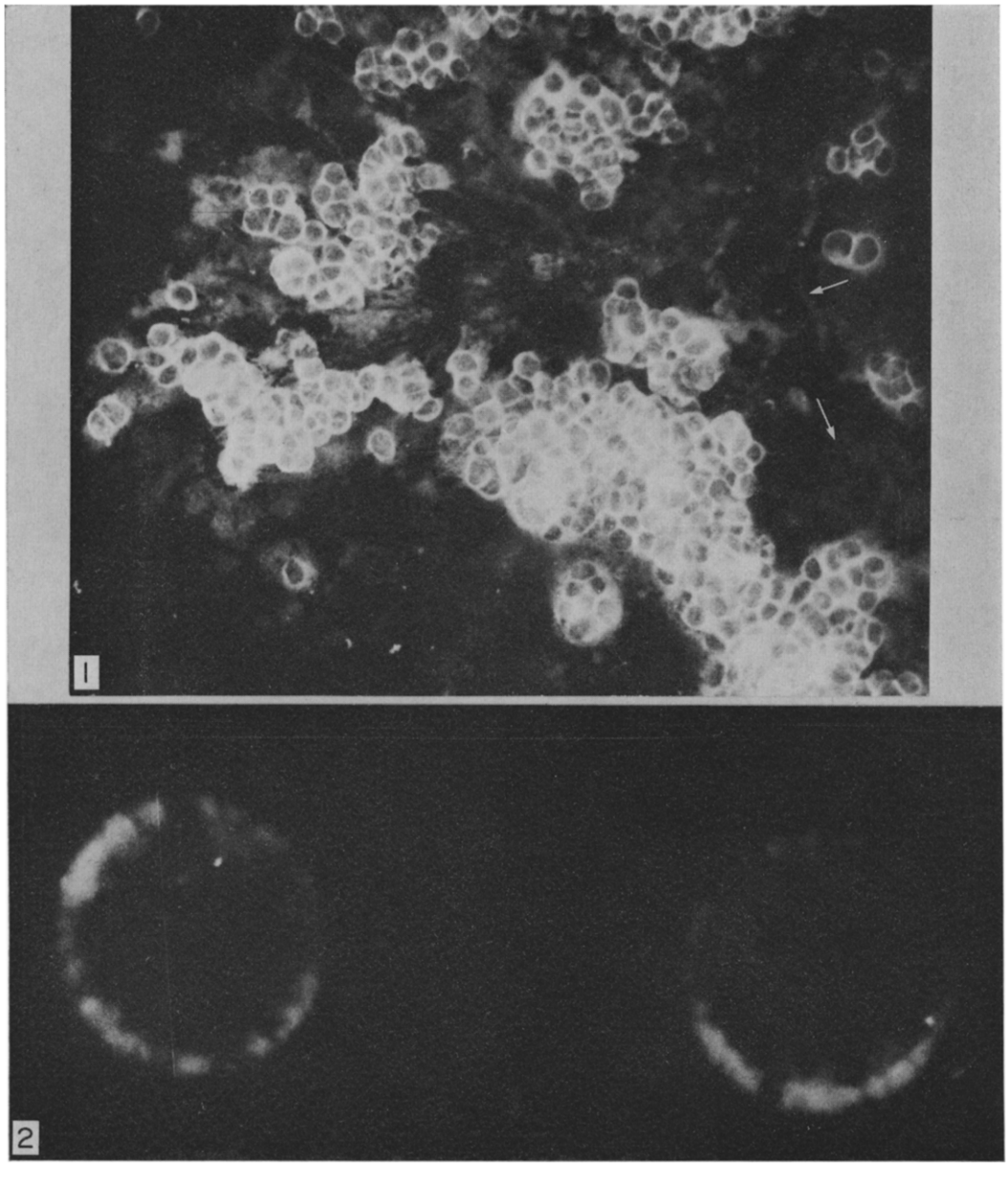


Fig. 1. *Fluorescence photomicrograph of a snap-frozen and thawed smear of malignant melanoma cells from patient D.A. stained by the "sandwich" method with a goat anti-melanoma serum (produced against M.M.'s tumor) absorbed with AB red cells, normal human tissue homogenates and malignant melanoma cell homogenate from S.F. (5 mg tumor homogenate/0·5 ml. serum). About half of the cells show cytoplasmic staining. Dark nuclear outline of unstained cells (marked with arrows) can also be seen.* × 280.

Fig. 2. *Fluorescence photomicrograph of a suspension of patient T.N.'s viable melanoma cells exposed first to a chlorambucil-bound cross-reacting goat-antimelanoma globulin and then to fluorescinated rabbit antigoat globulin. Discrete granular membrane staining of the viable melanoma cells shows that the anti-melanoma globulin retained its reactivity with the surface antigen of melanoma cells after binding with chlorambucil. Similar discrete granular membrane immunofluorescence of viable melanoma cells from this patient was also seen after exposure to the cross-reacting anti-melanoma globulins not bound to chlorambucil.* × 800.

(to face p. 322)

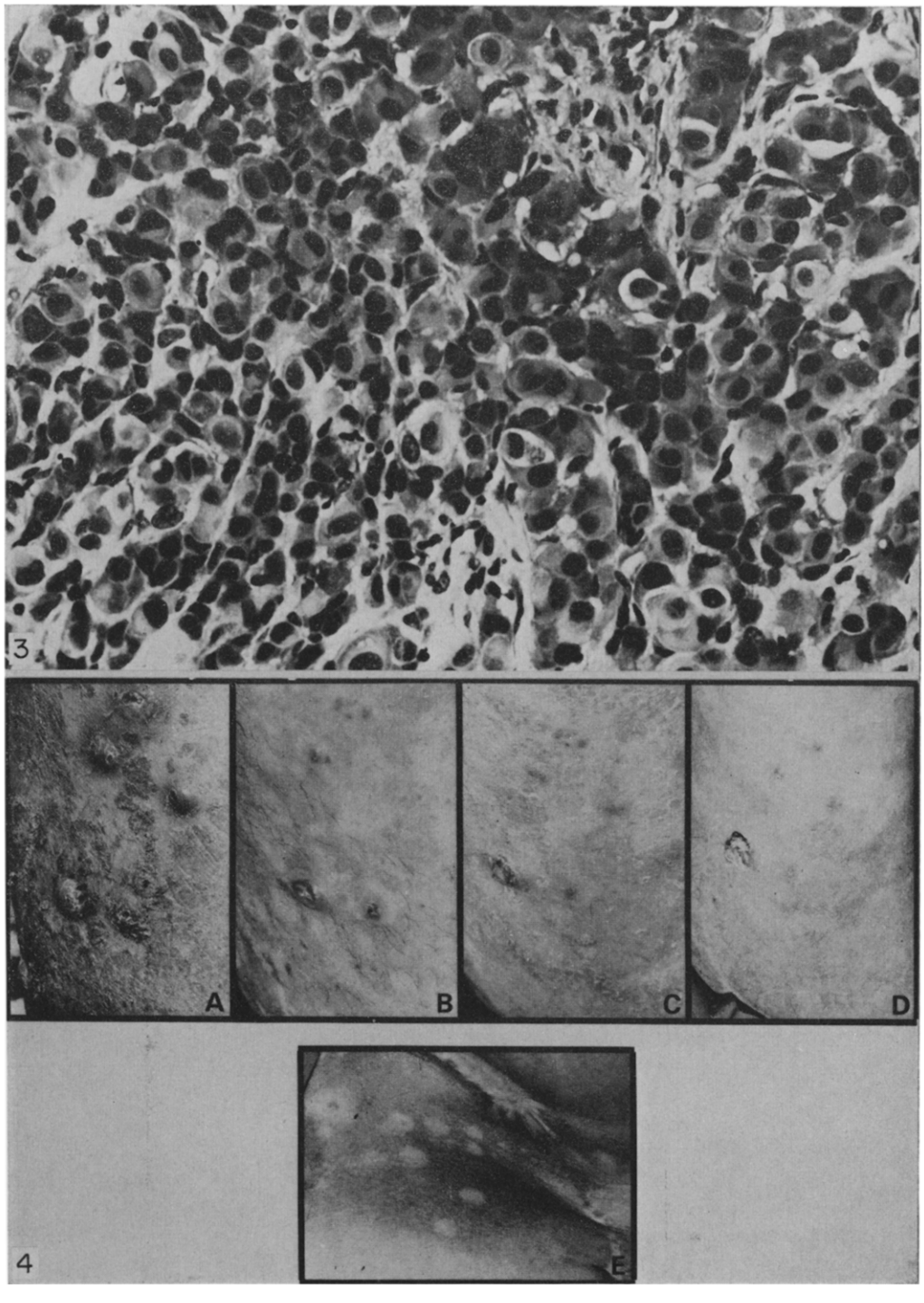


Fig. 3. *Photomicrograph of a section from the recurrent lesion in the patient's right heel showing cords and nests of round, ovoid and polyhedral cells with relatively large and hyperchromatic nuclei.* × 200.

Fig. 4. *Photographs of the five metastatic nodules on the anterior aspect of the right thigh of T.N., which were measured every week and photographed. A: just before immunochemotherapy, B: four months after the beginning of immunochemotherapy, C: six months after the beginning of immunochemotherapy, D: eight months after the beginning of immunochemotherapy, E: is the photograph of the right inguinal region of this patient taken six months after the beginning of immunochemotherapy showing depigmentation after regression of metastatic melanoma nodules. A total of about thirty metastatic nodules regressed during immunochemotherapy.*

immunizing tumour, they did not react with any other malignant melanoma cells. Absorption with appropriate amounts of homogenate of the majority of the cross-reacting tumours removed the serum's reactivity with the tumour used for absorption, but only reduced the reactivity of the serum with the immunizing tumour or with other tumours with which the serum reacted more strongly than the tumour used for absorption (Table 1). Occasionally, cross-absorption with a particular tumour homogenate either virtually did not affect the reactivity of the serum with some of the other cross-reacting tumours or revealed two populations of tumour cells; one reacting membrane immunofluorescence on melanoma cells (but not on the same patient's lymphocytes or fibroblasts) from 6 out of 7 melanomas demonstrated by each of these 4 anti-sera (none raised against the 7 test tumours) to possess a common cytoplasmic antigen. Viable cells from two immunizing melanomas were 4 and 9 times more effective than the cells from two cross-reacting melanomas in absorbing out the membrane immunofluorescence reactivity of two anti-malenoma sera with cells from their respective immunizing tumour. Prior exposure to autologous membrane localizing antibody from two patients could partially (though not completely) block the reactivity of the tumour

Table 1. *Comparison of the reactivity of a goat anti-melanoma serum with smears of melanoma cells from eight different patients, before and after absorption of this serum with tumour* (10 *mg tumour homogenate/ml serum) from these patients. This serum was produced against patient M.M.'s malignant melanoma and was rendered tumour-specific* (i.e., *the serum reacted with M.M.'s melanoma cells but not with* (*i*) *cryostat sections of M.M.'s skin* (*ii*) *M.M.'s skin fibroblasts* in vitro (*iii*) *M.M.'s peripheral leukocytes and* (*iv*) *cryostat sections of other normal adult human tissues), before this cross-reactivity study, by absorptions with human AB red cells* (*X*1), *and homogenates of normal human spleen* (*X*2), *liver* (*X*2), *lungs* (*X*2) *and kidney* (*X*4)

Tr. cells from:	Reciprocals of the highest dilution of the serum which reacted by immunofluorescence with melanoma cells after absorption of the serum with tumour homogenate from:								
	NONE	M.M.	E.R.	D.A.	O.S.	S.B.	H.R.	H.S.	S.F.
M.M.	>1024	—	4	128	64	64	64	128	256
E.R.	>1024	—	—	32	64	32	128	256	128
D.A.	1024	—	—	—	32	—	4	64 50% cells approx.	32 50% cells approx.
O.S.	1024	—	—	—	—	—	—	4	—
S.B.	512	—	—	—	—	—	—	—	—
H.R.	256	—	—	16	32	8	—	128	32
H.S.	256	—	—	4	—	—	—	—	4
S.F.	64	—	—	—	—	—	—	4	—

(— No reactivity detectable by immunofluorescence).

and the other not reacting with the absorbed serum (*e.g.*, cells from D.A. in Table 1 and Fig. 1). Absorptions of these sera with corresponding amounts of normal skin homogenate and frozen thawed peripheral blood lymphocytes from these patients did not affect their reactivity with melanoma cells.

Cross-reactivity of melanoma cell surface antigens

Membrane-immunofluorescence. All specific anti-melanoma sera showed discrete granular staining on the surface of viable melanoma cells from the immunizing tumour. One or more of the 4 anti-melanoma sera tested revealed cells with the heterologous membrane localizing antibodies.

None of the specific anti-melanoma sera reacted with either the smear or with the viable tumour cell suspensions from 4 human pulmonary squamous cell carcinomas, 2 hypernephromas and 2 breast adenocarcinomas.

Though all the sera prior to absorption were cytotoxic to melanoma cells and to cultures of a number of cell lines of human origin, no cytotoxic activity could be detected in these sera after being rendered melanoma-specific by absorption with AB red cells and human organ homogenates.

The case history of a melanoma patient treated by immunochemotherapy is given below:

Ten months prior to admission T.N., a 69 year old white male, had noticed a "wart" on his right heel, and four months later this lesion (not histologically examined) was excised. At admission, T.N. had an ulcer containing two reddish black heaped-up spherical nodules measuring 17 mm and 8 mm in their longest diameters, at the site of excision which had never healed. An incisional biopsy showed cords and nests of round and polygonal neoplastic cells with hyperchromatic nuclei and increased mitotic activity. Some of the cells contained a small amount of melanin (Fig. 3). After excision of the ulcer twelve days later, histology revealed tumour invasion of the lymphatics in the midcorium. Enlarged lymph nodes in the right groin were removed by radical dissection 25 days later. Several subinguinal nodes contained metastatic tumour. Examination 8 months later revealed histologically proven nodular recurrences of melanoma in the healed inguinal scar, an indurated mass above the inguinal ligament and edema of the right lower limb and external genitalia. Two months later about 30 metastatic nodules varying from the size of a pin head to spheres about 1 cm dia appeared in or under the skin of his right inguinal region, pubic ramus, the scrotum and the right lower limb. The patient at this time, did not react to the second strength of PPD (Connaught Laboratories, Toronto). Suspension of viable melanoma cells obtained from two metastatic nodules were tested for cytoplasmic and membrane immunofluorescence against a number of goat and rabbit anti-melanoma sera produced against malignant melanoma from other patients. One goat serum and two rabbit sera showed fairly strong (*i.e.*, titer 1/128) cytoplasmic and membrane immunofluorescence. When bound to chlorambucil, these anti-melanoma globulins retained alkylating activity as assessed by the Epstein method [6] as well as their reactivity with tumour cells (Fig. 2). The patient was injected i.v. with a total amount of 429 mg of γ-globulin bound to 41 mg of chlorambucil through nine separate injections administered on alternate days. Five subcutaneous metastases on the anterior aspect of his right thigh were measured weekly. The two largest diameters of these five nodules approximately at right angles to one another just before the beginning of treatment, were $1{\cdot}4 \times 1{\cdot}2$ cm, $1{\cdot}5 \times 1{\cdot}2$ cm, $1{\cdot}2 \times 1{\cdot}1$ cm and $1{\cdot}8 \times 1{\cdot}2$ cm. About 8 weeks after completion of the injections all of these five nodules and about 20 other nodules which were not regularly measured became smaller and after about 4 months, four of the five metastatic nodules under regular measurement and at least twenty other nodules in the inguinal region and the scrotum almost completely regressed (Fig. 4). Depigmentation was noticed around the regressing melanoma nodules. A month later, a new crop of nine metastases appeared in or under the skin. The patient again received, through 16 i.v. injections, a total of 800 mg of a cross-reacting goat anti-melanoma globulin bound to 80 mg of chlorambucil. The patient had a mild anaphylactic reaction after the last injection and all injections of goat gamma-globulin were stopped. From 7 days after the anaphylactic reaction, the patient received three i.v. injections containing a total of 175 mg of cross-reacting rabbit gamma-globulin bound to 17·5 mg of chlorambucil. However, after the last injection, the patient developed anaphylactic reactions to rabbit globulin as well and immunochemotherapy was stopped. During this period of immunochemotherapy, though, at least five other metastatic nodules completely disappeared leaving areas of cutaneous depigmentation, and those nodules which had already regressed did not reappear, four new metastatic nodules emerged as well. One nodule on the anterior aspect of his right thigh which was being measured regularly and which did never completely regress and another recent metastatic nodule were excised. Less than 5% cells from these two nodules reacted by membrane immunofluorescence with the rabbit and goat anti-melanoma globulins with which the patient was treated. At least 60% tumour cells in metastatic nodules excised prior to immunochemotherapy and stored in liquid nitrogen, showed membrane immunofluorescence when concurrently tested with these anti-melanoma globulins. About 80–90% of the stored melanoma cells and about the same proportion of cells from these excised nodules showed cytoplasmic immunofluorescence with these three anti-melanoma globulins.

The patient did not have at any time membrane localizing or cytoplasmic antibody or cytotoxic lymphocytes against his own tumour cells nor could any anti-rabbit or anti-goat globulin be detected in his serum by immunodiffusion.

After the stoppage of immunochemotherapy, in spite of treatment with large doses of BCG and a number of chemotherapeutic agents including DTIC, there has not been any further regression of tumour nodules. Existing

nodules have increased in size and a few have fungated.

DISCUSSION

The melanoma specificity of the appropriately absorbed heterologous anti-melanoma sera is supported by: 1) the absence of reaction by membrane or cytoplasmic immunofluorescence either with cryostat sections of the respective patients' own skin or with the smears and suspensions of viable autologous peripheral blood lymphocytes and cultures of autologous skin fibroblasts from these patients, 2) absorption of the reactivity of these sera with appropriate amounts of the immunizing or other cross-reacting melanoma tissues but neither with the corresponding amounts of homogenates of normal skin nor with skin fibroblasts or peripheral blood lymphocytes from these patients and, 3) absence of reactivity either with smears or viable cell suspensions from several human malignant tumours other than melanoma.

The tumour-specific melanoma cell-cytoplasmic antigen cross-reacts with sera from other malignant melanoma patients [3, 7–9]. Though Lewis *et al.* [3] are of the opinion that the melanoma cell-surface antigen which reacts with autologous serum is individual specific, other studies using membrane immunofluorescence [7, 11], cytotoxicity tests [11, 12] or lymphocytes [10, 13, 14] support our observation that there are tumour-specific cross-reacting antigens on the surface of human malignant melanoma cells. As prior exposure to the autologous membrane localizing antibody partially inhibited the reactivity of viable melanoma cells to the heterologous cell-surface localizing antibody, the antigen on the surface of malignant melanoma cells reacting with these two antibodies appears to be similar.

Our observation in several mouse tumour models, that chlorambucil or ^{131}I bound antibodies consistently cause much more tumour inhibition than antibody, chlorambucil or ^{131}I alone [1, 2, 15, 16] has now been confirmed by others [5, 17, 18]. However, as discussed by us before [2], the precise mechanism of increased tumour inhibition by antibodies attached to cytotoxic drugs or ^{131}I remains to be elucidated and could be explained on the basis of: (a) synergism between anti-tumour antibody and cytotoxic drugs [5, 18] or ionizing radiations [19], (b) antibody mediated preferential localization of cell damaging agents in tumour tissue [16] and (c) both [2].

None of the anti-melanoma globulins used in the treatment of this patient was cytotoxic to his tumour cells and it is unlikely that this antibody by itself caused tumour regression. Passive transfer of serum or plasma from cured patients has failed to cause regression of disseminated melanoma [20]. The complete or partial regression of a considerable number of metastatic melanoma nodules during immunochemotherapy (but at no time before immunochemotherapy) and the re-emergence and progressive growth of metastases after the cessation of immunochemotherapy but in spite of continuing chemotherapy and BCG administration, supports the contention that the regression of these metastases was not spontaneous but causally associated with immunochemotherapy.

Depigmentation has been observed after the regression of melanoma metastases in skin [20, 21]. No anti-melanoma cytoplasmic antibody (reported to be associated with the depigmentation around resolving "halo" nevus [22]) or antibodies against melanin containing cells in normal human skin could be detected in the serum of this patient before or after the appearance of these areas of depigmentation.

The results of our cross-reaction study are in keeping with the concept of antigenic diversity of tumour cells [23, 24] and suggest that the tumour-specific melanoma antigens have mosaics of determinants and that different combinations of these determinants are expressed in different tumours. The considerable decrease in the proportion of surface antigen containing melanoma cells in the metastasis which did not regress and the other which appeared during immunochemotherapy compared to metastases excised before immunochemotherapy, suggest selective suppression of the surface antigen containing cells and survival and proliferation of the antigen negative cells or antibody induced desensitization resulting either from the loss of antigen *i.e.*, "antigenic modulation" [25] or from the redistribution of antigen antibody complex on the cell membrane [26]. Such selective proliferation of antigen negative and/or drug-resistant tumour cells might be an impediment to the effective eradication of tumour cells by immunochemotherapy. Development of anaphylaxis against foreign globulins constitutes another limitation of immunochemotherapy. At present, attempts are being made to remove the antigenic Fc fragment of the immunoglobulin molecules prior to binding with chlorambucil.

Acknowledgement—We are grateful to Ms. Burroughs Wellcome and Co. for the free gift of chlorambucil (leukeran) and to Mrs. M. Mammen, P. Seaman and R. Sekaran for technical help.

REFERENCES

1. T. Ghose and S. Nigam, Antibody as carrier of chlorambucil. *Cancer* **29,** 1398 (1972).
2. T. Ghose, S. T. Norvell, A. Guclu, D. Cameron, A. Bodurtha and A. S. Macdonald, Immunochemotherapy of cancer with chlorambucil-carrying antibody. *Brit. med. J.* **3,** 495 (1972).
3. M. G. Lewis, R. L. Ikonopisov, R. C. Nairn, T. M. Phillips, G. Hamilton Fairley, D. C. Bodenhan and P. Alexander, Tumour-specific antibodies in human malignant melanoma and their relationship to the extent of the disease. *Brit. med. J.* **3,** 547 (1969).
4. T. M. Phillips and M. G. Lewis, A system of immunofluorescence in the study of tumour cells. *Rev. europ. études clin. et biol.* **15,** 1016 (1970).
5. D. A. L. Davies and G. J. O'Neil, *In vivo* and *in vitro* effects of tumour-specific antibodies with chlorambucil. *Brit. J. Cancer* **28** (suppl.), 285 (1973).
6. J. Epstein, R. W. Rosenthal and R. J. Ess, Use of γ-(4-nitrobenzyl) pyridine as analytical reagent for ethylenimines and alkylating agents. *Anal. Chem.* **27,** 1435 (1955).
7. D. L. Morton, R. A. Malmgren, E. C. Holmes and A. S. Ketcham, Demonstration of antibodies against human malignant melanoma by immunofluorescence. *Surgery* **64,** 233 (1968).
8. H. F. Oettgen, T. Aoki, L. J. Old, E. A. Boyse, E. DeHarven and G. M. Mills, Suspension cultures of a pigment-producing cell line derived from a human malignant melanoma. *J. natl. Cancer Inst.* **41,** 827 (1968).
9. N. M. Muna, S. Marcus and C. Smart, Detection by immunofluorescence of antibodies specific for human malignant melanoma cells. *Cancer* **29,** 88 (1969).
10. G. Fossati, M. I. Colnaghi and G. Della Porta, Cellular and humoral immunity against human malignant melanoma. *Int. J. Cancer* **8,** 344 (1971).
11. R. S. Metzgar, P. M. Bergoc, M. A. Moreno and H. F. Seigler, Melanoma-specific antibodies produced in monkeys with human melanoma cell lines. *J. natl. Cancer Inst.* **50,** 1065 (1973).
12. D. P. Goodwin, M. O. Hornung, S. P. L. Leong and E. T. Krements, Immune responses induced by human malignant melanoma in the rabbit. *Surgery* **72,** 737 (1972).
13. J. E. DeVries, P. Rumke and J. L. Bernheim, Cytotoxic lymphocytes in melanoma patients. *Int. J. Cancer* **9,** 567 (1972).
14. K. E. Hellstrom and I. Hellstrom, Immunity to neuroblastomas and melanomas. *Ann. Rev. Med.* **23,** 19 (1972).
15. T. Ghose, M. Cerini, M. Carter and R. C. Nairn, Immunoradiative agent against cancer. *Brit. Med. J.* **1,** 90 (1967).
16. T. Ghose and A. Guclu, Cure of a mouse lymphoma with radio-iodinated antibody. *Europ. J. Cancer.* In press (1974).
17. I. Flechner, The cure and concomitant immunization of mice bearing Ehrlich ascites tumours by treatment with an antibody-alkylating agent complex. *Europ. J. Cancer* **9,** 741 (1973).
18. R. D. Rubens and R. Dulbecco, Augmentation of cytotoxic drug action by antibodies directed at cell surface. *Nature (Lond.)* **248,** 81 (1974).
19. T. Ghose and M. Cerini, Radiosensitization of Ehrlich ascites tumour cells by a specific antibody. *Nature (Lond.)* **222,** 993 (1969).
20. T. C. Everson and W. H. Cole, *Spontaneous Regression of Cancer,* p. 197. Saunders, Philadelphia (1966).
21. W. C. Sumner and A. G. Foraker, Spontaneous regression of human melanoma. *Cancer* **13,** 79 (1960).
22. P. W. M. Copeman, M. G. Lewis, T. M. Phillips and P. G. Elliott, Immunological associations of the halo naevus with cutaneous malignant melanoma. *Brit. J. Derm.* **88,** 127 (1973).
23. J. Davidsohn, L. Y. Ni and R. Stejskal, Tissue iso-antigens A, B, and H in carcinoma of the stomach. *Arch. Path.* **92,** 456 (1971).
24. E. V. Sugarbaker and A. M. Cohen, Altered antigenicity in spontaneous pulmonary metastases from an antigenic murine sarcoma. *Surgery* **72,** 155 (1972).
25. E. A. Boyse, L. J. Old and S. Luell, Antigenic properties of experimental leukemias. II. Immunological studies *in vivo* with C57BL/6 radiation induced leukemias. *J. nat. Cancer Inst.* **31,** 987 (1963).
26. M. C. Raff and S. dePetris, Ligand induced redistribution of membrane macromolecules: Some possible implications for cancer. *J. clin. Path.* **27** (suppl. 7), 31 (1974).

Europ. J. Cancer Vol. 11, pp. 327–334. Pergamon Press 1975. Printed in Great Britain

Carcinogenicity of 6-Aminochrysene in Mice

G. LAMBELIN, J. ROBA, R. RONCUCCI and R. PARMENTIER*

Continental Pharma Research Laboratories, Steenweg op Haacht, 30, B-1830 Machelen, Belgium
**Laboratoire d'Anatomo-Pathologie, Faculté de Médecine de l'Université Libre de Bruxelles, Belgium*

Abstract—*Long term topical skin application of 6-aminochrysene (6-AMC) to mice induced development of benign skin tumours after 3 months and of skin malignancies after 7 months. Primitive cutaneous tumours arose at the site of application and other lesions arose elsewhere on the skin and in the viscera. Gross and histopathological examination revealed that 92% of mice in the 6-AMC treated groups carried tumours, compared to 6 and 5% respectively in vehicle-treated and untreated controls. In the 6-AMC treated mice, lung tumours were observed in 71% of the animals and liver lesions in 38%. Female mice responded earlier than males. Induction of skin tumours was more rapid when 6-AMC was applied ventrally instead of dorsally. Bioavailability studies with ^3H-6-AMC indicated that urinary excretion is about twice as high after skin application than after oral administration.*

INTRODUCTION

THE TUMOUR-INHIBITORY activity of 6-aminochrysene (6-AMC) was demonstrated for the first time by Rudali, Buu-Hoï and Lacassagne in 1953 [1], and then confirmed by several authors in various experimental models [2–5]. In man, the compound has been used for the treatment of splenomegaly of various origins and of myeloid leukemia [6–8], and more recently of advanced breast cancer in women [9]. The interest in 6-AMC has been relatively great, not necessarily because of its therapeutic effectiveness but because its anti-neoplasic activity was apparently not associated with carcinogenicity, which was considered exceptional for this kind of substance particularly among the polycyclic aromatic hydrocarbons. In mice, Rudali *et al.* reported that 6-AMC administered by topical applications on the skin or by i.m. injections was devoid of carcinogenic activity [1]. A study on rats to which the compound was added to the food and administered chronically also gave negative results [10]. On the other hand, in 1969, using neonatal mice, Roe and Carter [11, 12] found that 6-AMC induced hepatomas and pulmonary adenomas, but their technique was not widely accepted for carcinogenicity testing [13]. Thus, we found it interesting to conduct another study on 6-AMC using a more classical procedure, in the species apparently the most susceptible *i.e.*, the mouse, and adopting the most severe conditions, namely, the daily administration of high dosages of the compound added to an ointment providing an optimal percutaneous absorption.

Accepted 2 December 1974.

*Laboratoire d'Anatomo-Pathologie, Faculté de Médecine de l'Université Libre de Bruxelles, Belgium.

MATERIAL AND METHODS

(a) Bioavailability studies

6-AMC-12^{3}H (specific activity: 1930 μCi/g) was used for the assessment of the bioavailability of the compound after either oral or topical administration. Sixty male mice (CFLP strain, mean body weight ± 30 g) were divided into six groups of ten animals, each administered with the same dose, *i.e.*, 5 mg (see Table 1). Four groups (A through D) were used for the determination of the 0/48 hr ^{3}H-urinary excretion after single skin application of the ointment. The treatment consisted in rubbing into the

dorsal skin of the animals of the Groups A and B 100 mg of the 5 % 6-AMC-12^{3}H ointment of the following composition: liquid paraffin 11·25 %, cetylic alcohol 9·0 %, propylen glycol 9·0 %, Tween 60 1·62 %, Span 60 1·08 %, propyl-*p*-hydroxybenzoic acid 0·02 %, methyl-*p*-hydroxybenzoic acid 0·183 %, DL α tocopherol 0·813% distilled water 62·77%. Group B differed from Group A by the fact that Group B animals carried a thin parabolic "carcanet" around the neck. This "collar" considerably prevented mice from licking up the ointment, without limiting their normal activity. In Groups C and D the same procedure was adopted except that the ointment was rubbed into the ventral skin, animals of Group D bearing carcanets. In the two remaining Groups (E and F) the 0/48 ^{3}H-urinary excretion was measured after single oral administrations of 6-AMC-12^{3}H as mucilage (Group E) or as ointment (Group F). Animals of Group E received the radiolabelled compound by stomach tube as 1 % tragacanth gum mucilage (0.3 ml/animal). Animals of Group F also received the ^{3}H-compound by stomach tube but in the ointment form, of the same composition as used for topical application. Animals received food and water *ad libitum* and were housed in appropriate metabolic cages (10 animals/cage) for urine collection at the following intervals: 0/8, 8/24, 24/32 and 32/48 hr. Samples were kept frozen until analyzed. Analyses were achieved on the 0/24 and 24/48 hr urine pools of each group. ^{3}H-contents were determined by LSC (Tri-Carb, Packard Inst., model 3375) using a slightly modified Bray's solution as counting mixture.

The assay of the unchanged compound in urine was performed by GLC. 6-AMC was extracted from urine at neutral pH with methylisobutylketone (3 times, 1:2V/V). The solvent of the dried (Na_2SO_4) pooled extracts was evaporated and the residues dissolved in 1,4-dioxane. Derivatization of the unchanged 6-AMC was carried out with trifluoroacetylanhydride (Pierce Chem. Co.) and aliquots of 1–2 μl were injected (inlet temperature 270°C) into a gas chromatograph (Packard Inst., series 7400) equipped with a flame ionisation detector (working at 270°C). The column (2 m, 2 mm) was packed with 3 % SE on Supelcoport 80–100 mesh and maintained at 250°C. Nitrogen was used as carrier gas flowing at 30 ml/min. A similar technique has been described since by Belvedere *et al.* [14].

(*b*) *Carcinogenicity studies*

300 adult SPF mice of the CFLP strain (origin Carworth Europe, Huntingdon, U.K.), with a mean bodyweight of 25 g, were distributed randomly in 5 groups of 30 males and 30 females each.

Group I: Dorsal application of 6-AMC in a 5 % ointment.

Group II: Ventral application of 6-AMC in a 5 % ointment.

Group III: Dorsal application of the vehicle alone.

Group IV: Ventral application of the vehicle alone.

Group V: Untreated controls.

The mice were housed 5 per cage, in air conditioned rooms (21°C) and received food (A 04, UAR, France) and water *ad libitum*.

6-AMC was added at 5 % to an ointment as described above.

About 100 mg of the ointment was rubbed daily, 7 times a week, on either the dorsal or on the ventral skin. Mice were examined regularly for skin lesions. Half of the mice were treated for 6 months while treatment continued for 3 further months on the others. All the surviving mice were then sacrificed and submitted to gross and microscopic examinations.

RESULTS

1. *Bioavailability studies*

The results of the determinations of the ^{3}H-urinary contents in mice treated topically or orally with 6-AMC-12^{3}H are displayed in Table 1. Since no data are available concerning either the fecal or the biliary excretion, the ^{3}H amounts reported for urine represent the "minimal" absorption rates. The figures obtained show that the ointment gives rise to a relatively high excretion of tritium in the 0/48 hr urine.

Comparing ventral and dorsal applications, it appears that the former gives rise to higher mean ^{3}H excretion rates (*e.g.* 7·58 % in Group C vs 6·52 % in Group A).

Considering the figures obtained in the corresponding groups bearing carcanets, the minimal percutaneous absorption seems lower (5·18 % for ventral application (Group D) and 4·19 % for dorsal applications (Group B). These differences could be attributed possibly to the oral intake of part of the ointment by animals of Groups A and C. Indeed, results obtained in Group F confirm that 6-AMC-12^{3}H added to the ointment can be absorbed from the G.I. tract but at a lower rate than that obtained after oral administration of mucilage (Group E).

Finally, comparing the figures observed for "pure" percutaneous absorption (Groups B and

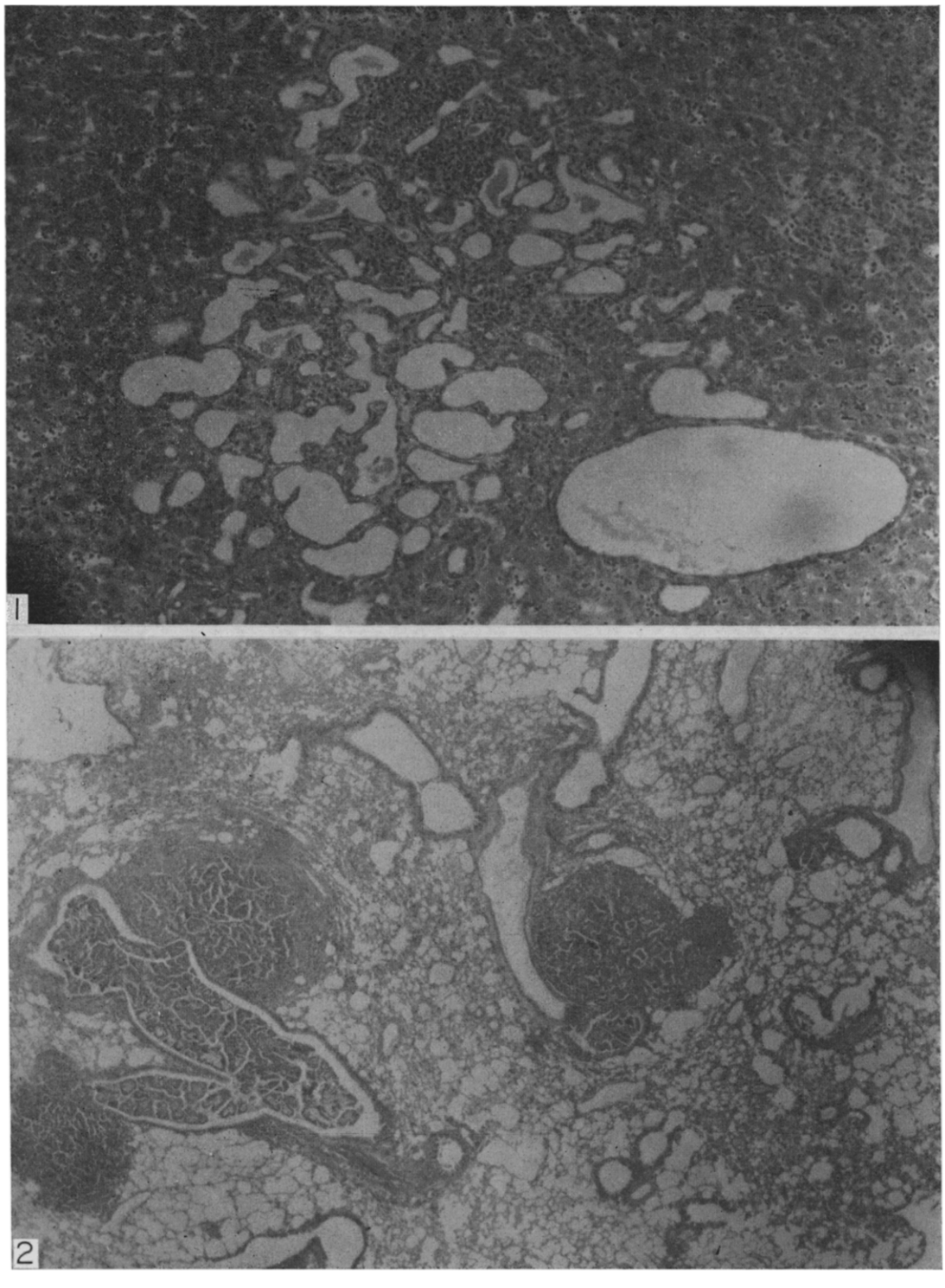


Fig. 1. *Aspect of hepatic peliosis with irregular cavities and cysts* (*No.* 7980, *female mouse No.* 115, *sacrificed after* 9 *months of ventral application of* 6-*AMC*). *Mouse bearing numerous skin papilloma* (*abdomen, eyelids, ears*) *and pulmonary tumours. Aqueous Bouin, hematoxylin-eosin.*

Fig. 2. *Pulmonary tumours as isolated nodules eventually expending in the bronchial lumen* (*No.* 7913, *male mouse No.* 4, *sacrificed after* 9 *months of* 6-*AMC application on the dorsal skin*). *Never found bearing skin tumours. Aqueous Bouin, hematoxylin-eosin.*

(to face p. 328)

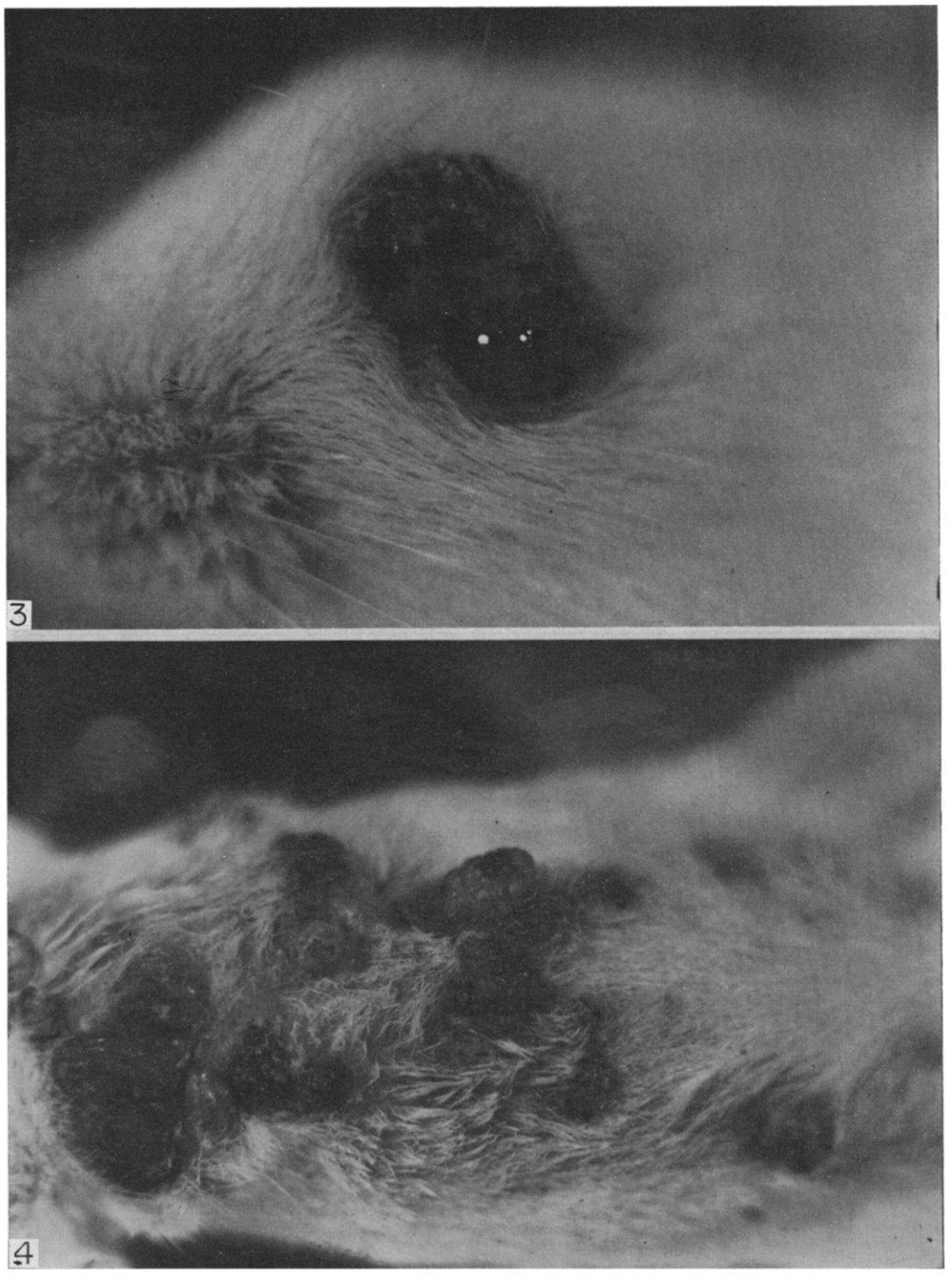


Fig. 3. *Skin tumour in male mouse No.* 27, *after* 8 *months of dorsal application of* 6-*AMC. First papilloma were observed on the dorsal skin after* 6 *months of experiment. At sacrifice at* 9 *months, skin epithelioma and lung tumour were noted.*

Fig. 4. *Skin tumour, papilloma and epithelioma, at the site of* 6-*AMC application in No.* 104 *female mouse after* 8 *months of administration. First skin tumours have been observed after* 5 *months of topical application.*

Table 1. *Mean* 0/48 *hr* ^3H*-urinary excretion in male mice after single administrations of* 5 *mg* (·65 *μCi*) 6-*AMC*-12^3H *by different routes*

Group	Number of mice	Preparation used	Route of administration	^{3}H-urinary excretion (% of the dose adm.) 0/24 hr	24/48 hr	Total 0/48 hr
A	10	5% ointment	topical (dorsal skin)	5·24	1·28	6·52
B with carcanet	10	5% ointment	topical (dorsal skin)	2·88	1·31	4·19
C	10	5% ointment	topical (ventral skin)	6·25	1·33	7·58
D with carcanet	10	5% ointment	topical (ventral skin)	3·71	1·47	5·18
E	10	mucilage	p.o.	2·16	1·16	3·32
F	10	5% ointment	p.o.	1·35	0·50	1·85

D) to those for oral absorption (Groups E and F) it appears that skin absorption is more efficient.

2. *Carcinogenicity studies*

(*a*) *Gross examinations*

Skin tumours. In the course of the third month of 6-AMC application, small cutaneous tumours of about 2 mm diameter developed in 1 male and 2 females of Group II (ventral application). Biopsy indicated the papillomatous nature of these tumours. Later, most animals developed similar lesions as shown in Table 2. The first evidence of tumours in mice treated by dorsal application with 6-AMC was obtained during the fifth month of the experiment in the females and during the sixth month in the males. After 7 months, some of the cutaneous tumours grew larger and appeared granulomatous. Biopsies showed them to be

Table 2. *Gross examinations: incidence of mice with one or more skin tumours in the treated groups I and II*

Time months	Group I Dorsal application of 6-AMC ♂	Group I Dorsal application of 6-AMC ♀	Group II Ventral application of 6-AMC ♂	Group II Ventral application of 6-AMC ♀
1	0/30	0/30	0/30	0/30
2	0/30	0/30	0/30	0/30
3	0/30	0/30	1/30	2/29
4	0/30	0/30	3/29	7/29
5	0/29	3/29	13/29	15/29
6	9/29	16/29	17/29	21/29
7	7/24	16/26	16/28	21/27
8	12/22	17/21	17/27	22/23
9	15/21	10/11	15/23	17/17

N.B. Skin tumours were never observed in the control groups III, IV and V.

spinocellular carcinomas. Obviously at this stage, carcinomas, self-inflicted lesions or infected papillomas is questionable. The first skin tumours arose at the site of application. Later, they grew elsewhere on the skin except on the limbs and tail. The scratching areas such as the head and particularly the lips, the eyelids and the base of the ears were very often affected.

Since there is no difference in incidence of skin tumours in mice in which 6-AMC application is interrupted after 6 months and in those treated for 9 months, the results are combined in Table 3. In vehicle-treated and in untreated mice, no skin tumours were observed.

Mortality. The mortality encountered during the experiment is shown in Table 3.

The death rate was strikingly higher in the 6-AMC treated mice (Groups I and II). Since there was no significant difference in mortality between males and females in the untreated and vehicle-treated groups, comparisons between treated and control groups were made on the combined results for the two sexes. For both sexes, mortality was significantly higher in Groups I and II than for the controls ($P < 0{\cdot}05$). Although the mortality seemed higher in Group I (dorsal application) than in Group II (ventral application) for both sexes, the difference was not significant. The mortality was also much higher in the 6-AMC treated females than in the males. The difference was significant in Group I ($P = 0{\cdot}01$) but not in Group II. In 6-AMC-treated female mice, the mortality increased particularly during the 9th month of the experiment. On the other hand, the mortality rate did not seem to vary between 6 or 9 months of treatment.

In 6-AMC treated mice, both for ventral and dorsal application, the first deaths, due

Table 3. Results of necropsy (gross and histological examinations)

Group	Sex	N	Incidence of tumours*					Number of mice		
			Skin						with one or more tumours	
			papilloma	carcinoma	lung	liver	other sites	without tumours	one or more benign but no malignant tumours	one or more malignant tumours
	♂ dead	9	5	2	3	4	2	3	3	3
I	sacrificed	21	17	9	21	9	3	0	0	21
6-AMC	total	30	22	11	24	13	5	3	3	24
(dorsal appl.)	♀ dead	18	15	5	10	5	5	2	5	11
	sacrificed	12	10	4	11	10	4	0	0	12
	total	30	25	9	21	15	9	2	5	23
II	♂ dead	7	5	4	2	0	2	2	0	5
6-AMC	sacrificed	23	17	5	20	5	1	1	2	20
(ventral appl.)	total	30	22	9	22	5	3	3	2	25
	♀ dead	13	11	5	3	1	2	2	4	7
	sacrificed	17	17	5	15	11	2	0	1	16
	total	30	28	10	18	12	4	2	5	23
III	♂ dead	1	0	0	0	0	0	1	0	0
Excipient	sacrificed	29	1	0	2	0	0	26	1	2
(dorsal appl.)	total	30	1	0	2	0	0	27	1	2
	♀ dead	2	0	0	0	0	0	2	0	0
	sacrificed	28	0	1	1	0	1	26	0	2
	total	30	0	1	0	0	1	20	0	2
IV	♂ dead	1	0	0	0	0	0	1	0	0
Excipient	sacrificed	29	0	0	2	0	0	27	0	2
(ventral appl.)	total	30	0	0	2	0	0	28	0	2
	♀ dead	1	0	0	0	0	0	1	0	0
	sacrificed	29	0	0	0	0	0	29	0	0
	total	30	0	0	0	0	0	30	0	0
V	♂ dead	5	0	0	0	0	0	5	0	0
Untreated	sacrificed	25	0	0	1	0	0	24	0	1
	total	30	0	0	1	0	0	29	0	1
	♀ dead	3	0	0	0	0	0	3	0	0
	sacrificed	27	0	0	2	0	0	25	0	2
	total	30	0	0	2	0	0	28	0	2

*The numbers refer to animals with one or more tumours in the sites indicated. If an animal had both malignant and benign tumours in a particular organ it appears only under the "malignant" heading.

probably to tumours, (pulmonary papillocarcinomas and/or metastatic carcinomas) occurred during the 7th month of the experiment, except for one female of Group I which died from a lymphoma at month 5 and never developed skin tumours. One mouse (No. 15, male, Group I) was found dead during the 8th month; it had a pulmonary tumour and myeloid leukemia but no cutaneous tumours. In other cases, mice with internal tumours always had skin tumours as well.

In mice of control Groups III, IV and V, which died during the experiment, no tumours were found. There were no obvious qualitative or quantitative differences in the distribution of the tumours between mice which died before the completion of the study and mice which were sacrificed after 9 months (see Table 3).

Necropsy of the mice sacrificed at the end of the study. Table 3 shows the incidence and distribution of the tumours.

In the 6-AMC treated mice, cutaneous tumours were found in most of the animals; in controls, only one vehicle-treated mouse bore a papilloma. Pulmonary tumours were also observed in practically all the necropsied mice of Groups I and II but in only 4% of the controls. It should be noticed that in the absence of cutaneous tumours, pulmonary and hepatic neoplasia were found in 8% of the mice of Groups I and II. Skin tumours appeared always in multiple, and pulmonary tumours were numerous in most of the cases. No attempt has been made to count the tumours in each tissue.

Tumours and metastases were found occasionally in different organs like heart (3 mice),

spleen (2 mice), kidney (3 mice), stomach (2 mice), etc.... In the 6-AMC treated groups, incidence and distribution of tumours were not influenced by the site of application of the compound. The final percentage of tumour bearing mice in the 6-AMC groups reached 92 %. The incidence of tumours was 6 % in the vehicle-treated and 5 % in the untreated controls. Several of the observed tumours were malignant.

(*b*) *Histopathological examinations*

All animals were submitted to an histopathological examination of the heart, liver, lung, spleen, kidney and skin. Other organs were examined whenever they showed macroscopical abnormality. Trivial inflammatory or haemorrhagic lesions were common but degenerative lesions and neoplasms were also encountered. A few mice, both treated and controls, had secondary amyloidosis involving, in order of decreasing importance, the spleen, the liver and the kidney. These animals always had suppurative lesions of the skin. Liver parenchyma alterations were very frequent in treated mice, especially females. Most often, hepatocytes had enlarged nuclei and granulous and basophilic cytoplasm. Lobulation became apparent and liver structure tended to become nodular, but fibrous proliferation was exceptional. In some cases mitosis were present and the normal trabecular pattern seemed distorted. Rare foci of steatosis were encountered. But the most significant lesions were necrosis of hepatocytes, isolated at their onset but extending progressively to adjacent cells. These cells underwent chromatolysis and hyalin condensation. Necrotic cells vanished leaving a hole in the parenchyma. As the process went on, cavities were thus created, which became increasingly larger to form cysts containing a liquid, which was sometimes blood-stained, and limited by a thin endothelium. A pseudo-angiomatous appearance, corresponding to that of peliosis, became therefore increasingly evident.

Tumoral pathology concerned almost exclusively the skin and the lung. Tumours in other organs were seldom encountered. A rumen papilloma appeared in a treated animal (Group I) and two cases of lymphosarcoma were found among the mice (Groups I, II) which died in the course of the experiment and which showed other tumours elsewhere.

In a control, a subcutaneous fibrosarcoma was observed (Group III).

Skin tumours only appeared in treated mice. The lesion firstly presented itself as a warty or a papillomatous proliferation. Sometimes the sebaceous glands were hypertrophied. Later on and in a high percentage of cases, those lesions underwent malignant metaplasia and gave rise to spindle-cells carcinoma which infiltrated progressively the dermis and sent metastasis especially to the lungs.

Primitive pulmonary tumours were seen in almost 70 % of treated mice, while they were scarce in the controls. They consisted of multifocal proliferations which seemed to make their origin in alveolar cells which agglutinated in small groups at the extremity of bronchioles. They soon organized themselves in a papillary fashion constituting round nodules of increasing sizes. The tumour showed a curious tendency to invade the lumen of the bronchi where it took a polypoid appearance. The obstruction of bronchi caused areas of secondary atelectasis and of parenchymal suppuration. Malignant histological features were hardly obvious: nuclei remained regular and homogenous, mitoses were few and normal and metastasis did not appear.

DISCUSSION

The data available at the moment do not allow definite conclusions about the bioavailability of 6-AMC to be drawn. Reviewing published data and those presented here, it appears that an estimation of this parameter can be achieved only on the basis of the urinary excretion of the compound.

In mice the results reported here show that the 0/48 hr ^{3}H-urinary excretion accounts for about 3 % of the dose (5 mg) given orally; this figure reaches 7 % after skin application. In rats given labelled 6-AMC, Grantham *et al.* [15] showed that the urinary excretion of the compound and its metabolites is also very low: i.p. injection of 6-AMC-^{14}C leads, in the ten-day urine to ^{14}C-recoveries of about 12 % of the dose (3 % are found during the 0/24 hr period). In humans [16] oral intake (2 g/day for several days) of 6-AMC-12^{3}H studied in four cancer patients gave rise to urinary excretions ranging between 3 and 5 %, 15 days after the first intake of the compound. After i.m. injections (150 mg) the excretion of the label in urine is very slow and reached maximum daily rates of 1·5 % of the dose.

Although these results are obtained under different experimental conditions (different species, different routes of administration, different dosages, different galenic preparations etc....) they all suggest that the bioavailability of the compound is rather low.

The poor bioavailability of 6-AMC has been

invoked to explain the problems that have arisen during the pharmacological [17] and the clinical evaluations of the compound. For example, the low rate of remission in woman breast cancer obtained with the compound was attributed to its low intestinal absorption [9] and justified a new chemical research program for better absorbed derivatives [13].

However since no systematic studies have been achieved yet on the bioavailability of 6-AMC, one may wonder if urinary excretion is a good criterion for the assessment of this parameter. Grantham *et al.* [15] report that in the rat ^{14}C-6-AMC i.p. administered gave rise to a very high ^{14}C-fecal excretion (55 % of the dose 10 days after a single dose). This behaviour indicates a very high biliary excretion in the rat. In humans [16], after oral intake of 6-AMC-12^{3}H, the fecal excretion is also very high (figures of 93 % of the dose have been found). These figures do not necessarily indicate that biliary excretion is involved, but if one takes into account the relatively high plasma levels encountered (max. values observed: about 30 μg/ml in terms of 6-AMC-12^{3}H equivalents) compared to the low urinary excretion, the biliary mode of excretion cannot be excluded in humans as well.

These observations seem to indicate that the bioavailability of 6-AMC is probably better than that expected on the basis of the urinary excretion rate. Consequently, they also reinforce the assumption that in our experimental conditions the percutaneous absorption of 6-AMC is high enough to allow the compound to exert its action. Indeed, 6-AMC undoubtedly appears to be a potent carcinogen, since 92 % of the mice treated with the compound bear tumours compared with 5 % of controls; the ointment base is devoid of effect (tumours found in 6 % of the animals of Groups III and IV).

The carcinogenic effect of 6-AMC is induced relatively rapidly by daily topical applications on the skin: malignant tumours are with certainty recorded 7 months after the start of the experiment, but in fact we cannot exclude that at least internal tumours occur earlier. Benign skin tumours already appear after a 3-month application (ventral). Cancer induction reaches its full development after 6 months of treatment: incidence and distribution of the tumours are entirely comparable in mice treated for 6 or 9 months.

Malignant transformation of chemically-induced cutaneous dysplasia is a well-known phenomenon in experimental carcinogenesis. The action of 6-AMC is in this regard similar to that of some other polycyclic hydrocarbons. As for benzopyrene for instance, wart-like lesions and papillomas appear very soon after the application of the drug on the skin and then become insidiously malignant after a period difficult to estimate, but not exceeding six months. After this time most of the tumours show invasive features and sometimes send metastasis to the lung. It should be emphazised that the primitive cutaneous tumours always occur at the site of application of the carcinogen while other kinds of abnormal growths develop elsewhere as is also observed with the most active carcinogenic benzene derivatives. Most characteristic in our animals are the lung tumours which occur simultaneously with the skin tumours and have been shown in most animals. Histologically these tumours which sometimes occur spontaneously in some strains of mice, do not show much evidence of malignancy. They consist of papillary growths, made up of uniform cylindro-cubic cells, taking place at the lower end of pulmonary lobules, and identical to those induced by urethan, triethylenemelamine and some coaltar derivatives [18, 19]. There are no metastasis in other viscera. However some features such as the multifocal appearance of the tumour, the presence of mitosis, sometimes numerous, and the tendency to invade the bronchi may indicate its potential malignancy. This is probably the reason why both terms, adenoma and adenocarcinoma, are used indifferently in the literature. Except for the absence of secretion its aspect and behaviour can be compared to that of the bronchio-alveolar carcinoma in man.

While it is tempting to imagine a relationship between the hepatic and the pulmonary lesions, one should point out that no delay could be determined between the time of appearance of the skin and the lung tumours that could be explained by a failure in the detoxification activity of the liver. Nevertheless 6-AMC proved very toxic to the liver parenchyma. Isolated cellular destruction apparently gives rise to this peculiar lesion consisting of the formation of irregular blood sinuses and corresponding to peliosis hepatis. This lesion is still poorly understood in human pathology and has been described in relation to the administration of certain steroids [20]. In our material it does not appear as a neoplastic growth similar to angioma but rather as a degenerative process as already suggested by Hamilton and Lubitz [21]. In some cases however, the liver parenchyma tends to assume a nodular aspect without fibrosis, suggestive of some regenerative

mechanism. It is sometimes difficult in these cases, owing to the trabecular distorsions and cellular irregularities and mitoses, to distinguish it from a true hepatoma.

In light of the results presented here proving the carcinogenicity of 6-AMC it could be of interest to compare them with those previously reported on this matter. In the experiment of Rudali *et al.* [1] mice were treated topically on the skin with one drop of an 0·3 % acetonic solution of 6-AMC, twice a week for 30 weeks. It seems possible that a lack of cutaneous absorption and a too-low dosage could explain the absence of tumours at the end of this year-long study: these authors observed, however, a splenic atrophy in most of the mice, which was not observed in the present study and was attributed to the drug. As regards the results obtained by the same authors after i.m. injection, the dosage could have been too low; furthermore we know that by this route, bioavailability is extremely slow and perhaps ineffective [16]. From this point of view it is relatively surprising that Roe and Carter obtained positive results after s.c. injection of 3 times 200 μg of 6-AMC in newborn mice [11, 12]. Considering the characteristics of this drug, one may wonder which is the exact amount effectively absorbed during the neonatal state. The evidence presented here establishes the receptivity of adult mice, provided adequate doses (5 mg/daily) and appropriate galenic preparations are used. It is worth noting that in adults, female mice appear slightly more sensitive than males, while according to Roe *et al.*, the newborn females are far less susceptible. On the other hand, in the experiment of Lambelin *et al.* [10], rats were treated with 6-AMC added to the food at various concentrations; in this case, a lack of absorption cannot explain the absence of carcinogenicity since toxic symptoms were noted at least in the animals treated with the highest doses (37 mg/kg/day) and especially in the females; this study was performed on three successive generations of rats and could, in this way, meet some of the up-to-date recommendations for carcinogenicity testing [22]. To develop possible explanations for these different susceptibilities, it is tempting to speculate on the possibility of species differences concerning the biodegradation of the compound. Unfortunately, the data available in this field are also rather scarce. However in mice, after oral intake, the GLC analyses carried out on the urine collected for the experiments reported in this paper show that the unchanged compound accounts for 25–30 % of the urinary ^{3}H-contents converted in 6-AMC equivalents. In rats [15] after i.p. administration, free water-soluble metabolites and conjugated molecules have been detected. The unchanged compound was found only in the feces. In humans [16] qualitative data indicate that unchanged 6-AMC is possibly present in urine. Finally, another finding in the present study is that the ventral applications of the ointment lead to an earlier apparition of skin tumours than the dorsal applications (a difference of nearly 2–3 months is observed). A definite explanation for such a phenomenon is not evident even if a higher ^{3}H-6-AMC-urinary excretion is observed after ventral application.

The main conclusion of this study is that 6-AMC exhibits, like the other polycyclic hydrocarbons, a carcinogenic activity in mice.

REFERENCES

1. G. Rudali, N. P. Buu-Hoï et A. Lacassagne, Sur quelques effets biologiques du 6-aminochrysène. *C.R. Acad. Sci. (Paris)* **236,** 2020 (1953).
2. Ch. Huggins, L. Grand and R. Kukunishi, Aromatic influences on the yields of mammary cancers following administration of 7,12-Dimethylbenz-(a)anthracene. *Proc. nat. Acad. Sci. (Wash.)* **51,** 737 (1964).
3. N. Back and E. A. Mirand, Inhibition by 6-aminochrysene of spleen transplants and leukemia-induced splenomegaly. In *Proceedings 9th International Cancer Congress Tokyo October* 23–29, 1966, p. 320. International Union Against Cancer, Genève (1967).
4. J. Gelzer and P. Loustalot, Chrysenex® in experimental advanced mammary cancer. *Europ. J. Cancer* **3,** 79 (1967).
5. G. Franchi, L. Moretti and S. Garattini, Experimental chemotherapy of a transplantable rhabdomyosarcoma in the rat. *Europ. J. Cancer* **6,** 441 (1970).
6. N. P. Buu-Hoï, Bui-Quoc-Huong et Tran-Lu-Y, Activité chimiothérapeutique du 6-aminochrysène dans les splénomégalies. *Bull. Acad. nat. Med. (Paris)* **146,** 313 (1962).

7. N. P. Buu-Hoï, Bui-Quoc-Huong, Tran-Lu-Y et Chau-Van-Tuong, Résultats préliminaires d'une tentative de chimiothérapie spécifique au moyen du 6-aminochrysène. *Chemotherapia (Basel)* **4,** 31 (1962).
8. N. P. Buu-Hoï, Bui-Quoc-Huong et Tran-Lu-Y, Action leucopéniante du 6-aminochrysène dans les leucémies myeloides chez l'homme. *Chemotherapia (Basel)* **4,** 413 (1962).
9. Groupe européen du cancer du sein, Induction par le 6-aminochrysène de rémission du cancer sein en phase avancée chez la femme. *Europ. J. Cancer* **3,** 75 (1967).
10. G. Lambelin, G. Mees and N. P. Buu-Hoï, Chronic toxicity studies of 6-aminochrysene in the rat. *Arzneimittel-Forsch.* **17,** 1117 (1967).
11. F. J. C. Roe, R. L. Carter and S. Adamthwaite, Induction of liver and lung tumours in mice by 6-aminochrysene administered during the first 3 days of life. *Nature (London)* **221,** 1063 (1969).
12. R. L. Carter and F. J. C. Roe, The carcinogenic effects of 6-aminochrysene administered to newborn mice. In *Proceedings Europ. Soc. for the Study of Drug Toxicity.* (Edited by S. E. de Baker, J. Jacob, J. Tripod), Vol. XI, p. 167. Excerpta Medica Foundation XI, Amsterdam (1970).
13. H. Tagnon, A. Coune, S. Garattini, R. Rosso, G. Lambelin, M. Gautier and N. P. Buu-Hoï, The antitumoral activity of some derivatives of 6-aminochrysene. *Europ. J. Cancer* **6,** 81 (1970).
14. G. Belvedere, G. Franchi and A. Frigerio, A new sensitive and specific gas chromatographic method for the determination of the carcinostatic agent 6-chrysenamine in biological samples. *J. Chromatogr.* **84,** 171 (1973).
15. P. H. Grantham, Giao Nguyen Ba, L. C. Mohan, E. K. Weisburger, N. P. Buu-Hoï and R. Roncucci, Metabolism of 6-aminochrysene in the rat. *Proceedings of the Annual Meeting of the American Society of Toxicology,* March 1974, *Toxicol. and Appl. Pharmacol.* **29,** 75 (1974).
16. R. Roncucci, M.-J. Simon, G. Lambelin and A. Coune, Unpublished data (1966).
17. G. Franchi, A. Forgione, S. Filipeschi, J. Csetényi and S. Garattini, A spectrophotometric method for the estimation of the carcinostatic agent 6-chrysenamine (EORTC 116) in biological fluids and tissues. *Europ. J. Cancer* **9,** 591 (1973).
18. H. L. Stewart, K. C. Snell, L. J. Dunham and S. M. Schlyers, In *Transplantable and Transmissible Tumors in Animals.* Armed Forces Institute of Pathology, Washington, D.C. (1959).
19. N. K. Clapp, An atlas of RF mouse pathology: disease descriptions and incidences. *United States Atomic Commission* (1973).
20. B. S. Gordon, J. Wolf, T. Krause and F. Shai, Peliosis hepatis and cholestasis following administration of norethandrolone. *Amer. J clin. Path.* **33,** 156 (1960).
21. F. T. Hamilton and J. M. Lubitz, Peliosis hepatis. Report of three cases, with discussion of pathogenesis. *Arch. Path.* **54,** 564 (1952).
22. *Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation,* Panel on Carcinogenesis report on cancer testing in the safety evaluation of food additives and pesticides. *Toxicol. appl. Pharmacol.* **20,** 419 (1971).

Europ. J. Cancer Vol. 11, pp. 335–341. Pergamon Press 1975. Printed in Great Britain

Some Properties of Adenovirus 12-induced Transplantation Antigen, as Measured by the Macrophage Migration Inhibition Test*

C. W. POTTER and R. C. REES†
The University of Sheffield Medical School, Department of Medical Microbiology, Beech Hill Road, Sheffield, England

Abstract—*Mice immunized with cell-free extracts of a transplanted adenovirus 12-induced tumour were relatively immune to subsequent challenge with live, homologous tumour cells. These extracts also stimulated macrophage migration inhibition factor (MIF) from spleen cells from immunized mice. Using the macrophage migration inhibition test as an assay procedure for antigenicity of tumour extracts, the antigens were found to be resistant to heating at 56°C for 2 hr, three extractions with ether and were not precipitated by dialysis at pH 5·0 or 6·0. In contrast, the antigens were destroyed by sodium deoxycholate and sodium dodecyl sulphate, and by dialysis at pH 4·0; the antigen could not be recovered from the precipitate formed by this last treatment. Similar properties were established for tranplantation antigens present in the tumour extracts using the transplantation immunity test to assay for antigen; however, tumour extracts dialysed at pH 5·0 were able to stimulate MIF but did not immunize mice against tumour cell challenge. The properties of the tumour antigen were very similar using the two different assay procedures, and the MMI test is considered to be an assay procedure for the transplantation antigens.*

INTRODUCTION

TUMOUR cells induced by adenovirus 12 have been shown to possess a specific, transplantation antigen [1], since specific immunity to tumour cell challenge can be produced by immunization with adenovirus 12 or killed or sublethal doses of homologous tumour cells [2, 3]. In addition, immunization with cell-free extracts of a transplanted adenovirus 12-induced tumour of CBA mice produced immunity to challenge with homologous tumour cells [4, 5]; three to five weekly inoculations were required before immunity could be demonstrated, and tumour extracts were found to vary in antigenicity. However, the presence of transplantation antigen(s) in cell-free tumour extracts afforded an opportunity to examine the properties of this antigen, and the sensitivity of the antigen to heat, detergents, enzymes and pH was determined by *in vivo* transplantation immunity studies [6]. These studies were limited by the large numbers of animals required to assay for the transplantation antigen(s).

Using adoptive transfer tests, the transplantation immunity induced with cell-free extracts of adenovirus 12-induced tumours was shown to be cell-mediated [7]. Cellular immune reactions were also demonstrated by the *in vitro* macrophage migration inhibition test [8]; thus, lymphocytes from CBA mice immunized with adenovirus 12-induced tumour extracts pro-

Accepted 1 March 1974.

*This work was financed partly by the Cancer Research Campaign and the Ernest and Minnie Dawson Cancer Research Fund, Preston, Lancashire.

†Present address: Cancer Research Campaign Laboratories, The University of Nottingham, University Park, Nottingham, NG7 2RD.

duced macrophage migration inhibition factor (MIF) when incubated with cell-free extracts of adenovirus 12-induced tumours, and this test can be used to assay transplantation antigen present in the tumour extracts. The present report describes some properties of the antigen which stimulates MIF production *in vitro*, and the results compared with studies using *in vivo* transplantation immunity tests, using tumour extracts treated in a similar manner.

MATERIAL AND METHODS

Adenovirus 12-induced tumour cells

Adenovirus 12-induced tumour cells were obtained from tumours serially transplanted in CBA mice. Tumours of 10–15 mm dia were removed, minced and treated with 0·25 % trypsin to give a single cell suspension. The cells were washed in two changes of Eagle's minimal essential medium containing 2 % inactivated foetal calf serum, resuspended in the same medium to a concentration of $5{\cdot}0 \times 10^6$ viable cells/ml, and used immediately. Each mouse was inoculated s.c. with 0·1 ml of the tumour cell suspension; this dose produced tumours in 90–100 % of animals within five weeks [4].

Tumour antigen extracts

Transplanted adenovirus 12-induced tumours of 10–20 mm dia were removed from groups of CBA mice and freed of necrotic tissue. The tumour tissue was cut into small fragments, washed in Hank's saline and centrifuged at 600 ***g*** for 15 min. The deposited tumour tissue was suspended in Hank's saline to a 20 % (v/v) concentration, briefly homogenized using a Waring blender and frozen at −80°. After thawing at 20°, the tumour extract was centrifuged at 100,000 ***g*** for 1 hr and the supernatant filtered through a Millipore filter of 0·45 μM pore size. The cell-free extracts were stored at −80°C.

Batches of antigen were tested for their ability to immunize CBA mice against transplanted homologous tumour cells, and to induce macrophage inhibiting factor from spleen cells from immunized CBA mice; only extracts which had these properties were used in the present study. Cell-free extracts were also prepared from normal CBA mouse tissue in the same manner.

Guinea pig macrophages

Guinea pigs (250–300g) were inoculated i.p. with 20 ml of sterile paraffin oil. Seven to ten days following inoculation, the animals were killed and the peritoneal cavity washed with 200–300 ml of Hank's saline. After allowing the paraffin oil to separate in a separating funnel, the macrophage suspension in Hank's saline was removed and centrifuged at 500 ***g*** for 5 min. The deposited macrophages were washed with three changes of medium "199", and resuspended in the same medium to a 5 % (v/v) concentration.

Spleen cell cultures

Groups of CBA mice were inoculated s.c. with three or five doses of 0·1 ml cell-free tumour antigen extract given at weekly intervals; previous studies have shown that this procedure produced a relative immunity to transplanted tumour cells [6, 7]. Five to seven days after the final inoculation, the mice were killed and the spleens removed; spleens were also taken from normal mice and treated in an identical manner. The spleens were finely chopped, further disrupted by extrusion through a 2·0 ml syringe without a fitted needle, and filtered through sterile cotton gauze. The cell suspension, which consisted mostly of single cells, was centrifuged at 1000 ***g*** for 10 min, and distilled water was added to the deposited cells to flash-lyse the red cells; 3·0 ml of water was added to 0·1 ml of cell deposit for 30 sec, and the medium was then restored to isotonicity with an equal volume of 0·3 M NaCl. The cell suspension was washed three times in medium "199", and the cells suspended in the same medium to a concentration of $1{\cdot}0 \times 10^7$ spleen cells/ml.

Treatment of antigen extracts

Heat stability. Antigen extracts were thawed, and placed in a water bath at 56°C for periods ranging from 30 min to 3 hr. Following treatment the extracts were stored at –80°C prior to testing for antigenicity.

Detergent treatment. Antigen extracts were dialysed for 18 hr at 4°C against 0·5 % or 0·05 % sodium deoxycholate (B.D.H. Chemicals Ltd., Poole, Dorset) dissolved in phosphate buffered saline, pH 7·4 (PBS). Batches of antigen were also dialysed against 0·5 % or 0·05 % sodium dodecyl sulphate in PBS. Following dialysis, the treated extracts were redialysed against PBS for 24 hr before testing for antigenicity.

Effect of pH. Tumour extracts were dialysed

at 4°C against 0·15 M NaCl buffered to pH 4·0 or 5·0 with 0·01 M citrate buffer, or 0·15 M NaCl buffered to pH 6·0 with 0·01 M phosphate buffer. After dialysis, the antigen extracts were centrifuged for 30 min at 2000 rev/min and the supernatant fluid redialysed against PBS pH 7·4. The precipitates were suspended in PBS to the original volume of tumour extract and also redialysed against PBS; the precipitate remaining after dialysis was removed by centrifugation. All the extracts were stored at −80°C.

Ether treatment. Cell-free extracts from tumour cells were treated with an equal volume of peroxide-free ether. After mixing for 10 min at 4°C the ther was removed by aeration at 37°C with 5 % CO_2 in air. Ether treatment was carried out three times, and the preparations were then stored at −80°C.

Macrophage migration inhibition (MMI) tests

These tests were carried out using the indirect macrophage migration inhibition test [9, 10]. Two-four ml of spleen cells (at a concentration of 1×10^7 cells/ml) from CBA mice immunized with tumour antigen extracts were placed in a 45 mm dia petri dish (Sterilin), and treated or non-treated control tumour cell extract was added to a final concentration of 10 % (v/v); a negative control spleen cell culture, which did not contain tumour antigen, was put up with every test. After incubation at 37°C for 72 hr in 5 % CO_2 in air, the culture fluids were collected; the control cultures were reconstituted after incubation with treated or non-treated tumour extract to a 10% (v/v) concentration, as used for the test culture. Culture fluids were centrifuged at 2000 ***g*** for 10 min, and the cell-free supernatant fluids dialysed for 48 hr against medium "199". After dialysis, inactivated foetal calf sera was added to a final concentration of 10 %, and the fluids tested for macrophage migration inhibition factor (MIF), using guinea pig peritoneal macrophages.

Guinea pig macrophage suspensions were placed in 10 µl haematocrit tubes and centrifuged at 500 ***g*** for 5 min. The tubes were cut at the deposited cell–liquid interface, and the packed cells were fixed to the bottom of individual wells of a migration chamber (Sterilin) with silicone grease. The chambers were completely filled with the fluids being assayed for MIF, and the wells sealed with a glass coverslip. The chambers were incubated at 37°C, and the area of macrophage migration was determined by planimetry after 24 and 48 hr incubation. Two to four tests were carried out for each fluid tested for MIF. The per cent migration inhibition was calculated from the formula:—

$$\% \text{ migration} = 100 \left(\frac{\text{(average migration in presence of test antigen}}{\text{(average migration in presence of control culture}} \times 100 \right)$$

The statistical significance of migration inhibition was calculated using Students *t* test.

RESULTS

Effect of heating at 56°C

Aliquots of tumour antigen extract were heated at 56°C for periods of 30 min, 1 hr and 3 hr, and then tested for the ability to stimulate MIF from spleen cells from immunized mice. The heated antigen extracts were also incubated with spleen cells from normal mice as controls. The results, which are given in Table 1, show that spleen cells from immunized mice produced MIF in the presence of tumour extract heated at 56°C for 30 min, since fluids from these cultures significantly inhibited the migration of guinea pig macrophages ($P = < 0{\cdot}01$); similar results were recorded after 24 and 48 hr migration (Table 1). MIF production was also shown for sensitized spleen cells incubated with tumour extract heated at 56°C for 1 hr and 3 hr; thus, tumour extracts were not inactivated by heating at 56°C for these times. Supernatant fluids from cultures of normal spleen cells incubated with heated tumour extracts did not inhibit guinea pig macrophage migration; these fluids occasionally produced a marked stimulation of migration.

Effect of detergent treatment

Tumour antigen extracts were dialysed against 0·5 or 0·05 % (w/v) sodium deoxycholate (DOC) or sodium dodecyl sulphate (SDS); after redialysis against PBS, the extracts were tested for their ability to stimulate MIF from spleen cells from CBA mice immunized with 3–5 inoculations of tumour extract. The results are shown in Table 2. Supernatant fluids from cultures of immune spleen cells incubated with untreated tumour antigen extract contained MIF, since these fluids inhibited the migration of guinea pig macrophages (Table 2); the inhibition was 30 % at 24 hr ($P = < 0{\cdot}05$), and 28 % at 48 hr ($P = < 0{\cdot}05$). The tumour antigen extract did not induce MIF production from normal spleen cells. The ability of tumour antigen extracts to stimulate MIF from sensi-

Table 1. *Production of MIF by spleen cells from normal and immunized CBA mice incubated with tumour extrac heated at* 56°C

Spleen cells from:	Treatment of tumour extract	Macrophage migration area (24 hr)	±	s.d.	% Inhibition	Macrophage migration area (48 hr)	±	s.d.	% Inhibition
Immunized CBA mice	Nil*	7·99	±	0·23	—	10·13	±	0·63	—
	56°C/½ hr	5·72	±	0·58	28‡	7·1	±	0·65	31‡
	56°C/1 hr	5·75	±	0·46	28‡	6·74	±	0·21	33‡
	56°C/3 hr	4·03	±	0·11	50‡	5·85	±	0·64	42†
Normal CBA mice	Nil*	2·43	±	0·04	—	2·55	±	0·07	—
	56°C/½ hr	4·58	±	0·78	—	5·64	±	0·40	—
	56°C/1 hr	5·45	±	1·10	—	9·98	±	0·07	—
	56°C/3 hr	6·21	±	0·23	—	6·58	±	0·33	—

*Spleen cell cultures reconstituted with 10% (v/v) tumour extract after incubation at 37°C for 3 days.
†Significant inhibition of macrophage migration ($P = < 0·05$).
‡Significant inhibition of macrophage migration ($P = < 0·01$).

Table 2. *Production of MIF by spleen cells from normal and immunized CBA mice incubated with tumour extract treated with SDS and DOC*

Spleen cells from	Treatment of tumour extract	Macrophage migration area (24 hr)	±	s.d.	% Inhibition	Macrophage migration area (48 hr)	±	s.d.	% Inhibition
	Nil*	2·22	±	0·20	—	5·47	±	0·40	—
Immunized CBA mice	Not treated	1·57	±	0·40	30†	3·97	±	1·06	28†
	SDS 0·5%	2·7	±	0·46	—	6·3	±	0·70	—
	SDS 0·05%	2·37	±	0·26	—	6·02	±	0·30	—
	DOC 0·5%	2·98	±	0·23	—	5·63	±	0·29	—
	DOC 0·05%	2·8	±	0·30	—	5·4	±	0·26	—
	Nil*	2·7	±	0·53	—	6·23	±	1·36	—
Normal CBA mice	Not treated	2·4	±	0·18	11	5·47	±	0·15	12
	SDS 0·5%	2·62	±	0·29	4	6·3	±	0·10	—
	SDS 0·05%	2·45	±	0·26	9	6·77	±	0·72	—
	DOC 0·5%	2·87	±	0·25	—	5·92	±	0·85	6
	DOC 0·05%	2·65	±	0·80	2	5·47	±	0·50	12

*Spleen cell cultures reconstituted with 10% (v/v) tumour extract after incubation at 37°C for 3 days.
†Significant inhibition of macrophage migration ($P = < 0·05$).

tized spleen cells was destroyed by all the detergent treatments tested; in most cases supernatant fluids from immune spleen cells incubated with treated tumour extracts produced slight stimulation of guinea pig macrophage migration. Culture fluids from detergent-treated tumour extracts and normal CBA mouse spleen cells did not affect the migration of guinea pig macrophages.

Effect of ether treatment

The effect of three extractions with ether on the ability of tumour antigen extracts to stimulate MIF from spleen cells from immunized mice is shown in Table 3. Supernatant fluids from cultures of immune spleen cells incubated with treated tumour extract inhibited guinea pig macrophage migration by 12% at 24 hr and by 32% at 48 hr; the inhibition recorded at 48 hr was statistically significant ($P = < 0·05$); this result indicated that the antigenicity was not inactivated by ether treatment.

Effect of pH treatment

Table 4 shows the results of macrophage migration inhibition tests using fluids from cultures of immune spleen cells incubated with supernatants or reconstituted precipitates from tumour extracts previously dialysed against buffers of different pH. Fluids from cultures of spleen cells incubated with supernatant fluids obtained by dialysis of tumour extracts at

Table 3. Effect of ether on adenovirus 12-induced tumour extract, and assayed by the macrophage mig ration inhibition test

Source of spleen cells	Treatment of tumour extract	Macrophage migration area (24 hr)	±	s.d.	% Inhibition	Macrophage migration area (48 hr)	±	s.d.	% Inhibition
Immunized mice	Ether X 3	3·28	±	0·60	12	4·65	±	1·20	32*
	Control†	3·65	±	0·78		6·85	±	0·78	
Normal mice	Ether X 3	4·85	±	0·35	—	7·60	±	0·57	—
	Control	3·88	±	1·73		6·40	±	2·55	

*Significant inhibition of macrophage migration ($P = < 0·05$).
†Spleen cell cultures reconstituted with ether-treated tumour extract after incubation at 37°C for 3 days.

Table 4. Production of MIF by sensitised spleen cells incubated with tumour extracts treated at different pHs

Exp. No.	Spleen cells from	Treatment of tumour extract	Macrophage migration area (24 hr)	±	s.d.	% Inhibition	Macrophage migration area (48 hr)	±	s.d.	% Inhibition
1	Immune CBA mice	Control*	4·70	±	0·84		8·78	±	1·73	
		pH 6·0 (supern)	1·84	±	0·11	65†	2·33	±	0·18	74†
		pH 6.0 (ppt)	5·40	±	0·28	—	8·10	±	0·28	8
		pH 5·0 (supern)	2·14	±	0·40	54†	2·70	±	0·14	69†
		pH 5·0 (ppt)	4·68	±	0·18	—	7·85	±	0·85	15
	Normal CBA mice	Control	4·81	±	1·36		10·03	±	1·52	
		pH 6·0 (supern)	5·03	±	0·25	—	8·35	±	0·35	17
		pH 6·0 (ppt)	5·58	±	0·46	—	10·18	±	0·32	—
		pH 5·0 (supern)	4·08	±	0·46	15	7·8	±	0·00	22
		pH 5·0 (ppt)	5·78	±	0·18	—	8·38	±	0·38	16
2	Immune CBA mice	Control	5·33	±	1·03		5·55	±	0·00	
		pH 4·0 (supern)	5·20	±	0·17	2	7·13	±	0·51	—
		pH 4·0 (ppt)		NT		NT		NT		NT
	Normal CBA mice	Control	5·7	±	0·56		7·13	±	0·51	
		pH 4·0 (supern)	4·83	±	0·49	15	5·20	±	0·56	27
		pH 4·0 (ppt)		NT		NT		NT		NT
3	Immune CBA mice	Control	5·33	±	1·03		5·55	±	0·00	
		Nil	2·40	±	0·36	55†	2·15	±	0·21	61†
	Normal CBA mice	Control	5·7	±	0·56		7·13	±	0·51	
		Nil	4·9	±	0·61	14	5·60	±	1·35	21

*Supernatant from control cultures not reconstituted with tumour extract.
†Significant inhibition of macrophage migration ($P = < 0·05$).
NT—Not tested.

pH 5·0 or 6·0 produced MIF. In both cases, the inhibition recorded after 24 and 48 hr was greater than 50 %, and was statistically significant ($P = < 0·05$); thus, the relevant antigens present in the tumour extracts were not precipitated at these pH values (Table 4, Exp. 1). The reconstituted precipitates from tumour extract dialysed at pH 6·0 and 5·0 did not stimulate MIF from immune spleen cells. Since 60 % of the protein was precipitated by dialysis at pH 5·0, this represente a preliminary step in the purification of the antigens from tumour extracts.

The supernatant fluids from tumour extracts dialysed against buffer at pH 4·0 did not stimulate MIF from immune spleen cells (Table 4, Exp. 2). Thus, the tumour antigen extracts were destroyed or precipitated by this treatment, and the antigen could not be reconstituted by dialysis at pH 7·0. Control, untreated extract produced MIF when incubated with sensitized spleen cells (Table 4, Exp. 3).

Correlation of MMI tests and transplantation immunity tests

The ability of cell-free extracts of adenovirus 12-induced tumour cells to induce transplantation immunity after treatment with heat, ether, detergents and dialysis at different pH values has been reported previously [6]. Groups of CBA mice were immunized at weekly intervals for three weeks with an 0·1 ml volume of treated tumour extract preparation; a control group of mice received an extract of normal CBA mouse tissue treated in an identical manner. Two weeks following the third inoculation, all the mice were inoculated s.c. with $5{\cdot}0 \times 10^5$ viable adenovirus 12-induced tumour cells in an 0·1 ml vollme. The incidence of tumours in the mice five weeks after tumour cell inc culation is shown in Table 5. Since the treatments of the tumour extracts were identical for the transplantation immunity and the MMI studies, the results of the two tests are compared.

Tumour antigen extracts heated at 56°C for 30 min or extracted three times with ether were able to induce MIF production from sensitized spleen cells, and to immunize mice against transplanted tumour cells (Table 5). In contrast, tumour antigen extracts previously dialysed against 0·5 % DOC or SDS did not immunize against tumour cell challenge or induce MIF production from spleen cells from immune mice. Immunization with supernatant fluids from tumour extracts dialysed at pH 4·0 or 5·0 did not induce transplantation immunity, and the antigens could not be recovered from the precipitates by redialysis. In contrast, the supernatant fluid obtained by dialysis at pH 5·0 was found to stimulate MIF production. The supernatant obtained after dialysis at pH 4·0 did not stimulate MIF from sensitized spleen cells (Table 5). Thus, the results of the two tests agree, except that obtained for dialysis at pH 5·0; after this treatment, the supernatant fluids were able to induce MIF production, but did not immunize mice against transplanted tumour cells.

Table 5. Properties of tumour antigen extracts, as measured by MIF tests and by transplantation immunity tests

Treatment of tumour antigen	Incidence of tumours following challenge of immunized mice (5×10^5 viable adenovirus 12-induced tumour cells)				Significant inhibition	MIF production following treatment
	Tumour antigen	(%)	Normal antigen	(%)		
Heat (56°C/30 min)	7/20	(35)	19/20	(95)	$+(P = < 0{\cdot}001)$	$+(P = < 0{\cdot}01)$
SDS (0·5%)	17/19	(89)	20/20	(100)	—	—
DOC (0·5%)	18/19	(95)	19/21	(90)	—	—
Ether × 3	8/20	(40)	18/19	(95)	$+(P = < 0{\cdot}001)$	$+(P = < 0{\cdot}05)$
pH 6·0 (supern)	5/18	(28)	20/20	(100)	$+(P = < 0{\cdot}001)$	$+(P = < 0{\cdot}05)$
pH 6·0 (ppt)	18/20	(90)	18/19	(94)	—	—
pH 5·0 (supern)	19/20	(95)	18/19	(94)	—	$+(P = < 0{\cdot}05)$
pH 5·0 (ppt)	19/19	(100)	19/19	(100)	—	—
pH 4·0 (supern)	19/19	(100)	18/19	(94)	—	—
pH 4·0 (ppt)	17/18	(94)	20/20	(100)	—	NT
—	5/19	(26)	17/18	(94)	$+(P = < 0{\cdot}001)$	$+(P = < 0{\cdot}05)$

DISCUSSION

Previous studies have shown that mice immunized with cell-free extracts from a transplanted adenovirus 12-induced tumour of CBA mice gave significant transplantation immunity against challenge with viable tumour cells [4, 5]. The extracts were prepared by freezing and thawing, centrifugation at 100,000 *g* for 1 hr and filtration of the supernatant fluids through a Millipore filter of 0·45 μM pore size; thus, the transplantation antigen present in the cell-free extract may have been in a soluble form [4]. In this form, it is possible to examine the properties of these antigen(s), and to investigate methods for the purification and further analysis of the transplantation antigens. By using an *in vivo* transplantation immunity test as an assay procedure, some of the properties of the transplantation antigen have been investigated [6]. However, this test is both costly and time-consuming; furthermore, for the assay of fractions obtained by immunochemical methods this method was impractical. Since the transplantation immunity induced in CBA mice by immunization with tumour extracts was shown to be cell-mediated [7], the present study was

carried out to determine if the MMI test could be used to assay the same transplantation antigen(s). For the MMI test to be suitable as an alternative assay method, the test would have to give results similar to those obtained using the transplantation immunity test.

The transplantation immunity test and the MMI test gave similar results for the effect on the tumour extract of heating at 56°C, ether extraction and detergent treatment. Antigen activity was detected in the supernatant after dialysis against buffer at pH 6·0 by both tests. Both tests showed that the antigen in the tumour extract was destroyed or precipitated by dialysis at pH 4·0, and that the antigenic activity could not be demonstrated in the reconstituted precipitate. In contrast, supernatant fluids obtained by dialysis at pH 5·0 were able to stimulate MIF production from sensitized spleen cells, but did not immunize mice against transplanted tumour cells; this was the only contradiction between the two tests found in the present study. The reason for this discrepancy is not known; however, since the amount of antigen required for the two tests may be quantitatively different, the results may be due to the amount of antigen present in the supernatant after dialysis at pH 5·0. Thus, the results indicate that the antigen present in tumour extracts is precipitated at about pH 5·0; partial precipitation of the antigen could diminish the antigen content significantly for the transplantation immunity test but not for the MMI test.

With the above exception, the transplantation immunity test and the MMI test showed the same properties for tumour extracts, and this result indicated that the two tests measure the same antigen. Since, by definition, the transplantation immunity test measures the transplantation antigen, the MMI test represents a more convenient assay for the same antigen(s). This latter test can be used to determine the properties of the transplantation antigen and provides a more practical assay method to monitor purification of these antigens

Acknowledgements—We would like to thank Professor Sir Charles Stuart-Harris and Professor M. G. McEntegart for their advice and criticism, and Mr. A. Clegg for excellent technical assistance.

REFERENCES

1. L. W. Law, Studies of the significance of tumour antigens in induction and repression of neoplastic diseases: Presidential Address. *Cancer Res.* **29,** 1 (1969).
2. J. J. Trentin and Estelle Bryan, Immunization of hamsters and histogenic mice against transplantation of tumours induced by human adenovirus type 12. *Proc. Amer. Ass. Cancer Res.* **5,** 64 (1964).
3. L. D. Berman, On the nature of transplantation immunity in the adenovirus tumour system. *J. exp. Med.* **125,** 988 (1967).
4. C. W. Potter and J. S. Oxford, Transplantation immunity following immunization with extracts of adenovirus 12 tumour cells. *Int J. Cancer* **6,** 410 (1970).
5. R. C. Rees and C. W. Potter, Specific enhancement of transplantation immunity with heat-killed Mycobacterium butyricum and immunizing extracts from adenovirus 12-induced tumour cells. *Brit. J. Cancer* **26,** 139 (1972).
6. E. G. Brown, C. W. Potter and J. S. Oxford, Properties of transplantation antigen present in cell-free extracts of adenovirus 12. *Europ. J. Cancer* **8,** 9 (1971).
7. R. C. Rees and C. W. Potter, *In vivo* studies of cell-mediated and humoral immune responses to adenovirus 12-induced tumour cells. *Arch. ges. Virusforsch.* **41,** 116 (1973 a).
8. R. C. Rees and C. W. Potter, Immune response to adenovirus 12-induced tumour antigens, as measured *in vitro* by the macrophage migration inhibition test. *Europ. J. Cancer* **9,** 497 (1973 b).
9. M. George and J. H. Vaughan, *In vitro* cell migration as a model for delayed hypersensitivity. *Proc. Soc. exp. Biol. (N.Y.)* **111,** 514 (1962).
10. J. R. David, S. Al-Askari, H. S. Lawrence and L. Thomas, Delayed hypersensitivity *in vitro*—I. The specificity of inhibition of cell migration by antigens. *J. Immunol.* **93,** 264 (1964).

Europ. J. Cancer Vol. 11, pp. 343–349. Pergamon Press 1975. Printed in Great Britain

Methotrexate, Cyclophosphamide and Vincristine (MEV Regimen) for non-Hodgkin's Lymphoma

FRANCESCO LAURIA, MICHELE BACCARANI, MAURO FIACCHINI, MARCO GOBBI and SANTE TURA

Insegnamento di Ematologia dell'Università e Servizio di Ematologia dell'Ospedale Sant'Orsola di Bologna, Via G. Massarenti, 9, 40138 *Bologna, Italie*

Abstract—*Five courses of combination chemotherapy with methotrexate, cyclophosphamide and vincristine (MEV regimen) were given on an outpatient basis to sixteen previously untreated patients with lymphocytic lymphoma (L.L.) and to fourteen previously untreated patients with histiocytic lymphoma (H.L.) Clinical and haematological tolerance was such that the interval between each course was never prolonged, nor was drug dosage reduced (vincristine excepted).*

Of the 16 *cases with L.L., there were* 9 *complete remissions,* 5 *of them being continuously unmaintained complete remissions for* 5 *to* 24 *months. Of the* 14 *cases with H.L., there were* 13 *complete remissions,* 7 *being continuously unmaintained complete remissions for* 10 *to* 34 *months.*

It is suggested that the combination of cell-cycle phase-specific drugs with an alkylating agent may prove to be more than additive in effect in non-Hodgkin's lymphoma. The very high remission rate in H.L. warrants further investigation.

INTRODUCTION

COMBINATION chemotherapy, mainly the MOPP regimen, has been shown to be much more effective for Hodgkin's disease than single drug administration [1, 2]. Survival of stage IV patients ineligible for curative radiotherapy has been markedly improved by MOPP and a significant number of these patients are disease-free five years after treatment [3]. In non-Hodgkin's lymphoma a number of clinical trials assaying different polychemotherapeutic regimens have been established [4–9]. The results have been superior to chemotherapy with single agents, but are not as impressive as in Hodgkin's disease. Moreover, few, if any cases of non-Hodgkin's lymphoma with a prolonged disease-free survival have been selected. Cyclophosphamide has been the fundamental drug in most trials, and has been given in different schedules, mainly in combination with vincristine and prednisone. From animal tumor system studies, the suggestion has come that combination of an alkylating agent with a cell-cycle active drug may be more than additive, and may be synergistic [10–12]. Preliminary results of a combination including methotrexate, cyclophosphamide and vincristine (MEV regimen) have been encouraging [13]. The purpose of the present paper is to report on 30 previously untreated patients with non-Hodgkin's lymphoma submitted to MEV regimen.

Accepted 10 February 1975.

Correspondence to: Dr. Francesco Lauria, Servizio di Ematologia, Policlinico S. Orsola, Via G. Massarenti, 9, 40138 Bologna, Italy.

MATERIAL AND METHODS

All adult patients first seen at the Division of Haematology, Bologna, since September 1971, with biopsy-proven non-Hodgkin's lymphoma and ineligible for radical radiotherapy, entered the trial. All received five consecutive

courses, at intervals of twelve to fifteen days, of cyclophosphamide (800 mg/m^2 in 4 hr infusion on the first day), methotrexate (20 mg/m^2, i.m., on the third day) and vincristine (2 mg/m^2, i.v., on the fourth day).

Complete blood counts were repeated at the start of every cycle.

The histopathologic classification was done according to Rappaport [14]. Staging was the same as for Hodgkin's lymphoma, i.e., according to Ann Arbor criteria [15]. The staging was clinical (complete blood counts, marrow and bone biopsy, chest and G.I. X ray, i.v. pyelography, lower extremity lymphangiography, liver and spleen ^{199}Tc scan, serum alkaline phosphatases, SGOT, SGPT, ESR, serum protein electrophoresis) in 24 patients, and pathological (laparotomy and splenectomy) in 6 patients.

Pertinent data are listed in Tables 1 and 2. Sixteen patients with previously untreated L.L. and 14 patients with previously untreated H.L. entered the trial and completed MEV therapy at least by May, 1974. Most patients with either types of lymphoma had diffuse subtypes. There were a total of 4 patients in stages I and II, who were considered ineligible for radical radiotherapy because of advanced age. However, 2 of them, who had localized supradiaphragmatic disease, received local radiotherapy. Two patients with H.L. and one with L.L. were given total nodal irradiation (TNI) during MEV-induced complete remission. Neither maintenance nor reinduction therapy was given to all the other patients who entered complete remission.

Complete remission (C.R.) has been defined as the complete absence of any clinical, radiological and biochemical evidence of lymphoma. Incomplete remission (I.R.) has been defined as a good and prolonged response, but with perceptible disease nevertheless. Remission duration and survival have been calculated from the end of treatment.

RESULTS

Results of MEV therapy in L.L. are tabulated in Table 1. Nine patients of 16 achieved C.R.; 4 of these have relapsed after 2, 5, 6 and 32 months respectively. Five are in C.R. after 5, 13, 13, 24 and 24 months respectively. Two of the patients who relapsed died 5 and 19 months after completion of MEV, while 2 are alive after 18 and 35 months. Five patients achieved I.R.: 1 of them died after 15 months, and 4 are alive 14 to 17 months after their I.R. Two patients failed to respond and both died 7 months after treatment.

In contrast, in patients with H.L. no failure has been recorded (Table 2). Thirteen cases out of 14 achieved C.R. Six of them have relapsed

Table 1. *The effect of MEV therapy in* 16 *previously untreated patients with lymphocytic lymphoma*

Patient	Age	Sex	Clinical stage	Histology	Response	Duration of C.R. (months)	Survival after therapy complete (months)	Additional radiotherapy
D.M.	65	F	IV-B	NWD	I.R.	/	17+	
C.G.	56	M	*IV-A	NPD	C.R.	32	35+	
V.G.	33	M	IV-A	NPD	F.	/	7	
R.M.M.	68	M	III-A	DWD	C.R.	5	19	
A.R.	62	M	I-A	DWD	C.R.	13+	13+	local RX
B.G.	63	M	IV-B	DWD	I.R.	/	15	
D.V.G.	72	M	IV-A	DWD	I.R.	/	15+	
Z.B.	48	M	IV-A	DWD	I.R.	/	17+	
P.J.	43	F	III-A	DPD	C.R.	24+	24+	
M.A.	50	F	III-A	DPD	C.R.	24+	24+	TNI
A.G.	58	M	IV-B	DPD	C.R.	5	18+	
M.G.	40	M	IV-A	DPD	C.R.	5+	5+	
N.C.	39	M	*IV-A	DPD	C.R.	13+	13+	
P.F.	48	M	III-A	DPD	C.R.	2	5	
C.S.	71	M	III-A	DPD	I.R.	/	14+	
S.N.	53	F	IV-B	DPD	F.	/	7	

* = Pathological Stage

NWD = nodular well differentiated, NPD = nodular poor differentiated, DWD = diffuse well differentiated, DPD = diffuse poor differentiated. C.R. = complete remission, I.R. = incomplete remission F. = failure. TNI = total nodal irradiation. + = still in C.R. and/or surviving.

after 2, 2, 8, 10 and 17 months respectively. Seven are still in C.R. after 10 to 24 months. One of the patients who relapsed died after 18 months, while the other 5 are still alive after 7 to 31 months. The only patient who achieved I.R. is alive 13 months after MEV.

Two of 10 relapsed patients have been given another course of MEV therapy: one C.R. and one I.R. were achieved.

The complete response-rate for both lymphomas according to histologic subtypes and to the stage, is shown in Table 3.

Blood counts were made before every course or more frequently, if necessary. Mild neutropenia (1000–1500/μl) was recorded in 4 patients, who recovered promptly, so that in no case was MEV discontinued, nor was the interval between each course prolonged. In no case was the drug dosage reduced, with the exception of vincristine in some cases, because of the development of neurotoxicity. Thrombocytopenia under 100,000/μl was never recorded. Haemoglobin levels did not change significantly during MEV therapy.

DISCUSSION

The remission rate obtained with MEV therapy has been very high in H.L. and the difference with respect to the remission rate in

Table 2. The effect of MEV therapy in 14 previously untreated patients with histiocytic lymphoma

Patient	Age	Sex	Clinical stage	Histology	Response	Duration of C.R. (months)	Survival after therapy complete (months)	Additional radiotherapy
F.F.	38	M	IV-A	Nodular	C.R.	10	29+	
B.O.	64	F	I-A	Nodular	C.R.	16+	16+	local Rx
M.L.	68	F	II-B	Nodular	C.R.	16+	16+	
P.A.	67	F	II-A	Nodular	I.R.	/	13+	
C.R.	58	M	III-A	Diffuse	C.R.	34+	34+	
G.E.	62	F	*IV-B	Diffuse	C.R.	32+	32+	
C.A.	18	F	*III-B	Diffuse	C.R.	21+	21+	TNI
B.P.	32	M	IV-B	Diffuse	C.R.	16+	16+	
M.A.	44	F	III-A	Diffuse	C.R.	10+	10+	
B.M.	45	M	III-A	Diffuse	C.R.	8	18	
P.D.	53	F	III-B	Diffuse	C.R.	2	21+	
D.M.A.	46	F	*IV-A	Diffuse	C.R.	8	21+	TNI
F.M.	52	F	IV-A	Diffuse	C.R.	17	31+	
T.M.	35	F	*III-B	Diffuse	C.R.	2	7+	

* = Pathological Stage.
C.R. = complete remission. I.R. = incomplete remission. TNI = total nodal irradiation. + = still in C.R. and/or surviving.

Table 3. Complete remission rate according to histologic subtypes, and to clinical stages

		I-A	II-A	II-B	III-A	III-B	IV-A	IV-B	*Total
Lymphocytic lymphoma	NWD	/	/	/	/	/	/	0/1	0/1
	NPD	/	/	/	/	/	1/2	/	1/2
	DWD	1/1	/	/	1/1	/	0/2	0/1	2/5
	DPD	/	/	/	3/4	/	2/2	1/2	6/8
TOTAL		1/1	/	/	4/5	/	3/6	1/4	9/16
		I-A	II-A	II-B	III-A	III-B	IV-A	IV-B	*Total
Histiocytic lymphoma	Nodular	1/1	0/1	1/1	1/1	/	/	/	3/4
	Diffuse	/	/	/	1/1	4/4	3/3	2/2	10/10
TOTAL		1/1	0/1	1/1	2/2	4/4	3/3	2/2	13/14

* = Number of patients in C.R./Number of patients.

L.L. is impressive, although several factors may account for it. Sex distribution was not identical, males being predominant in the L.L. group, and females in the H.L. group. Although better prognosis has been demonstrated in Hodgkin's disease for females [16], no sex difference has been found in the prognosis of non-Hodgkin's lymphoma [17, 18]. Secondly, the distribution according to clinical stage (or to pathological stage) was different, as there were 10 out of 16 patients in stage IV in the L.L. group, but only 5 of 14 patients in stage IV in the H.L. group. Most of this difference was accounted for by the more frequent bone-marrow involvement in L.L., as commonly observed [18, 19]. However, the C.R. rate in stage IV patients was still much higher in H.L. (5/5) than in L.L. (4/9). The difference is less impressive or even absent when considering the duration of C.R. and the relapse rate (Table 4). The relapse rate seems identical in L.L. and H.L., but due to the higher remission rate, survival is slightly prolonged in H.L.

Table 4.

	Lymphocytic lymphoma	Histiocytic lymphoma
No. C.R./No. entered MEV trial	9/16	13/14
Mean duration of C.R. (months)	14+	15+
No. in continuous C.R./ No. C.R.	5/9	7/13
Mean duration of continuous C.R. (months)	16+	21+
No. surviving/No. entered MEV trial	11/16	13/14
Mean survival of all patients (months)	15·5+	20·3+

The criteria for the definition of C.R. may have affected these results in that they possess a certain intrinsic inadequacy. It is well known that only the surgical exploration of the abdomen, with splenectomy, and multiple open biopsies (liver, bone, lymph nodes) can offer an adequate picture of the extension of the disease [20–22]. However, clinical, long-lasting C.R., though not pathologically checked, corresponds excellently to a condition of minimum residual disease. In this sense, a long healthy survival is the first and very encouraging step in achieving a cure [23].

As previously stated, 2 patients (1 with L.L. and 1 with H.L.) entered MEV trial while in clinical stage I-A (left high cervical, and left subaxillary nodes, respectively) as they were not eligible either for pathological staging or for extended radiotherapy. Both also received local radiation therapy and both are still in C.R. after 13 and 16 months. One patient with L.L. received TNI during MEV-induced C.R. and is still in C.R. after 24 months. Two patients with H.L. received the same therapy: one of them is still in C.R. (21 months), while the other has relapsed (after 8 months). The longest, and still present remissions, have been obtained in patients who have had MEV as the only treatment.

The efficacy of MEV therapy in obtaining a second remission has not been tested sufficiently, as only 2 of 10 relapsed patients have been given MEV in a second course.

Survival has been shown to be correlated with histologic subtypes [17, 18]. Most of our patients presented with the type diffuse with poorer prognosis, but the remission rate was still encouraging: 8/13 in L.L. and 10/10 in H.L. Ironically, the only patient with the prognostically more favourable subtype (L.L. nodular well differentiated) failed to achieve C.R. Schein *et al.* [9] have emphasized recently the long-term disease-free survival of diffuse H.L. patients who have entered C.R. The very high C.R. rate obtained with MEV in H.L. patients, in comparison with previously published data (Table 5), is provocative enough to warrant further investigation on MEV effect in H.L. In contrast, the results obtained with MEV in L.L. do not look different from those obtained with other drug combinations (Table 5).

The three drugs employed display different, not additive, extra-haematological toxicity, in contrast with several other combinations, *e.g.*, MOPP. The possible immunodepressive effect of MEV regimen has not been investigated in the present trial. However, no infectious complications, bacterial, viral or fungal, have been recorded. Though it could be explained by the disease "per se" and not by treatment, we have been impressed by the lack of severe lymphocytopenia and by the absence of varicella-zoster complications, as compared with Hodgkin's disease patients treated by MOPP [24, 25]. The lack of infectious complications and of marrow depression, as well as the good non-haematological tolerance, have made it possible to give all but the first MEV courses on an out-patient basis. Occurrence of bone-marrow depression has been very low, often absent, during and after MEV regimen. Higher doses, as well as supplementary courses given during C.R., are expected to be well tolerated. This could provide better results as

Table 5.

Reference	Therapy	Histiocytic Lymphoma No. C.R./ No. of patients	% C.R.	Lymphocytic Lymphoma No. C.R./ No. of patients	% C.R.
Hoogstraten *et. al.* 1969 [5]	COP	8/26	31%	13/37	35%
Lowenbraun *et al.* 1970 [6]	MOPP	3/8	37%	7/15	47%
Luce *et al.* 1971 [7]	COP	26/66	39%	37/74	50%
Bagley *et al.* 1972 [7]	CVP	/	/	20/35	57%
Levitt *et al.* 1972 [8]	EDX + Vincristine + Methotrexate + Arabinosyl-C	9/15	60%	/	/
Present study	MEV	13/14	93%	9/16	56%

to remission duration and relapse rate. Alternatively, MEV regimen could be given prior to, or after total body irradiation. This has been shown to be very promising in non-Hodgkin's lymphoma [26]. Both possibilities are currently being tested in a new clinical trial.

The rationale for MEV treatment was the combination of a non-cell-cycle phase-specific agent (cyclophosphamide), and two phase-specific drugs (vincristine, methotrexate) [27]. Moreover, these three drugs show a low combined haematological toxicity, as vincristine is one of the less myelotoxic drugs, and methotrexate, given in a single dose, even at weekly intervals, does not produce a significant marrow depression [28]. The use of methotrexate in the therapy of non-Hodgkin's lymphomas deserves consideration. Methotrexate has rarely been adequately explored for induction or maintenance treatment of lymphomas. Moxley *et al.* [29] have reported good results in Hodgkin's disease at different stages, by combining radiotherapy with a polychemotherapeutic regimen including methotrexate. Later, Levitt *et al.* [8] obtained 60% C.R. in H.L., using methotrexate in combination with vincristine, cyclophosphamide and arabinosyl cytosine. Moreover, a number of theoretical considerations [30], and experimental studies [10–12] have provided a rationale for the combination of a non-cell-cycle phase-specific agent with a specific one. In the present trial, cyclophosphamide was given first, with the hope of killing a consistent number of cycling and slowly-cycling cells, and to recruit previously quiescent cells in the cell-cycle, the rationale being to offer a higher fraction of sensitive cells to methotrexate and vincristine. This rationale has not been demonstrated as yet, and it is possible that different timing and scheduling could provide even better results. The number of the variants is so high, however, that *in vitro* or animal tumor system studies are requested, as recently emphasized [31].

REFERENCES

1. J. E. Ultmann and D. D. Nixon, The therapy of lymphoma. *Semin. Hematol.* **6,** 376 (1969).
2. V. T. De Vita and G. P. Canellos, Treatment of the lymphomas. *Semin Hematol* **9,** 193 (1972)
3. V. T. De Vita, G. P. Canellos and J. H. Moxley III, A decade of combination chemotherapy of advanced Hodgkin's disease. *Cancer (Philad.)* **30,** 1495 (1972)
4. C. M. Bagley, V. T. De Vita, C. W. Berard and G. P. Canellos, Advanced lymphosarcoma: intensive cyclic combination chemotherapy with cyclophosphamide, vincristine and prednisone. *Ann. intern. Med.* **76,** 227 (1972).
5. B. Hoogstraten, A. H. Owens, R. E. Lenhard, O. J. Glidewell, L. A. Leone, K. B. Olson, J. B. Harley, S. R. Townsend, S. P. Miller and C. L. Spurr, Combination chemotherapy in lymphosarcoma and reticulum cell sarcoma. *Blood* **33,** 370 (1969).

6. S. Lowenbraun, V. T. De Vita and A. A. Serpick, Combination with nitrogen mustard, vincristine, procarbazine and prednisone in lymphosarcoma and reticulum cell sarcoma. *Cancer (Philad.)* **25,** 1018 (1970).
7. J. K. Luce, J. F. Gamble, H. E. Wilson, R. W. Monto, B. L. Isaacs, R. L. Palmer, C. A. Coltman, Jr., J. S. Hewlett, E. A. Gehan and E. Frei III, Combined cyclophosphamide, vincristine and prednisone therapy of malignant lymphoma. *Cancer (Philad.)* **28,** 306 (1971).
8. M. Levitt, J. C. Marsh, R. C. De Conti, M. S. Mitchell, R. T. Skeel, L. R. Farber and J. R. Bertino, Combination sequential chemotherapy in advanced reticulum cell sarcoma. *Cancer (Philad.)* **29,** 630 (1972).
9. P. S. Schein, B. A. Chabner, G. P. Canellos, R. C. Young, C. Berard and V. T. De Vita, Potential for prolonged disease-free survival following combination chemotherapy of non-Hodgkin's lymphoma. *Blood* **43,** 181 (1974).
10. I. Kline, Potentially useful combinations of chemotherapy detected in mouse tumor system. *Cancer Chemother. Rep.* Part 2, **4**(1), 33 (1974).
11. F. M. Schabel, Jr., *In vivo* leukemia cell kill kinetics and "curability" in experimental system. In *The Proliferation and Spread of Neoplastic Cells.* Published for the University of Texas M. D. Anderson Hospital, Williams & Wilkins, Baltimore, 379 (1968).
12. F. M. Schabel, Jr., H. E. Skipper, H. W. Trader, W. R. Laster, Jr. and J. B. Cheeks, Combination chemotherapy for spontaneous AKR lymphoma. *Cancer Chemother. Rep.* Part 2, **4**(1), 53 (1974).
13. F. Lauria, M. Baccarani, E. Barbieri, M. Fiacchini and S. Tura, Polichemioterapia dei linfomi non-Hodgkin con methotrexate, Endoxan e vincristina (M.E.V.). *Tumori* **59,** 401 (1973).
14. H. Rappaport, Tumors of the haematopoietic system. *Atlas of Tumor Pathology*, Section III—Fascicle 8. Armed Forces Institute of Pathology, Washington (1966).
15. P. P. Carbone, H. S. Kaplan, K. Musshoff, V. W. Smithers and M. Tubiana, Report of the committee on Hodgkin's Disease staging classification. *Cancer Res.* **31,** 1860 (1971).
16. M. V. Peters, Study of survival in Hodgkin's Disease treated radiologically. *Amer. J. Roentgenol.* **63,** 299 (1950).
17. S. E. Jones, Z. Fuks, M. Bull, M. E. Kadin, R. F. Dorfman, H. S. Kaplan, S. A. Rosenberg and H. Kim, Non-Hodgkin's lymphomas. IV. Clinicopathologic correlation in 405 cases. *Cancer (Philad.)* **31,** 806 (1973).
18. C. D. Bloomfield, A. Goldman, F. Dick, R. D. Brunning and D. J. Kennedy, Multivariate analysis of prognostic factors in the non-Hodgkin's malignant lymphomas. *Cancer (Philad.)* **33,** 870 (1974).
19. S. E. Jones, S. A. Rosenberg and H. S. Kaplan, Non Hodgkin's lymphomas. I. Bone-marrow involvement. *Cancer (Philad.)* **29,** 954 (1972).
20. S. A. Rosenberg, A critique of the value of laparotomy and splenectomy in the evaluation of patients with Hodgkin's disease. *Cancer Res.* **31,** 1737 (1971).
21. S. Tura, F. Lauria and M. Baccarani, La splenectomia nella terapia del linfoma di Hodgkin. *Haematologica* **57,** 465 (1972).
22. D. R. Goffinet, R. A. Castellino, H. Kim, R. F. Dorfman, Z. Fuks, S. A. Rosenberg, T. Nilsen and H. S. Kaplan, Staging laparotomies in unselected previously untreated patients with non-Hodgkin's lymphomas. *Cancer (Philad.)* **32,** 672 (1973).
23. D. S. Martin, R. Fugmann, R. Stolfi and P. Hayworth, Rationale of combined modality therapy for cancer. *Cancer Chemother. Rep.* Part 2, **4**(1), 13 (1974).
24. D. R. Goffinet, E. J. Glatstein and T. C. Merigan, Herpes Zoster-varicella infection and lymphoma. *Ann. intern. Med.* **76,** 235 (1972).
25. S. Tura, F. Lauria and M. Gobbi, Influenza della splenectomia e della terapia radiante linfonodale totale sull'incidenza dell'Herpes Zoster nei pazienti con Linfoma di Hodgkin. *Gazz. Sanitaria.* In press (1975).
26. R. E. Johnson, G. T. O'Connor and D. Levin, Primary management of advanced lymphosarcoma with radiotherapy. *Cancer (Philad.)* **25,** 787 (1970).
27. V. H. Bono, Jr., Biochemical rationales for the selection of combination of chemotherapeutic agents. *Cancer Chemother. Rep.* Part 2, **4**(1), 131 (1974).

28. D. Pinkel, J. Simone, M. D. Hustu and R. J. A. Aur, Nine years experience with "total therapy" of childhood acute lymphocytic leukemia. *Pediatrics* **50,** 246 (1972).
29. J. H. Mosley III, V. T. De Vita, K. Brace and E. Frei III., Intensive combination chemotherapy and X-Irradiation in Hodgkin's disease. *Cancer Res.* **27,** 1258 (1967).
30. R. W. Brockman, Biochemical aspects of drug combination. *Cancer Chemother. Rep.* Part 2, **4**(1), 115 (1974).
31. S. K. Carter, Planning combined therapy. The interaction of experimental and clinical studies. *Cancer Chemother. Rep.* Part 2, **4**(1), 3 (1974).

Europ. J. Cancer Vol. 11, pp. 351–357. Pergamon Press 1975. Printed in Great Britain

Effect of Transplanted Syngeneic Myelomas on the Antibody Response of Mice*

G. W. WARR, N. WILLMOTT and K. JAMES†
Department of Surgery, University Medical School, Teviot Place, Edinburgh EH8 9AG

Abstract—*The responses to alum precipitated bovine serum albumin (alum BSA) and sheep erythrocytes (SRBC) were assessed in Balb/c mice bearing transplanted syngeneic myelomas.*

The growth of all the myelomas tested significantly depressed the response of mice to alum BSA in terms of the antigen-binding capacity of serum, but had no effect on the relative binding affinity of the antibody produced.

The influence of early or established tumour on the response to SRBC, in terms of splenic PFCs of IgM, IgG1, IgG2a and IgG2b specificities was variable. Early growth of MOPC 47A (IgA), MPC 25 (IgG1) and ADJPC 5 (IgG2a) myelomas depressed certain IgG responses, especially IgG1, while IgM responses were unaffected. In contrast, the early growth of an IgM-secreting myeloma (MOPC 104EM) enhanced both IgM and IgG responses. The established myelomas had no effect on the IgM responses to SRBC, although MOPC 47A and ADJPC 5 gave some enhancement of certain IgG responses.

INTRODUCTION

PREVIOUS studies from our laboratory [1, 2] have shown that CBA mice bearing transplanted methylcholanthrene-induced fibrosarcomas exhibit a reduced response to bovine serum albumin (BSA). In addition, these studies have shown that mice bearing established tumours can, under certain conditions, show an enhanced response to sheep erythrocytes, but that early in its growth the tumour may selectively depress the IgG1 splenic plaque-forming cell response [2].

In view of the observed susceptibility to infection, and poor antibody responses, of multiple myeloma and macroglobulinaemia patients [3–5] and reports of immunodepression in mice bearing myelomas [6–8], we decided to investigate the antibody response to alum precipitated BSA and sheep erythrocytes (SRBC) in mice bearing a number of myelomas which are maintained in our laboratory. The effect of myeloma growth on the anti-SRBC response was analysed in terms of splenic PFCs of IgM, IgG1, IgG2a and IgG2b specificities, since the differential effects of these tumours on the various Ig class and subclass responses in mice have not, to our knowledge, been reported previously.

Accepted 27 January 1975.

*This work was supported by a grant from the Cancer Research Campaign.

†Correspondence to: Dr. K. James, Department of Surgery, University Medical School, Teviot Place, Edinburgh, EH8 9AG.

MATERIAL AND METHODS

Animals

Inbred Balb/c mice of both sexes, age 10–12 weeks at the start of an experiment, were used. These mice were bred in the Department of

Surgery from mice obtained from the M.R.C. Laboratory Animals Centre, Carshalton, Surrey. In any one experiment mice of one sex were used, and controls were age matched.

Tumours

MOPC 47A (IgA), MPC 25 (IgG1), MOPC 104EM (IgM) and ADJPC 5 (IgG2a) myelomas were used in these studies. These mineral oil- or adjuvant-induced tumours were obtained through the courtesy of Dr. D. Dresser, National Institute for Medical Research, Mill Hill, and Dr. H. S. Micklem, Department of Zoology, University of Edinburgh. The tumours were routinely passaged by the s.c. injection of from $1–250 \times 10^6$ cells obtained by the homogenization of small pieces of tumour tissue in Eagles Minimal Essential Medium, using an homogenizer with a loosely fitting plunger. The class of Ig secreted by the tumours was confirmed by immunoelectrophoretic and gel-diffusion analysis (using specific antisera) of samples of serum (or urine, in the case of MOPC 47A).

Antigens, and assessment of the antibody response

The antibody response to alum BSA was assessed as described elsewhere [9,10]. Both the antigen-binding capacity (ABC) and relative binding affinity (RBA) were measured [11].

Sheep erythrocytes (SRBC) were prepared from sheep's blood in Alsever's solution purchased from Wellcome Reagents Limited, Beckenham, Kent, England. The SRBC were washed 3 times in phosphate buffered saline (PBS) pH 7·2 before injection. Splenic plaque-forming cells (PFC) were determined as described elsewhere [12]. Indirect PFCs were developed using sheep antisera to mouse IgG1, IgG2a and IgG2b, kindly supplied by Dr. S. Sell, University of San Diego, California, U.S.A. Details of their preparation and specificity have been recorded elsewhere [13].

Immunization

Standard immunizing doses of 1 mg BSA in alum or 3×10^8 SRBC in 0·3 ml. PBS were used. All injections were by the i.p. route.

Mice received alum BSA 5 days after tumour transplantation. Immunization with SRBC was either at the time of tumour transplantation or when the tumour was established. In this latter case immunization was delayed until it was judged that the mice were within 10–14 days of succumbing to the lethal effects of tumour growth. Owing to variations in growth rate, depending on (i) number of cells transplanted (see Table 2), and (ii) the growth characteristics of the differing tumours, this was not at a standard time after tumour transplantation.

Calculation and presentation of results

The tumour size was expressed as the geometric mean diameter (mm) or geometric mean weight (as a percentage of body weight). In some cases the limits of one standard error from the mean were also recorded.

The ABC values (as assessed using 0·2 μg. BSA nitrogen) were expressed as geometric means together with the limits of one standard error from the mean.

The RBA's were expressed as mean $\pm$ s.e.

PFC data were expressed per total spleen, and not per 10^6 nucleated cells for the following reasons. Firstly, total PFC levels give a better indication of the overall immune response of an animal. Secondly, MOPC 104EM and ADJPC 5 showed a tendency to metastasize to the spleen (either as discrete nodules or growing diffusely) and the presence of neoplastic cells would have distorted the normal ratio of specific PFCs to other nucleated cells. The splenic PFC counts were assessed for individual animals and calculated for a group of mice as mean $\log_{10} \pm$ s.e. Geometric means were also calculated, and the results plotted as

$$\frac{\text{geometric mean experimental group}}{\text{geometric mean control group}} \times 100,$$

since control values varied from experiment to experiment.

The significance of differences between values for control and experimental groups was assessed by the standard two-tail Student's t test method.

RESULTS

Antibody response to alum BSA

In two experiments, the myelomas were inoculated into groups of mice 5 days before administration of alum BSA. The antibody response was assessed 14 days after immunization, and the results are shown in Table 1. The growth of all the myelomas significantly depressed the antibody response to alum BSA as measured in terms of the antigen-binding capacity. However, the relative binding affinity of the antibody produced was not reduced by the growth of any tumour, and was even slightly (although not significantly) increased by MPC 25.

Splenic PFC response to SRBC

The immune response to SRBC in mice simultaneously transplanted with viable tumour

Table 1. *Influence of transplanted myelomas on the response to alum BSA*

Exp.	Myeloma*	Anti-BSA response		Tumour size¶		Number of animals
		ABC†	RBA‡	Diameter (mm)	% Body weight	
A	None	2·61 (2·17–3·14)	26·7 ± 1·9			10
A	MOPC 104EM (IgM)	1·27§ (1·10–1·48)	23·5 ± 1·1	25·2 (23·5–26·9)	17·4 (15·5–19·7)	7
A	ADJPC 5 (IgG2a)	0·67§ (0·55–0·82)	27·9 ± 4·7	22·7 (21·2–24·3)	18·0 (15·0–21·6)	5
B	None	3·53 (3·23–3·86)	26·0 ± 3·2			6
B	MOPC 47A (IgA)	1·31§ (1·09–1·58)	24·4 ± 3·4	15·5 (13·9–17·4)	6·85 (5·0–9·3)	6
B	MPC 25 (IgG1)	1·01§ (0·88–1·17)	39·7 ± 6·3	22·3 (21·6–23·1)	14·3 (12·8–15·9)	7

Values are for groups of Balb/c mice 14 days after 1 mg alum BSA given i.p.
*Inoculated s.c. 5 days before administration of alum BSA.
†Antigen binding capacity (0·2 μg BSA nitrogen per test) as geometric mean together with the limits of one standard error from the mean.
‡Relative binding affinity (arithmetic mean ± S.E.).
§Values significantly less than those for controls ($P < 0·01$ to $P < 0·001$).
¶At time of sacrifice.

cells, or bearing established tumours, was determined and the results of this experiment are shown in Fig. 1.

Early during tumour growth MOPC 47A, MPC 25 and ADJPC 5 all significantly depressed the IgG1 response, and in the case of MOPC 47A and ADJPC 5 this was also accompanied by significant suppression of the IgG2b and IgG2a responses respectively. The IgM response was unaffected by the early growth of MOPC 47A, MPC 25 and ADJPC 5. In contrast to the effect of these three tumours, MOPC 104EM had an enhancing effect on the splenic PFC response early during its growth, this being manifest in significantly increased IgM, IgG2a and IgG2b responses.

During the late growth of all the tumours tested, the IgM response remained unaffected. None of the IgG responses was altered significantly by the growth of MPC 25 or MOPC 104EM, although established MOPC 47A enhanced all of the IgG subclass responses and ADJPC 5 significantly enhanced the IgG1 response.

Data on the tumour size at time of immunization and assay is shown in Table 2. At the time of assay the tumour size corresponded to between 5 and 12% of body weight (data not shown). As can also be seen from Table 2, none of the tumours had produced significant splenomegaly 7 days after inoculation, but all except MOPC 47A induced splenomegaly during their later growth. In this experiment relatively small changes in spleen weight induced by tumour growth may have been obscured by the splenomegaly induced by antigen challenge.

DISCUSSION

The results presented clearly demonstrate that all the transplanted syngeneic myelomas tested can depress the response of mice to alum BSA. However, their influence on the response to SRBC is highly variable, depending on the tumour and the stage of growth. Early during the growth of MOPC 47A, MPC 25 and ADJPC 5, depression of the IgG1 splenic PFC responses was observed (in some cases accompanied by depressed IgG2a and IgG2b responses). A similar depression of IgG1 responses early during the growth of a transplanted fibrosarcoma has been reported [2]. In contrast to the above effects, the IgM, IgG2a and IgG2b responses were significantly enhanced early during the growth of MOPC 104EM. A similar variability in the effects of myelomas on the response to SRBC has been demonstrated by Zolla [8], who observed that not all the tumours tested were immunosuppressive, and that enhancement of both IgM and IgG responses sometimes occurred. It should be stressed however that part of the

Table 2. Myeloma growth and splenomegaly in mice challenged with SRBC (See Fig. 1)

Myeloma	Early tumour growth				Late tumour growth				
							Tumour Diameter (mm)		
	No. of cells transplanted	Day* of assay	Tumour diameter (mm)	Splenomegaly†	No. of cells transplanted	Day* of assay	Day of SRBC challenge	Day of assay	Splenomegaly† at day of assay
MOPC 47A	$2{\cdot}5 \times 10^8$	7	<1	1	$2{\cdot}5 \times 10^8$	17	12·0	13·5	1
MPC 25	8×10^7	7	1·7	1	8×10^7	14	10	11·7	2·3
MOPC 104EM	3×10^6	7	<1	1	1×10^6	41	7·6	9·8	1·7
ADJPC 5	3×10^7	7	<1	1	1×10^6	21	9·5	14·2	2·7

*Day after tumour inoculation on which antibody response was assessed. Sheep erythrocytes (3×10^8) were injected i.p. 7 days before the response was measured.
†Ratio of spleen weight (tumour-bearing group): spleen weight (control group).

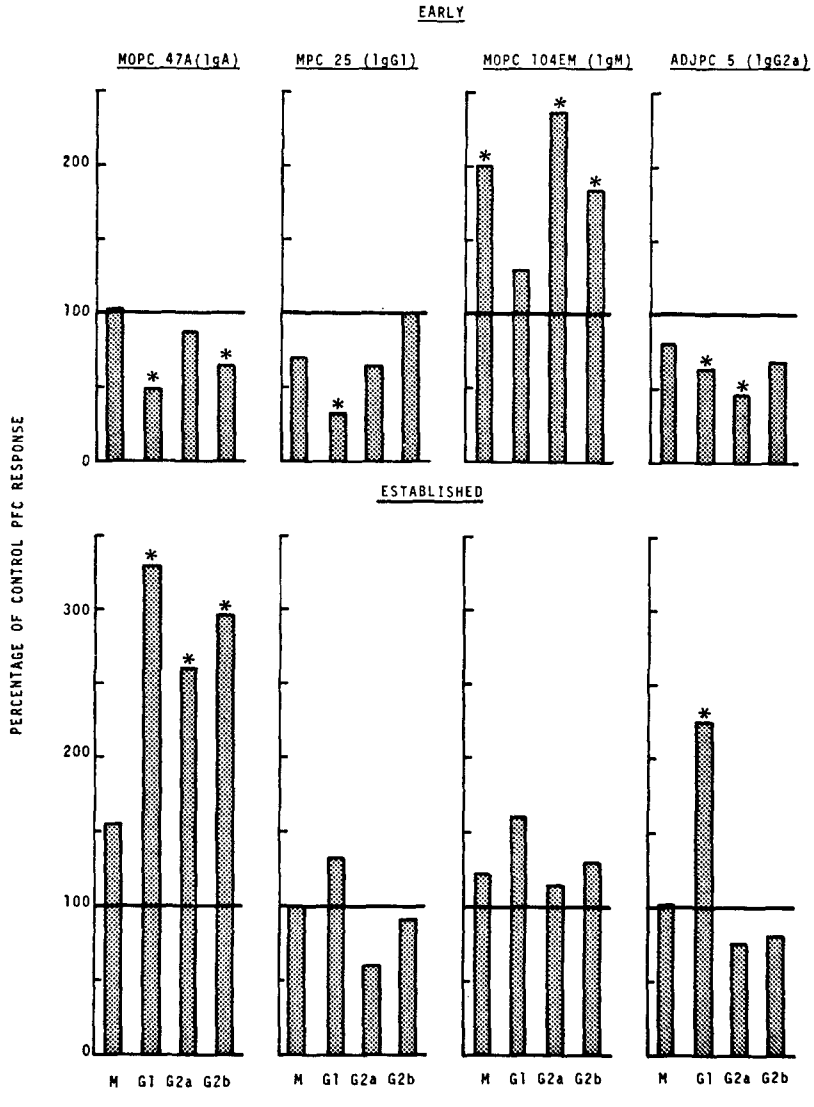


Fig. 1. Influence of myeloma growth on the response of mice to SRBC. The splenic response, in terms of PFCs of IgM, IgG1, IgG2a and IgG2b specificity, was measured 7 days after 3×10^8 *SRBC were given i.p. to groups of 4 or 5 Balb/c mice. The response was expressed as a percentage of the control value. SRBC were injected at the time of tumour inoculation (early) or at a time when the tumour had grown (established, see Table 2). Responses significantly different from those of controls* ($P < 0{\cdot}05$ *to* $P < 0{\cdot}001$) *are denoted by* *.

variation observed in the present experiments may have been due to differences in the number of tumour cells initially transplanted this being governed by the growth characteristics of the tumour under study.

We could find no clear indication that the effects of the myelomas on the class or subclass of PFC produced were influenced by the class of Ig secreted, confirming indirectly the observation of Zolla [8] that feedback by paraprotein is unimportant in determining the effects of myelomas on immune responsiveness.

Our observations that established myelomas failed to suppress the primary IgG response to SRBC in mice are in agreement with those previously reported [8]. (It should be noted, however, that a depressed IgG response has been observed in humans with multiple myeloma [4]). On the other hand, the normal IgM response observed in mice with established myelomas in the present study contrasts with previously published results [7, 8, 14–16] showing a marked suppression of this response. These differences may have resulted from a number of causes, including:

(1) The possibility that the particular tumours used in this study (which were not, to our knowledge, used in any of the other reported studies discussed here), may not exert a potent suppressive effect on the response

to SRBC (c.f. 8). If this is the case, it is unclear why these tumours should have varying effects on the responses to alum BSA and SRBC. However, this distinction between depressed anti-alum BSA and unaltered or enhanced anti-SRBC responses in mice bearing established tumours is identical to that seen with a transplanted syngeneic fibrosarcoma in CBA mice [1, 2].

(2) The tumours may not have been sufficiently large to exert an immunosuppressive effect. Indeed, immunosuppression has been shown to be related to tumour size [6–8, 14], and this explanation remains a possibility which was not investigated.

(3) The dose of antigen (SRBC) may not have been optimal for the demonstration of immunodepression. Although Zolla [14] has shown that low doses are necessary for maximal suppression to be observed, other workers have demonstrated suppression with doses of SRBC similar to, or greater than, the one used in the studies reported here [6, 15, 16].

Further experiments are required to distinguish between these possibilities.

The manner in which myelomas and other tumours bring about their effects on humoral immunity is not clear, and neither is it clear whether the various tumours can be considered as a single group, since immunosuppressive properties vary widely. However, one possibility that has been raised is that tumour may impair T (thymus-derived) lymphocyte function [17, 18]. Defective T-cell helper function has been demonstrated in some tumour-bearing mice [17, 18], and in this connection it is of interest that early tumour growth can sometimes depress the IgG1 response (2, and present results), since the IgG1 response is believed to be the most thymus-dependent [19, 20]. It is apparent, however, that the effects of myelomas are not restricted to T-cells, for they can depress thymus-independent antibody responses [6, 14]. Zolla [14] has postulated that myelomas affect the maturation and differentiation of antibody-forming cells or their precursors. Irrespective of the lymphoid cells affected, the immunosuppression noted in myeloma bearing animals appears to be dependent upon the presence of actively growing tumour [7, 8, 21], a phenomenon also observed with the immunosuppression induced by other tumours [2, 22–24]. Nevertheless, further studies are required to elucidate fully the nature and significance of tumour-induced immunosuppression.

Acknowledgements—We would like to thank Mr. I. Milne for his skilful technical assistance, and Professor Sir Michael Woodruff for his encouragement.

REFERENCES

1. K. James, A. Ghaffar and I. Milne, The effect of transplanted methylcholanthrene induced fibrosarcomata and *Corynebacterium parvum* on the immune response of CBA and A/HeJ mice to thymus dependent and independent antigens. *Brit. J. Cancer* **29,** 11 (1974).
2. K. James, I. Milne and G. Warr, Further studies on the effect of a transplanted methylcholanthrene-induced fibrosarcoma on humoral immunity in CBA mice. *Clin. exp. Immunol.* **18,** 243 (1974).
3. J. L. Fahey, R. Scoggins, J. P. Utz and C. F. Szwed, Infection, antibody response and gamma globulin components in multiple myeloma and macroglobulinaemia. *Amer. J. Med.* **35,** 698 (1973).
4. J. Harris, R. Alexanian, E. Hersh and P. Migliore, Immune function in multiple myeloma: impaired response to keyhole limpet hemocyanin. *Canad. med. Ass. J.* **104,** 389 (1971).
5. L. Cone and J. W. Uhr, Immunological deficiency disorders associated with chronic lymphocytic leukaemia and multiple myeloma. *J. clin. Invest.* **43,** 2241 (1964).
6. J. L. Fahey and J. H. Humphrey, Effect of transplantable plasma-cell tumours on antibody response in mice. *Immunology* **5,** 110 (1962).
7. F. Smith, M. M. Grenan and J. Owens, Effect of a transplanted plasma-cell tumor on antibody formation. *J. nat. Cancer Inst.* **25,** 803 (1960).
8. S. Zolla, The effect of plasmacytomas on the immune response of mice. *J. Immunol.* **108,** 1039 (1972).
9. K. James and I. Milne, The effect of antilymphocytic antibody on the humoral immune response in different strains of mice. I. The response to bovine serum albumin. *Immunology* **23,** 897 (1972).
10. R. S. Farr, A quantitative immunochemical measure of the primary interaction between ^{131}I-labelled bovine serum albumin and antibody. *J. infect. Dis.* **103,** 239 (1958).

11. K. James and V. S. Jubb, Effect of anti-rat lymphocyte antibody on humoral antibody formation. *Nature (Lond.)* **215,** 367 (1967).
12. G. W. Warr and K. James, The effect of antilymphocytic antibody on the humoral immune response of different strains of mice. V. Effects on the class and subclass of antibody produced, and the antibody responses of congenitally athymic mice. *Clin. exp. Immunol.* **18,** 571 (1974).
13. S. Sell, A. B. Park and A. A. Nordin, Immunoglobulin classes of antibody-forming cells in mice. I. Localized hemolysis-in-agar plaque-forming cells belonging to five immunoglobulin classes. *J. Immunol.* **104,** 483 (1970).
14. S. Zolla, Cellular basis of immunodepression in mice with plasmacytomas. *J. Immunol.* **112,** 2068 (1974).
15. P. J. Carlson and F. Smith, Effect of plasma cell tumor on antibody production by mouse spleen cells. *Proc. Soc. exp. Biol.* **127,** 212 (1968).
16. H. Hirano, Y. Imamura, F. Takaku and K. Nakeo, Immune response in mice with plasma cell tumour (X 5563) assayed by agar plaque technique. *Blood* **31,** 252 (1968).
17. K. Takatsu, T. Hamaoka, U. Yamashita and M. Kitagawa, Suppressed activity of thymus-derived cell in tumor-bearing host. *Gann* **63,** 273 (1972).
18. G. F. Rowland, A. J. Edwards, M. R. Sumner and C. M. Hurd, Thymic dependency of tumor-induced immunodepression. *J. nat. Cancer Inst.* **50,** 1329 (1973).
19. G. Torrigiani, Quantitative estimation of antibody in the immunoglobulin classes of the mouse. II. Thymic dependence of the different classes. *J. Immunol.* **108,** 161 (1972).
20. D. W. Dresser and J. M. Phillips, The cellular targets for the action of adjuvants: T-adjuvants and B-adjuvants. In: *Immunopotentiation,* Ciba Foundation Symposium *18*, p.1. Associated Scientific Publishers, Amsterdam (1973).
21. F. Smith, P. J. Carlson and S. Koenig, Plaque-forming spleen cells following irradiation of X5563 tumor. *Proc. Soc. exp. Biol.* **130,** 530 (1969).
22. I. Kamo and N. Ishida, Immune response in tumour-bearing mice. *Gann* **62,** 453 (1971).
23. G. F. Rowland, A. J. Edwards, C. M. Hurd and M. R. Sumner, Changes in lymphoreticular tissues during growth of a murine adenocarcinoma. III. Plaque-forming cell response in lymph nodes and spleen. *J. nat. Cancer Inst.* **47,** 321 (1971).
24. P. Mocarelli, M. Luisa Villa, G. Garotta, C. Porta, G. Bigi and E. Clerici, Reversibility of the immunodepression due to Ehrlich ascites carcinoma. *J. Immunol.* **111,** 973 (1973).

Europ. J. Cancer Vol. 11, pp. 359–363. Pergamon Press 1975. Printed in Great Britain

Electrophoretic Study of Phosphofructokinase Isoenzymes from Human Tissues and Tumours of the Nervous System*

M. J. BENNETT, C. B. TAYLOR† and W. R. TIMPERLEY
Department of Neuropathology, The Royal Infirmary, Sheffield S6 3DA, U.K.

Abstract—*The distribution of phosphofructokinase isoenzymes was investigated in extracts of a variety of normal human adult tissues, 17 gliomas and foetal brains ranging in age from 14 to 42 weeks, by polyacrylamide disc gel electrophoresis. At least four forms of enzymes were observed. None of the adult tissues was identifiable by a single species of enzyme. Foetal brain up to 28 weeks contained one band (band III). Liver contained the two most cathodal enzymes (bands III and IV), whilst skeletal and cardiac muscle contained the three most anodal bands (I, II and III). Brain contained the two intermediate bands (II and III). Seven out of eight grade IV astrocytomas contained bands II, III and IV, whereas the better differentiated gliomas showed the same pattern as adult normal brain. This change in enzyme profile appears not to be a reversion to a foetal form.*

INTRODUCTION

MULTIPLE forms of phosphofructokinase (ATP: D-fructose-6-phosphate-1-phosphotransferase. EC 2.7.1.11.) have been shown to occur in the tissues of rat, man and rabbit. Using DEAE–cellulose column chromatography it has been suggested that there are a total of four types of phosphofructokinase in human and rat tissues [1–3]. There has been some controversy over the heterogeneity of rat hepatic phosphofructokinase. Two isoenzymes initially were reported in rat liver, [4] but subsequent authors found only one form [2, 3, 5]. Recently, it has been reported [6] that there are at least nine forms of phosphofructokinase in the tissues of the rat chromatographically, five of these being hepatic and readily separable into two groups designated L_1 and L_2 [7, 8].

Electrophoretically, little work has been performed with the isoenzymes of phosphofructokinase. Human skeletal muscle and erythrocytes were each reported to have a single but different species of the enzyme after electrophoresis on starch gel, and cellulose acetate membrane [9]. Single non-identical species were also proposed for rabbit liver and muscle, with intermediate distributions in other tissues and a third possible isoenzyme in brain after separation on cellulose acetate. [10–12].

In this present study a method is described for polyacrylamide gel electrophoresis of phosphofructokinase isoenzymes. The distribution of these enzymes has been studied in a variety of adult normal human tissues, seventeen gliomas and human foetal brains ranging in age between 14 and 42 weeks.

Accepted 10 January 1975.

*We are indebted to the endowment funds of the Trent Area Health Authority (Teaching) for the provision of financial support (Grant code No. 212).

†Department of Biochemistry,
University of Sheffield,
Sheffield S10 2TN, U.K.

MATERIAL AND METHODS

All biochemicals used in this study were purchased from Boehringer Mannheim with the exception of D-Glyceraldehyde-3 phosphate dehydrogenase (Sigma Chemical Co.). Other chemicals were obtained from British Drug Houses, with the exception of dithiothreitol (DTT) and phenazine methosulphate (PMS), which were obtained from Sigma Chemical Co.

Human tissues were obtained at autopsy within fifteen hours of death. Brain and brain tumours were obtained directly after surgical removal. All tissues were either used immediately or stored at −20°C. No changes were observed in the isoenzyme patterns after storage of whole tissues for up to one month, although crude homogenates rapidly lost all phosphofructokinase activity after storage. Samples were homogenised over ice in a bench homogeniser (Silverson U.K.) for two periods of 30 sec with a one-minute interval. The homogenising medium consisted of 100mM potassium phosphate buffer pH 7·4 containing 2mM EDTA and 0·2mM DTT. Liver, renal cortex, cerebral cortex and white matter and glioma were homogenised in one volume of the medium; skeletal and cardiac muscle in two volumes. The samples were centrifuged at $20{,}000 \times g$ for 45 min and the supernatant filtered through gauze prior to electrophoresis.

Zonal disc electrophoresis was performed on polyacrylamide gels modified after the method of Davis and Ornstein [13]. A continuous buffer system of 40mM Barbitone pH 8·6 containing 5mM Dipotassium hydrogen phosphate was used with a gel consisting of 6·65% w/v acrylamide, 0·35% w/v, NN-methylene-bis-acrylamide, 0·01% w/v ammonium persulphate and 0·02% v/v 3-dimethylaminoproprionitrile (3-DMAP) in precibor tubes (Shandon Scientific Instruments U.K.) 7·5cm long and 5mm diameter. The electrophoresis was carried out in a disc electrophoresis tank (Shandon Scientific Instruments U.K.). The use of a spacer gel was eliminated by incorporating the sample (approx. 20μl) under 50% glycerol and performing a preliminary run of 0·5mA per gel for 30 min. This procedure ensured that all of the applied sample entered the gel. Electrophoresis was then carried out for 4 hr at 3·75 mA per gel in the cold, by which time a marker of bovine serum albumen containing bromophenol blue had migrated 4·5cm. In order to resolve the isoenzyme bands of the liver and also those of the brain tumours it was necessary to add the following stabilizers to the upper buffer reservoir, 1mM ATP, 1mM AMP and 0·1mM DTT. These were not necessary in the case of other tissues.

The isoenzymes were visualised on the gels by a stain adapted from Layzer *et al.* (1969) [9] containing the reagents necessary for completion of the phosphofructokinase reaction, coupled to the production of NADH from glyceraldehyde-3-phosphate dehydrogenase and subsequent production of insoluble blue formazan bands from nitro blue tetrazolium (NBT). The stain was modified by the use of 2mM fructose-6-phosphate, 0·5% agarose, the addition of 1 unit per ml. of triosephosphate-isomerase and stained at room temperature until band development. The bands associated with enzymic activity were photographed after fixation in 5% acetic acid, and scanned on a Vitatron Densitometer (Fisons Ltd. U.K.) with a 578nm filter.

A total of five liver samples were run which showed consistent patterns and three samples each of skeletal muscle, cardiac muscle and renal cortex. Of seven normal brain samples tested, there were two each of cerebral cortex and white matter from autopsy specimens, and three samples of brain tissue removed surgically, along with gliomas, showing no macroscopical evidence of tumour. Seventeen gliomas were analysed which included the following types (numbers in parentheses). Astrocytoma grade IV (8); grade III (6); grade II (1); microcystic astrocytoma (1); giant cell glioblastoma (1).

Twenty foetal brains ranging in age from 14 to 42 weeks were also analysed.

Control gels were run alongside the test gels and stained in the same medium, but lacking substrate (F6P or ATP). Under the above conditions no bands appeared.

RESULTS

At least four bands of phosphofructikonase activity were detected in normal human tissue samples (Fig. 1). They have been numbered from I to IV in order of decreasing negative charge. Thus, the fastest moving band under the conditions used was type I, whilst the slowest was type IV, with two intermediate bands II and III. Of three skeletal muscle samples studied, one contained bands I and II only, another contained bands I, II and a trace of III, whilst the third contained I, II, III and possibly a trace of IV. All samples of cardiac muscle and renal cortex contained types I, II and III, heart containing proportionally more of type III than kidney cortex (Fig. 2(a)).

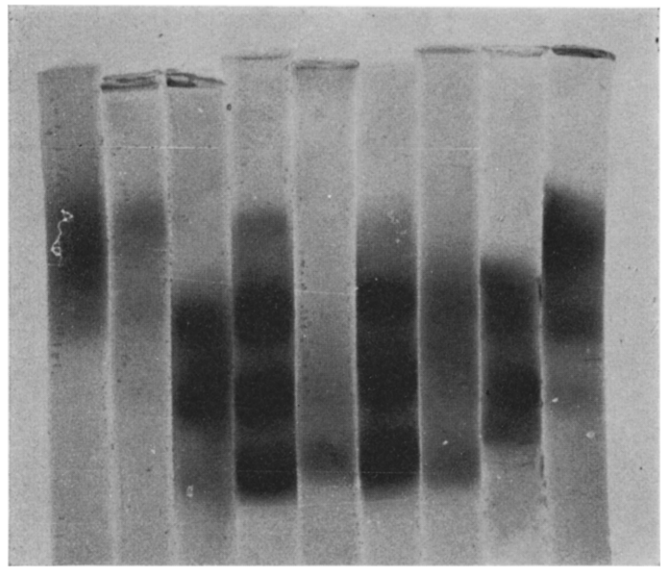


Fig. 1. *Photograph of polyacrylamide disc gels stained for human phosphofructokinase isoenzymes. Seven per cent polyacrylamide gels in* 40*mM Barbitone buffer, pH* 8·6, *were electrophoresed for* 4 *hr at* 3·75 *mA per gel. The origin (cathode) of the gels is at the top and the anode at the bottom. From left to right the samples are:* 1, 2 *liver;* 3, 4, 5, *skeletal muscle;* 6, *cardiac muscle;* 7, *renal cortex;* 8, *normal brain;* 9, *brain tumour. (Astrocytoma Grade IV).*

(to face p. 360)

Table 1. *Relative distribution of phosphofructokinase isoenzyme in human tissues*

Tissue \ Isoenzyme	I	II	III	IV
Liver			+	+ +
Brain				
(Adult)		+ +	+ +	
Foetal (0–28/52)			+	
Foetal (28/52–Term)		+	+	
Renal cortex	+	+	+	
Heart	+ +	+ +	+ +	
Skeletal muscle	+ +	+ +	+	?
+				

+ + large amount; + small amount; ? trace, questionable.

Liver contained predominantly isoenzyme type IV and some of type III although a clear distinction between the two bands was only obtained when ATP, AMP and DTT stabilizers were added to the upper buffer reservoir.

All samples of normal cerebral grey and white matter contained bands II and III only, in about the same proportions (Fig. 2(b)).

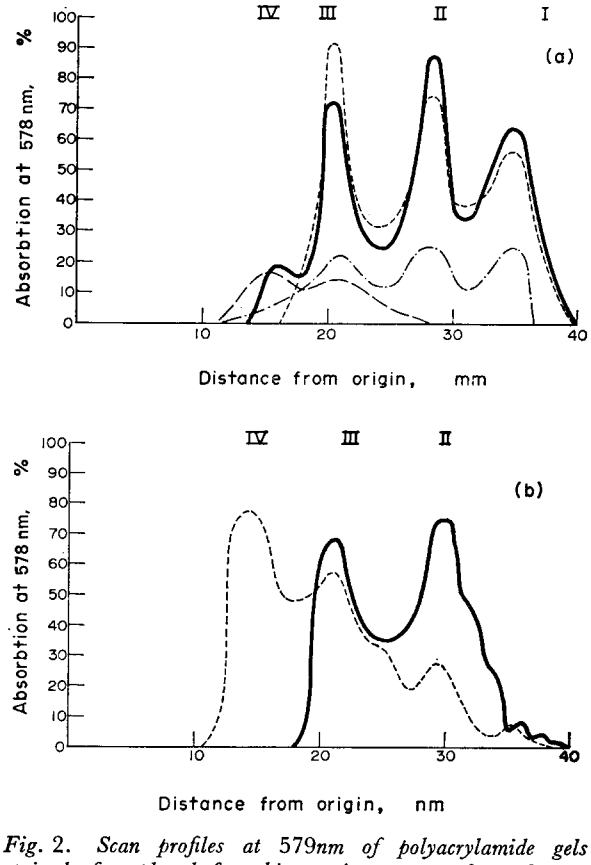


Fig. 2. *Scan profiles at* 579*nm of polyacrylamide gels stained for phosphofructokinase isoenzymes from human tissues. Experimental details described for Fig.* 1. (*a*) *Scan profiles for skeletal muscle* (————), *cardiac muscle* (– – – – –), *renal cortex* (—— ——) *and liver* (+ + + + +) (*b*) *Scan profile of normal brain* (————) *and Astrocytoma grade IV* (– – – – –).

The proportionate amounts of each isoenzyme in these various normal tissues is shown in Table 1.

The distribution of these isoenzymes in the gliomas (Table 2) showed that there was a change of isoenzyme profile in 7 out of 8 grade IV tumours. These contained type IV isoenzyme, a band which is not present in other less malignant astrocytomas or in normal non-neoplastic cerebral tissues. One astrocytoma grade II contained only band III. A giant cell glioblastoma also showed the isoenzyme profile II and III.

Of the foetal brains studied, those up to 28 weeks of development showed band III only, whilst after this time both adult isoenzymes were present.

DISCUSSION

The results of the present work agree with the chromatographic findings of Layzer and Conway [13] inasmuch as 4 forms of human phosphofructokinase have been demonstrated, but differ from them in showing heterogeneity for both muscle and liver enzymes. The liver phosphofructokinase of other species is now known to have at least two forms and three chromatographic components of both muscle and heart enzyme have been described by Kirby and Taylor [6]. The overlap between the muscle and liver isoenzyme profiles has not been reported previously and is in contrast to the results for other species; Kirby and Taylor found only 5% immunological cross reaction and little overlap between the chromatographically distinguishable forms of phosphofructokinase extracted from these two tissues. This probably reflects true species difference rather than inconsistencies between the techniques of electrophoretic, chromatographic and immunological analysis of isoenzymes. Despite

Table 2. Relative distribution of phosphofructokinase isoenzymes in tumours of the human nervous system

Tumour \ Isoenzyme	I	II	III	IV
Astrocytoma grade IV		+ +	+ +	+
		+ +	+ +	+
		+	+ +	+ +
	+	+ +	+ +	+ +
			+ +	+ +
		+ +	+ +	
		+ +	+ +	+
		+	+ +	+ +
Astrocytoma grade III		+ +	+	
		+ +	+	
		+ +	+ +	
		+	+	
		+ +	+ +	
		+ +	+ +	
Astrocytoma grade II			+ +	
Microcystic astrocytoma		+	+	
Giant cell glioblastoma		+	+	

+ + large amount; + small amount.

these species differences the gradation in the tissues isoenzyme profiles for human phosphofructokinase shown here by electrophoresis, namely, muscle, heart, kidney, brain and liver, is essentially the same as that demonstrated by Kirby and Taylor for rat tissues.

Changes in phosphofructokinase isoenzyme patterns have been reported in tumours, mostly hepatomas [3, 5, 14]. In some cases it has been claimed that isoenzymes not found in the normal tissues arise in the tumours but more recently it has been suggested that the normal spectrum of isoenzymes occurs in the tumours but in abnormal proportions. No previous reports are available on phosphofructokinase isoenzymes in tumours of the brain. A shift in isoenzyme profile towards the liver pattern is demonstrated clearly here for the more malignant astrocytomas tested while the less malignant forms of tumour tend to retain the pattern characteristic of the normal tissue. The loss of the type II isoenzyme was noted in an astrocytoma Grade II.

It has been reported that certain isoenzymes revert to foetal forms in the neoplastic state [15–17]. Preliminary studies on foetal patterns of phosphofructokinase suggest that this is not the case in human brain tumours. During brain development up to 28 weeks, only one band (III) was observed, whilst after this time and up until term, the normal adult profile was observed. Band III could therefore be described as the foetal type.

The appearance of "hepatic type" isoenzymes in the more malignant tumours can be related to changes in other glycolytic isoenzymes. Studies have been performed on human brain lactate dehydrogenase [18] which have shown a similar relationship to the one observed with phosphofructokinase, wherein there was observed a parallelism between increased malignancy and increased proportion of the liver type isoenzymes. A relationship has also been observed between increased malignancy and an increase in phosphofructokinase activity, and in glycolytic rate [19] which may also be linked to the appearance of liver type isoenzymes in the more malignant tumours.

Acknowledgements—The authors would like to thank Mr. J. Hardman, Mr. A. A. Jefferson and Mr. D. Forster, Consultant Neurosurgeons, Sheffield Royal Infirmary, for providing surgical material; Dr. S. Hill, Department of Pathology, Jessop Hospital for Women, Sheffield, for kindly providing foetal materials; and Dr. W. Kirby, Department of Biochemistry, University of Sheffield, for helpful advice.

REFERENCES

1. R. B. LAYZER and M. M. CONWAY, Multiple isoenzymes of human phosphofructokinase. *Biochem. biophys. Res. Commun.* **405,** 1259 (1970).
2. T. TANAKA, T. ANN and Y. SAKAUE, Studies on multimolecular forms of phosphofructokinase in rat tissues. *J. Biochem.* (*Tokyo*) **69,** 609 (1971).
3. T. TANAKA, K. IMAMURA, T. ANN and K. TANIUCHI, Multimolecular forms of pyruvate kinase and phosphofructokinase in normal and cancer tissues. *Gann monograph on Cancer Research* **13,** 219 (1972).
4. C. B. TAYLOR and M. BEW, The distribution of two chromatographically distinguishable forms of phosphofructokinase in the tissues of the rat. *J. Biochem.* **119,** 797 (1970).
5. N. KURATA, T. MATSUSHIMA and T. SUGIMURA, Multiple forms of phosphofructokinase in rat tissues and rat tumours. *Biochem. biophys. Res. Commun.* **48,** 473 (1972).
6. W. KIRBY and C. B. TAYLOR, Multiple forms of phosphofructokinase in rat tissues. *Int. J. Biochem.* **5,** 89 (1974).
7. W. KIRBY and C. B. TAYLOR, Differences in the properties of phosphofructokinase isoenzymes of rat tissues. *Biochem. Soc. Trans.* **546,** (2), 491 (1974).
8. G. A. DUNAWAY and G. WEBER, Rat liver phosphofructokinase isoenzymes. *Arch. Biochem.* **162,** 620 (194).
9. R. B. LAYZER, L. P. ROLAND and W. J. BANK, Physical and kinetic properties of human phosphofructokinase from skeletal muscle and erythrocytes. *J. Biochem.* **244,** 3823 (1969).
10. R. G. KEMP, Rabbit liver phosphofructokinase, comparison of some properties of muscle type. *J. biol. Chem.* **246,** 245 (1971).
11. M. Y. TSAI and R. G. KEMP, Hybridization of rabbit muscle and liver phosphofructokinase. *Arch. Biochem.* **150,** (2), 407 (1972).
12. M. Y. TSAI and R. G. KEMP, Isoenzymes of rabbit phosphofructokinase; Electrophoretic and immunochemical studies. *J. biol. Chem.* **284,** 785 (1973).
13. W. R. TIMPERLEY, D.M. Thesis, University of Oxford. Oxford, U.K. (1970).
14. W. KIRBY, H. P. MORRIS and C. B. TAYLOR, A comparison of the phosphofructokinase isoenzymes profile of transplantable hepatomas and normal liver. *Europ. J. Cancer* **10,** 629 (1974).
15. S. WEINHOUSE, Glycolysis, respiration and anomalous gene expression in experimental hepatomas. G. H. A. Clowes Memorial Lecture. *Cancer Res.* **32,** 2007 (1972).
16. F. FARRON, H. H. T. HSU and W. E. KNOX, Fetal-type isoenzymes in hepatic and non-hepatic rat tumours. *Cancer Res.* **32,** 302 (1972).
17. M. SUDA, T. TANAKA, S. YANAGI, S. HAYASHI, K. IMAMURA and K. TANIUCHI, Dedifferentiation of enzymes in the liver of tumor bearing animals. *Gann monograph on Cancer Res.* **13,** 79 (1972).
18. A. L. SHERWIN, F. E. LEBLANC and W. P. MCCANN, Altered LDH isoenzymes in brain tumours. *Arch Neurol.* (*Chic.*) **18,** 311 (1968).
19. M. WOLLEMAN, Biochemistry of brain tumours. In *Handbook of Neurochemistry.* (Edited by A. LAJTHA), Vol. VII, p. 524. Plenum Press, London (1971).

Europ. J. Cancer Vol. 11, pp. 365–371. Pergamon Press 1975 Printed in Great Britain.

Cancer and Altitude. Does Intracellular pH Regulate Cell Division?

ALAN C. BURTON

Department of Biophysics, University of Western Ontario, London, Ontario, Canada

Abstract—*Tissue culture growth rate is very sensitive to changes in pH of the external medium (H. Eagle), suggesting that the concentration in cells of ^{+}H or ^{-}OH might be the key factor controlling synthesis and eventual mitosis in normal and cancerous tissue. Since physiological acclimatization to higher altitudes produces changes in alkali-reserve in man and animals remaining at altitude, a possible correlation with statistics on cancer has been investigated.*

Available data on registrations of cancer (International Committee Against Cancer) and of cancer deaths (World Health Organization) have been analyzed for possible correlation of age-specific rates with a population-weighted mean altitude for each region surveyed. There is no "altitude-effect" below 60 or 65 years, but a statistically significant negative correlation ($r > 0{\cdot}5$. $P < 0{\cdot}05$ in 8, $< 0{\cdot}01$ in 7) was found for older ages in 15 of 16 sets of independent data. The drug acetazolamide has been used to produce "artificial acclimatization", producing similar acid-base changes, and is reported (Evans) to have produced relief of intractable pain in terminal cancer patients. Some diseased states, such as achlorhydria and emphysema, in which there are chronic disturbances of acid-base relations, exhibit unexpected cancer rates.

Some other possible explanations of the apparent "altitude-effect", particularly that it is related to inefficiency of collection of data at high altitudes, seem implausible.

INTRODUCTION

SINCE IT IS thought that there are multiple causal factors in the many different types of cancer, significant correlations of the incidence of cancer in general, with any single environmental factor could hardly be expected, and very few have been found. The dominant correlation is, of course, with the age of the subject. The total age-specific incidence, including all types of cancer, rises almost exponentially, with comparatively very small rates below age 35 (as rates per 100,000 population of specific age groups, e.g. 30–35 years). There is also a significantly lower incidence, for the total of all types of cancer, for females than males above 50 years.

In research on growth and cellular division in tissue culture there is much evidence of the marked effect of changes in pH of the external medium. Eagle and his co-workers [1] have accumulated results on the rate of protein synthesis vs pH for a large number and variety of normal and tumour cells. The curves vs pH are usually triangular in shape with a sharp maximum of growth rate at a certain pH, characteristic of the particular cell line, falling off sharply for pH values on either side of the maximum. Sensitivity to changes of pH is such that 0·2 pH units is usually sufficient to reduce the growth rate to half the maximum value. Little is known of the corresponding changes in pH within the cell. If the cell is well provided with "buffers", it would likely be less than the 0·2 units of the external medium.

In the mammal, the pH of blood is of course

Accepted 12 December 1974.

Address correspondence to: Professor Alan C. Burton, Department of Biophysics, Univ. of Western Ontario, 1151 Richmond Street, London, Ontario N6A 3K7, Canada.

kept to relative constancy (by regulation of breathing and by the kidney). Nevertheless, there are some chronic conditions in which the intracellular pH may be abnormal. One of these is in the acclimatization to high altitude of men and other animals. The change is initiated as a response to hypoxia at high altitude, *i.e.*, hyperventilation results in excessive loss of carbon dioxide and a rise of blood pH. In about a week, the blood pH may return to normal. However, as long as the animal stays at high altitude, the alkali reserve of the body is markedly abnormal [2] so there is very probably a disturbance of acid–base relations within the cell. The receptor area for hypoxia response is, of course, the chemoreceptors of the carotid body, and Heath and his co-workers [3, 4] have shown, in animals and man residing at high altitudes, and in man at low altitudes suffering from disease accompanied by chronic hypoxemia, that there is a very marked increase in weight and size of the carotid body. Nature has thus provided an "experiment" in which we might see if chronic disturbances of acid-base balance affect the incidence of cancer. We have, therefore, examined the data available to us on rates of incidence of cancer in different parts of the world, looking for a correlation of age-specific rates with altitude. The data is, of course, disappointingly very small, compared to what we would wish, since results on the very large populations of Asia living at a variety of altitudes are not available, except in a very few scattered instances.

We knew of only two truly independent sources of statistical data of sufficient numbers of geographical sites and sufficient variation of altitude to be expected to show significant correlations.

The first source was the publication of the International Union Against Cancer (U.I.C.C.) on the incidence (registration) of cancer in five continents [5]. This gives figures for some 30 regions, varying from cities like Liverpool and Birmingham, England, to some provinces in Canada, to whole countries like Sweden, South Africa, or New Zealand. Very specific information is given about the criteria of registration, hospital facilities, demographic and geographic limits of the particular survey, with critical comments as to the adequacy of medical services and diagnosis. In some cases the validity of the data is questionable, for various reasons given in the text, but for the correlation analysis we included all regions listed (which would make it harder, rather than easier to obtain significant correlations). The tables give the rates per 100,000 population for 5-year age groups, for some 35 groups of different types of cancer according to the scheme of international classification, and the totals of all types of cancer, age-specific.

Our second major source of data was the report of W.H.O. on total deaths, rather than registration of cancer, in 36 different countries for the year 1965 [6]. Population of the regions is given in a separate set of tables, which allowed calculation of the rates per 100,000 population. Similar classifications and age-groups are given, but there is no critical assessment of reliability of the surveys, the data being accepted without question from official sources and governments. Ten of the regions coincide, at least in name, with those in the data on registration, and this gave an opportunity to compare the reported death rates with the reported "incidence". In these, the registration was always higher than the mortality rate, varying from 10 to 100 % greater. Fortunately, the mortality data include several new regions of altitude above 500 m. The regions surveyed vary from single cities to large countries, in which the population lives at a great variety of altitudes. We needed some sort of population-weighted "mean altitude", and this was done by a Mr. Edward Avey, who studied the distribution of populations between the larger cities and rural areas of a given country or region, with the known altitudes of the different regions. His estimates were, of course, "blind" *i.e.*, not influenced by knowledge of the cancer statistics. His weighted-mean values were accepted without change. In several countries considered to be of high altitude, his weighted-mean altitudes were lower than expected, because populations tend to live in the valleys rather than in the mountains. The range of mean altitudes calculated varied from sea level to 1780 m (Johannesburg). In all, the lists contained 13 regions with calculated population-mean altitudes over 500 m. (See Appendix).

A third possible source of data was the publication of the Department of Public Health, Tohoku University, Japan, on cancer mortality in 24 selected sites [7]. The data here might not be considered as independent of the W.H.O. data on mortality, except for differences in the years surveyed. Also, the list included only two regions of mean population altitude above 500 m, thus the regression with altitude could not be expected to be significant.

We made one further attempt to find the correlation, in data for the total death rates from cancer in the counties of California whose mean altitudes differ greatly. A plot of the

overall cancer rate vs altitude showed no regression at all and normal distribution of scatter at all altitudes. This is to be expected from the smallness of these geographic units and probable mobility of their populations. On the other hand, too large a geographic unit, including both high and low altitudes, is disadvantageous, since altitude effects will be less evident after the averaging. There is obviously an optimal size of unit for our purpose. The best possible data for our purpose would be, of course, from a large sub-continent like India, including age-specific rates for regions of high vs low altitude, collected by a single agency. This is not available to us.

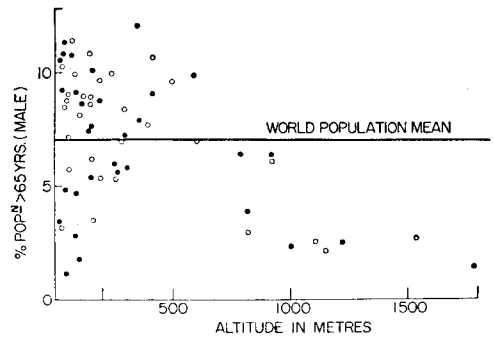


Fig. 1. Distribution of ages in the populations vs altitude. The percentage of the population above 65 years of age is taken as an index of population-age-distribution (P.A.D.). The data is shown by solid dots from U.I.C.C. [9], *that by open circles from W.H.O.* [10].

Evidence of an "altitude effect"

A purely preliminary exploration was made of the correlation of total rates (all age groups included) vs mean-population-altitude. There was very great scatter at the low altitudes, in which most of the data clusters, but a quite remarkable and significant negative regression (less cancer at higher altitudes) of total cancer rates with altitude evident from the scatter graphs (only above 500 m) in the two sources of data (U.I.C.C. and W.H.O.). The total cancer rate appeared to decrease on the average by about 100 cases per 100,000 population for each 1000 m of altitude. For the highest altitudes in the lists the rates were one-half to one-third those at low altitudes. The absence of any "altitude effect" at altitudes below 600 m would, of course, be consistent with the response to anoxia (acclimatization), which does not occur until there is a considerable "threshold" of anoxia. However, it was considered unjustifiable to calculate correlation coefficients and regression slopes which excluded the data at lower altitudes, though this would obviously yield higher values of correlation coefficients. In all the statistics, all of the very low altitudes were included.

However, since the age distribution of the populations might well be very different, with "younger" populations at higher altitudes, this apparent regression might well be due to this factor alone, and not a true altitude effect. This caution proved to be justified. From the data, an index of "population-age" was calculated as the percentage of the total population that was over 65 years of age. Figure 1 gives the result, as well as indicating the range of mean-altitudes available in our data. It is certainly true that for altitudes above 500 m the index of age distribution lies below the world average, and we must, therefore rely entirely on age-specific rates to establish an altitude effect, as other than indirect, due to differences in population-age-distribution.

It is obviously impossible to present all the data, for all age-specific groups (five-year intervals) in a concise form. We therefore arranged the lists of regions in order of their estimated population-mean altitudes, and took the six regions of highest vs the six of lowest altitude in the lists, calculating the mean cancer rates and SE of these means, for all of the age-specific groups. The results are presented in Figs. 2 and 3, which represent four independent sets of data. The rates for low vs high altitude are remarkably similar, and certainly not significantly different, up to age 60 or 65, but the differences become increasingly statistically significant (note lack of overlap of areas of mean ± 1 S.E.M.) at higher ages.

This technique of comparing six at the top with six at the bottom of the order of altitudes was useful, in showing that any correlation

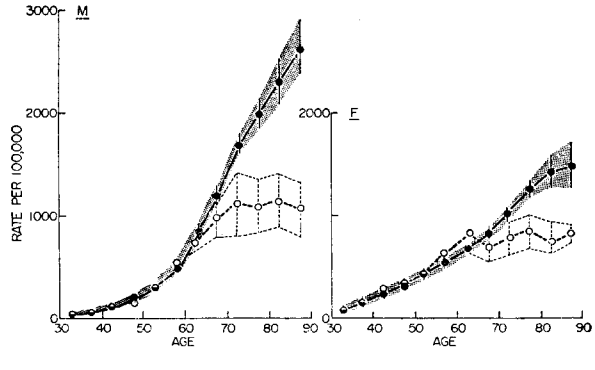


Fig. 2. Age-specific rates of "incidence" of cancer vs age for the six places of highest vs the six places of lowest altitude (U.I.C.C. data). The SE of the means are shown by the vertical bars. Note the significant differences at ages above 65.

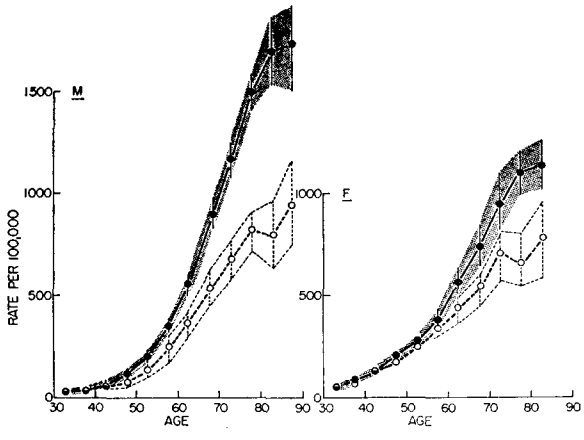


Fig. 3. *As in Fig.* 2, *but for W.H.O. data on death rates.*

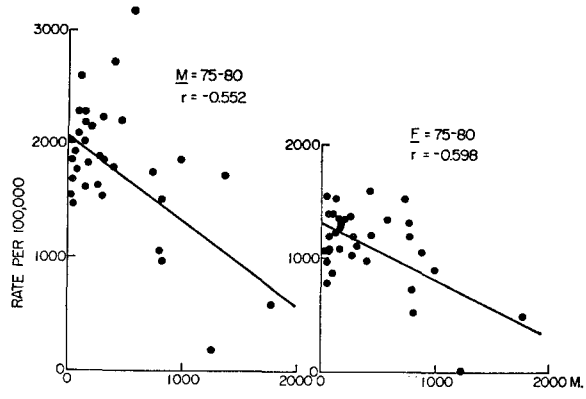


Fig. 4. *Illustrating one of the negative correlations of Table* 1 (*columns* 3 *and* 7) *of age-specific rates with altitude. The straight line is the calculated regression, including all the points, and the correlation coefficient is significant to the* 99% *confidence level.*

with altitude was confined to the older ages, but, of course, it is inefficient in that it utilized less than half of the data, as it omitted the data for all the intermediate altitudes. Accordingly, the regression lines and coefficients of correlation were computed for age groups 65–70, 70–75, 75–80, and over 80 in the four sets of data. The results are shown in Table 1. Of the 16 independent correlations, eight were significant at the 99% level of confidence, seven at the 95% level, and only one at a lower level. However "unreliable" the data may be considered to be, the chance that without some factor associating cancer with altitude in the data, such a set of apparent correlation coefficients would turn up is very small indeed, to the verge of impossibility. Most of the correlation coefficients for males exceeded 0·5, which would mean that more than 25% of the variance in cancer rates, for these ages, was associated with the altitude (75% with the many other random factors affecting cancer rates). This is remarkable for any one factor in cancer statistics, and distribution of age in the populations could play only a very small role indeed in these groups with narrow ranges of age. Figure 4 gives a typical example of one of these regressions for a specific age group. Analysis of age-specific data down to ages 50–55 still shows some significant correlations with altitude, but at lower ages there is very little indication of any reduction of cancer rates except in the regions of very high altitude.

Dependence of altitude effect on types of cancer

The data used gives the age-specific and total rates for some 40 different types of cancer, according to an international classification. We used the device of calculating the percentage of all cancers for a given region that were in a given group of types of tumours (reduced from 40 to 10 broad categories), to give a "profile" (histogram) of types of cancer. This was done for the six places of highest vs the six of lowest altitude in the U.I.C.C. data. The profiles were remarkably similar and there was no indication of a significant difference between

Table 1. *Statistical evaluation of correlations of age-specific rates*/100,000 *with altitude*

Statistic	Age	Incidence rates U.I.C.C. 65–	70–	75–	> 80	Death rates W.H.O. 65–	70–	75–	> 80
Coefficient of correlation r*	M	−0·508	−0·499	−0·552	(−0·214)	−0·608	−0·590	−0·580	−0·613
	F	−0·291	−0·410	−0·598	−0·352	−0·291	−0·398	−0·344	−0·334
Intercept A†	M	1306	1688	2107	2270	1023	1342	1638	2006
	F	830	1034	1306	1408	560	757	999	1288
Standard error of estimate	M	±255	±391	±470	±771	±180	±274	±330	±483
	F	±167	±218	±274	±414	± 83	±140	±187	±310
Regression Slope B*	M	−0·376	−0·562	−0·767	−0·406	−0·376	−0·562	−0·767	−0·406
	F	−0·127	−0·244	0·505	−0·375	−0·127	−0·244	−0·505	−0·375

*Where underlined, the confidence in r is > 99%, where not underlined > 95%, and where in brackets < 95%.
†A and B in the regression equation. Rate/100,000 = A+B (Altitude in m).

the means for any category. For example, the greatest percentage of all cancer was for the "gastrointestinal tract", and the mean percentages were $25{\cdot}5 \pm 4{\cdot}9$ S.E.M. for the six high altitude, cf. $24{\cdot}7 \pm 2{\cdot}2$ S.E.M. for the six low altitude regions. The next highest percentage was for lungs and thorax, $11{\cdot}6 \pm 2{\cdot}0$ vs $9{\cdot}7 \pm 1{\cdot}5$. The "altitude effect", whatever its cause, is apparently a non-specific reduction in reports of cancers of all types.

Other correlations, cancer and altitude

We know of one other report of a strong negative correlation of cancer with altitude. This is in the case of Burkitt's lymphoma, which is confined to a region of Central Africa, and to children. Burkitt [8] states "Altitude seems to be the determining factor.... The only district in Uganda from which we have recorded no single case is the very densely populated southwest, which is mountainous country at an altitude of 5000 feet (1500 m)." Since the annual rainfall is much less than average in this region, the correlation might be attributed to many other vectors than altitude *per se*, e.g., distribution of an insect vector.

Medical missionaries penetrating to remote regions very often have reported an absence of cancer cases, but as "civilization" advanced into these regions, the cancer rates usually were found not to be unusual [9]. The State of Hunza, with a population over 20,000 seems to be an exception, since over 60 years of reports by Medical residents have failed to find a single case (according to Stefansson) whereas one would expect at least 20 cancer deaths per year, from the mean values for low altitude elsewhere. Hunza is in the Himalayas, with no part of the State below 15,000 feet (4600 m).*

Simulated acclimatization to altitude, acetazolamide (Diamox)

Acclimatization to altitude takes a week or more, and rapid transport to high altitude can produce "altitude sickness". Pre-treatment with several drugs has been tried to alleviate this and to produce the acclimatized state sooner. Acetazolamide (Diamox) is one of the most successful. It is an anti-carbonic anhydrase, and produces changes in acid-base relations (reduction in alkali reserve) similar to those in acclimatization to altitude [10]. We therefore looked for evidence in the literature that this drug had effects on cell division and on cancer. Evans [11] used it, on the theory that pain-sensitivity was affected by acid–base relations, on a large group of patients with intractable pain, nearly all of whom happen to be "terminal cancer patients". He verified the changes in acid–base produced by the drug, and there was evidence of considerable relief of pain. The relief might, of course, not only be due to an effect on pain-sensitivity, but also on a decrease in rate of growth of the tumours. Many of the patients on the drug in the test period continued its use, and some have survived many years more than expected, though this cannot be reliably related to the drug alone because radiation and chemotherapy was given in addition. (Personal communication by Dr. Evans).

In large doses acetazolamide is known to be "embryocidal", and there is a report in the clinical literature [12], of a 73-year old man who died from a suppression of cells of all types in the bone marrow after taking 250 mg/day of Diamox.

DISCUSSION

The fact of the strong and statistically significant negative correlation of cancer with altitude in the data used, for the age groups above 60 years, must be accepted, but of course, the interpretation of this fact in terms of causal relations will be highly controversial. The suggestion most often made by colleagues to whom the correlation has been pointed out, is that it results from inefficiency of cancer surveys at high altitude compared to lower altitudes. Communications, medical services, and cancer registration of the populations are, of course, likely to be less well developed at high altitudes. However, there are three reasons why this explanation does not seem plausible: (1) for the very high altitude regions the degree of inefficiency would have to be such that half the cases (e.g. cancer deaths) or more were missed; (2) the list of high altitude regions, while it includes some underdeveloped regions, also includes places where medical care is relatively sophisticated, as in Johannesburg, Colombia, Venezuela, Mexico, or Alberta, Canada; (3) advocates of inefficiency of surveys at altitudes as the explanation, would have to advance a reason why at ages below 60 the surveys were apparently efficient, but became inefficient for the older people. (Figs. 2 and 3).

*While this paper was in press, a paper appeared showing a remarkable negative correlation of death by leukemia in the United States with altitude. Over 500 geographical locations were used and segregated into altitude increments of 400 ft [ECKHOFF *et al. Health Physics* **27,** 377 (1974)].

We also might be asked to explain why the "altitude effect" is seen only for the advanced ages. The small proportion of cancers detected at young ages are usually rapidly growing malignant forms, while the majority of all cancers are, perhaps, very slow growing tumours, which might take as long as 30 years of "incubation" before they are clinically detected or lethal.

Another very reasonable suggestion is that there may be a "selection effect" on the part of the older people in the population, In that the "weaker and more diseased" elderly people might not be able to cope with the rigours of high altitude life and may tend to reside at lower altitudes. Here then is an assumption that cancer in general attacks the "weaker and more diseased", more than it attacks the strong and otherwise healthy. This assumption may not be generally acceptable. If the selection were specific rather then general, *i.e.* those who cannot cope with life at high altitude do not live there, this explanation of the correlation is similar to ours. We know that successful coping with long-term residence at high altitude depends on the physiological changes in acclimatization to altitude, involving acid-base readjustments. The suggestion of a "selection factor" in the population leads therefore, to the same explanation, namely that acclimatization to altitude may involve resistance to cancer.

If it is suggested that the correlation is related causally to some other environmental factor closely associated with altitude, there would be, of course, a wide choice other than the acid-base change in acclimatization to altitude. One might postulate the absence of some toxic atmospheric factor, or an insect vector, or some physical factor like humidity or temperature that might be well correlated with altitude. The existence of the correlation with altitude merely is consistent with our theory that acid-base changes in acclimatization to altitude might be responsible. "Circumstantial" evidence from conditions where disturbed acid-base relations exist, other than related to acclimatization to altitude, is required to strengthen the case. The very strong evidence in tissue culture has already been discussed in the Introduction.

There are only a few diseases where marked changes in pH persist for long periods (e.g. the acidosis of diabetes is removed by insulin therapy). The best example is achlorhydria, which accompanies pernicious anaemia. Treatment of the anaemia does not remove the chlorhydria. The stomach cells of the patient continue not to secrete the strong acid of the corresponding normal stomach cells, so these cells must be supposed to have abnormal acid-base conditions. It is encouraging to our hypothesis to note that the report of a Research Committee of W.H.O. on a conference on cancer of the gastrointestinal tract [13] states—"The rate of gastric cancer in pernicious anaemia patients has greatly exceeded expectations many times, as have rates for achlorhydria; from four to five times the expected number of gastric cancers are found in achlorhydric patients".

Chronic emphysema is another example, for the cells of the lungs are exposed to unusual concentrations of carbon dioxide and are likely to be abnormally acid. The incidence of cancer of the lung is, of course, very high indeed in this disease. Since most of the patients are heavy smokers of cigarettes, the increased incidence is usually attributed to this, but it might well be mediated by an acid-base disturbance.

What is needed is "prospective" rather than "retrospective" research at the clinical investigative level on the effect of manipulations of acid-base relations, by drugs like acetazolamide, or even ammonium chloride, which are used freely, in the case of acetazolamide as a mild diuretic on all kinds of patients. Investigations should be on patients with forms of cancer where quantitative measurement of the effect, if any, can be made from day to day (e.g. in leukemia). One would think that merely on the basis of the impressive work of Eagle [1] on tissue culture, such clinical investigation would be indicated. The suggestive additional evidence cited in this article should give a further stimulus to research on how changes in pH may underlie the control of cellular division in health, and be used in controlling neoplastic disease.

We undertook this investigation because of our pursuit of a speculative, very general theory of how the exchange between cells of a "key substance" or substances, whose concentration intrinsically cycles in each cell, could control cellular division ("contact inhibition") [14]. We had already suggested that H^+ or OH^- ions would be excellent candidates for such "key substances" [15]. Recent evidence on fibroblast proliferation [16] suggested that the effect of pH of the external medium is greatest in the confluent stage of culture, *i.e.*, on intercellular reactions and "contact inhibition". The finding of these remarkable and significant correlations of cancer with altitude in the data available supports that suggestion.

Acknowledgements—Without the excellent work of Mr. Edward Avey in estimating the mean altitude for the populations surveyed, the correlation with altitude would not have been reliably established. Dr. Peter Canham, of this department, did much of the computing of regression and correlations, in which also Mr. Peter Higgins, graduate student in Biophysics, assisted. I am greatly indebted to Dr. K. Stavraky, of the Department of Epidemiology, for finding the sources of data for me, and to Dr. Wanklin and Professor Carol Buck of the same department, for helpful suggestions.

APPENDIX

List of regions in C.I.C.C. and in W.H.O. data with their estimated population-weighted mean altitude.

The regions are arranged in descending order of altitude (given in metres).

C.I.C.C. (Incidence of cancer)

Johannesburgh, S. Africa, 1780; Uganda, 1214; Cali, Colombia, 1000; Yugoslavia, 910; Chile, 810; Alberta, Canada, 760; Saskatchewan, Canada, 580; Manitoba, Canada, 410; Iceland, 300; Newfoundland, Canada, 300; Israel, 255; Jamaica, 250; New York State, U.S.A., 195; Puerto Rico, 156; Finland, 150; Norway, 150; New Zealand, 140; Connecticut, U.S.A., 105; New Brunswick, Canada, 100; Birmingham, England, 100; Liverpool, England, 80; Hawaii, 80; Sweden, 60; Japan, 32; London, England, S.W. Region, 30; London, England, S. Metro, 30; Denmark, 26; Hamburg, Germany, 25; Netherlands, 10.

W.H.O. (Death rates from cancer)

Mexico, 1540; Venezuela, 1150; Mauritius, 1100; Colombia, 1100; Yugoslavia, 910; Chile, 810; Spain, 600; Switzerland, 500; Austria, 420; Czechoslovakia, 395; U.S.A., 295; Canada, 290; Israel, 255; German Fed. Rep., 234; Portugal, 233; Poland, 180; France, 180; Ceylon, 160; Finland, 150; Norway, 150; Italy, 140; New Zealand, 140; Hungary, 120; Greece, 100; S. Ireland, 90; Belgium, 88; U.K. England, Wales, 60; Japan, 60; Sweden, 60; Australia, 60; Scotland, 50; W. Berlin, 50; N. Ireland, 32; United Arab Republic, 26; Denmark, 26; Netherlands, 10.

REFERENCES

1. H. Eagle, The effect of environmental pH on the growth of normal and malignant cells. *J. cell Physiol.* **82,** 1 (1973).
2. F. A. Hitchcock, Animals in high altitudes: the aerosphere. In *Adaptation to the Environment* (Edited by D. B. Dill, E. F. Adolph and G. C. Weber) Sec. 4, Chaps. 52–57, p. 835. Amer. Physiol. Soc., Washington, D.C. (1964).
3. D. Heath, C. Edwards and P. Harris, Postmorten size and structure of the human carotid body. *Thorax* **25,** 129 (1970).
4. C. Edwards, D. Heath, P. Harris, Y. Castillo, H. Kruger and J. Arias-Stella, The carotid body in animals at high altitude. *J. Pathol.* **104,** 231 (1971).
5. R. Doll, P. Payne, J. Waterhouse, Editors, Cancer incidence in five continents. A technical report. *Int. Union Against Cancer (U.I.C.C.)*. Springer-Verlag, Berlin-Heidelberg, New York (1966).
6. World Health Organization, *Mortality from Malignant Neoplasms.* 1955–1965. Div. of Editorial and Reference Services, W.H.O., Geneva, Switzerland (1970).
7. Cancer mortality for selected sites in 24 countries. No. 3. 1960–1961. M. Segi and M. Kurchava, Dept. Public Health, Tohoku Univ. School of Medicine, Scandai, Japan, 1964.
8. D. Burkitt, A lymphoma syndrome in African children. *Ann. roy. Coll. Surg., Engl.* **30,** 211 (1962).
9. V. Stefansson, *Cancer, Disease of Civilization?* An *Anthropological and Historical Study* **17,** p. 147. Hill & Wing, New York (1960).
10. S. A. Forward, M. Landowne, J. N. Follansbee and J. E. Hansen, Effect of acetozolamide on acute mountain sickness. *New Engl. J. Med.* **279,** 839 (1968).
11. R. J. Evans, Acid-base changes in patients with intractable pain and malignancy. *Canad. J. Surg.* **15,** 37 (1972).
12. L. C. Underwood, Bone-marrow depression after treatment with acetazolamide (Diamox). *J. Amer. med. Ass.* **161,** 1477 (1966).
13. E. L. Wynder, S. Graham and H. Eisenberg, Conference on the etiology of cancer of the gastrointestinal tract. Report of the Research Committee W.H.O. *Cancer* **19,** 1501 (1966).
14. A. C. Burton, Cellular communication, contact inhibition, cell clocks, and cancer: The impact of the work and ideas of W. R. Loewenstein. *Perspect. Biol. Med.* **14,** 301 (1971).
15. A. C. Burton and P. B. Canham, The behaviour of coupled biochemical oscillators as a model of contact inhibition of cellular division. *J. theor. Biol.* **39,** 555 (1973).
16. J. E. Froehlich and T. P. Anastassiades, Role of pH in Fibroblast Proliferation. *J. cell. Physiol.* **84,** 253 (1974).

Europ. J. Cancer Vol. 11, pp. 373–379. Pergamon Press 1975. Printed in Great Britain

Cytosine Arabinoside Uptake by Tumour Cells *In Vitro*

J. H. MULDER* and K. R. HARRAP
Department of Applied Biochemistry, Institute of Cancer Research, Clifton Avenue, Belmont, Surrey, England

Abstract—*The uptake of cytosine arabinoside (Ara-C) by Yoshida ascites sarcoma cells is a facilitated diffusion process operating in two concentration ranges with a K_m of 400 μM and 2 mM. Passive diffusion becomes significant only at concentrations greater than 1 mM. The uptake of cytosine arabinoside is competitively inhibited by deoxycytidine (CdR). Comparisons of the transport kinetics of the two nucleosides (K_m and maximum velocity) and the K_m/K_i (Michaelis constant for substrate and inhibitor) ratios for the compounds indicate that Ara-C and CdR are transported by the same carrier. The existence of two facilitated diffusion processes is also demonstrated in L1210 and L5178Y cells.*

INTRODUCTION

CYTOSINE ARABINOSIDE inhibits the growth of tumour cells in culture [1–3], and finds extensive use in the treatment of acute myeloid leukaemia. The metabolic interconversions of this compound are summarised in Fig. 1. The drug is phosphorylated to arabinofuranosyl cytidine monophosphate (Ara-CMP) by deoxycytidine kinase [4–6], and further phosphorylated to arabinofuranosyl cytidine diphosphate (Ara-CDP) and arabinofuranosyl cytidine triphosphate (Ara-CTP), the latter being the major intracellular form of the drug [4, 5].

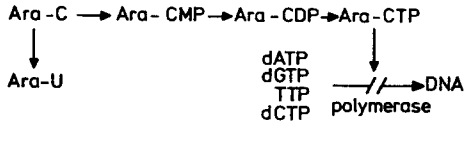


Fig. 1. Metabolism and major locus of action of Ara-C. (dATP stands for deoxyadenosine, dGTP for deoxyguanosine, dTTP for deoxycytosine triphosphates).

Ara-CTP competes with deoxycytosine triphosphate (dCTP) for the binding site on DNA polymerase, which is thought to be the major locus of action of the drug [7, 8].

Sensitivity of tumour cells to Ara-C appears to be related to their ability to phosphorylate the drug, while acquired resistance is accompanied by impaired phosphorylating ability [4, 9–11]. A further limitation on the effectiveness of Ara-C is its deamination by deoxycytosine deaminase to the inactive arabinofuranosyl uridine (Ara-U) especially in the liver [12]. As a result, the half-life of the drug in man is of the order of 15 min [13, 14]. Resistance to Ara-C has also been accompanied by an accumulation of the metabolite dCTP [15].

While the uptake of Ara-C is apparently not impaired in drug-resistant cells [4], it is likely that the rate at which cells accumulate the drug in relation to its extracellular catabolism will determine in large measure the resultant cytotoxicity. A further consideration is the extent to which other nucleosides, and in particular, deoxycytosine, are able to modify the cellular uptake of Ara-C. This latter possibility may be of considerable importance when a previously successful course of chemotherapy has produced cell autolysis leading to elevated circulating (or local in the case of a solid tumour) levels of nucleosides. It is known, for example, that nucleoside uptake occurs by facilitated diffusion

Accepted 18 October 1974.

*J.H.M. gratefully acknowledges financial support from the British Council and from the Dutch Cancer Research Council (Koningin Wilhelmina Fonds). Present address: Radiotherapeutisch Instituut, Groen Hilledijk, Rotterdam, Holland.

[16, 17] and that many nucleosides at mM concentrations inhibit the uptake of heterologous nucleosides [18]. In the present communication we have studied the kinetics of Ara-C uptake in three tumour cell lines and investigated the ability of nucleosides to modify these processes.

MATERIAL AND METHODS

5-tritiated Ara-C (14 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. and unlabelled Ara-C from the Upjohn Co. Ltd., Crawley, Sussex, U.K. Deoxycytidine (CdR) was purchased from Sigma, St. Louis, U.S.A. Fischers medium (for L5178Y) and RPMI-1640 medium (for L1210 cells) was supplied by Grand Island Biological Co., New York, U.S.A.

The Yoshida Sarcoma was passaged in the ascites form in female Chester Beatty strain Wistar rats at 6 weeks of age (body weight approx. 120 g) The animals were killed by cervical dislocation between the 3rd and 5th day after tumour transplantation when the tumour was growing logarithmically. The peritoneal contents were aspirated with 10 ml ice cold PBS, centrifuged at 0°, 600 *g*, resuspended in PBS to a concentration of 10^6 cells/ml and used immediately for the experiments described below. The L1210 and L5178Y leukaemia cells were grown in appropriate medium containing 10 % foetal calf serum. In all cases, logarithmically-growing cells were adjusted to a concentration of 10^6/ml in PBS and exposed to varying concentrations of labelled Ara-C and unlabelled Ara-C, dissolved in PBS. The ratio between labelled and unlabelled Ara-C was adjusted to give sufficient radioactivity in the cell pellet.

Uptake studies

Uptake studies were performed as follows: 0·5 ml aliquots of cells were incubated in centrifuge tubes containing 0·5 ml of Ara-C solution (concentration range 15 nM–25 mM). At timed intervals thereafter, 5 ml ice-cold PBS was added and the tube spun at 0°, 600 *g* for 90 sec. The pellet was resuspended twice in 5 ml ice-cold PBS and respun in order to remove the extracellular, trapped Ara-C. Finally, 10 ml of scintillation fluid (Butyl PBD 5 g, Naphtalene 8 g, Toluene 400 ml, Dioxan 400 ml, 90 % Methanol 200 ml) was added to the pellet. Further washing failed to remove additional Ara-C. The samples were counted in an Intertechnique SL40 liquid scintillation spectrometer.

Explanation of terms used

Passive diffusion is regarded as the diffusion of a substrate through the cell membrane as if the latter were an inert barrier. Facilitated diffusion or carrier mediated transport is a process in which the membrane is dynamic and contains a component (carrier) which increases the rate of uptake, to which the substrate binds stereospecifically, and which is saturable by the substrate. The in- and outward movement is called influx and efflux respectively, and the overall uptake is the result of the balance between these. The intial uptake velocity of Ara-C is the difference in ct/min between two time points converted to n-mole/min/10^9 cells at a stated temperature. The kinetic parameters are V_{max}, the maximum initial uptake velocity of the substrate and K_m, the substrate concentration at which $\frac{1}{2}V_{max}$ is achieved. K_i is the calculated Michaelis constant for the inhibitor.

RESULTS

Time course of uptake by Yoshida cells

The uptake of Ara-C at concentrations below 1 mM and at 37° in Yoshida cells reached a plateau within 90 sec; a typical curve for 400 μM Ara-C is shown in Fig. 2. At 19° the

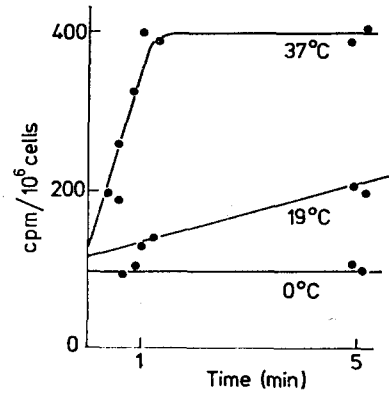


Fig. 2. Time course of Ara-C uptake in vitro *by Yoshida ascites sarcoma cells at different temperatures: extracellular Ara-C concentration* 400 μM.

uptake was linear over an interval of at least 5 min and subsequent work was confined to this temperature. To determine the initial uptake velocity we measured the ct/min at 15 sec and at 120 sec and determined the slope through these points. Extrapolation of this line

to zero time gave an intercept which was always above the origin of coordinates and was proportional to the extracellular Ara-C concentration up to a level of about 1 mM. This instantaneous reaction was independent of temperature and occurred even at 0°, while the second reaction was temperature sensitive. Passive diffusion was apparent at 0° at concentrations of Ara-C in excess of 800 μM (Fig. 3). It was apparent that two processes were occurring: an instantaneous accumulation of Ara-C by the cells, followed by further uptake of drug.

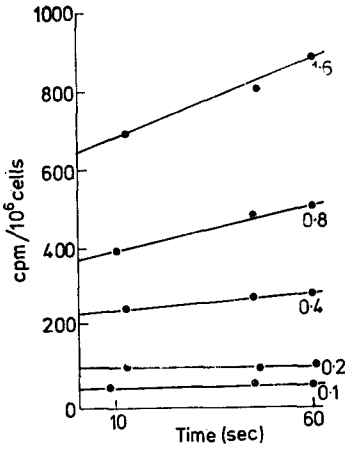


Fig. 3. Accumulation of Ara-C by Yoshida ascites sarcoma cells exposed to varying concentrations (0·1–1·6 mM) of the drug at 0°. Cells at 19° were spun at 0°, 600 g for 90 sec, resuspended in ice-cold PBS and immediately used for the experiment.

Relationship between initial uptake velocity and Ara-C concentration in Yoshida cells

The uptake velocity was measured at different concentrations of Ara-C (in the range of 60 nM–8 mM at 19°). Two saturable processes were detected (Figs. 4, 5). The first process was characterised by a K_m of 400 μM and a V_{max} of 100–130 n-mole/min/10^9 cells at 19°. The second process had a K_m of 2 mM and a V_{max} between 0·2 and 0·3 μ-mole/min/10^9 cells at 19°. At the plateau of Ara-C accumulation, the intracellular concentrations of drug (based on an intracellular volume of 2 μl per 10^6 cells —P. G. Riches, unpublished results) never exceeded those in the suspension medium; hence it would appear that Ara-C is not actively transported.

Effects of deoxycytidine on Ara-C uptake by Yoshida cells

1 mM concentrations of purine and pyrimidine deoxyribonucleosides inhibited Ara-C

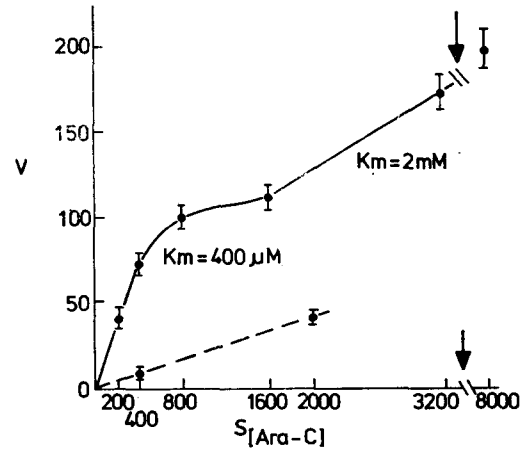


Fig. 4. Relationship between S *and* v *for Ara-C uptake by Yoshida ascites sarcoma cells. The curve is a composite of several separate experiments within the range 60 nM–8 mM. The dotted line represents passive diffusion of Ara-C determined by blocking its facilitated diffusion with 16 mM CdR.* S = *Ara-C concentration, μM;* v = *initial uptake velocity of Ara-C, n-mole/min/10^9 cells at 19°.*

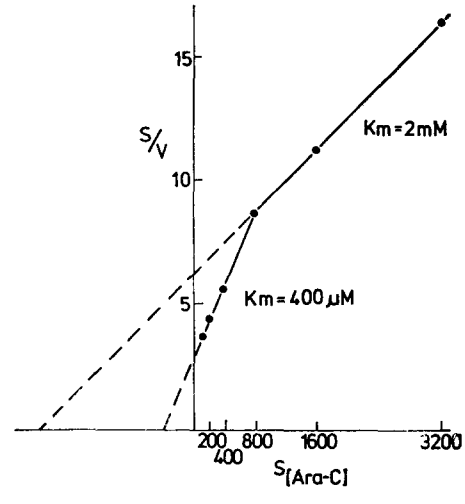


Fig. 5. S/v *against* S *plot for Ara-C uptake by Yoshida ascites sarcoma cells.* S = *Ara-C concentration, μM;* v = *initial uptake velocity of Ara-C, n-mole/min/10^9 cells at 19°.*

(400 μM) uptake (Fig. 6). Subsequent studies concentrated on the effects of CdR on Ara-C influx. Cells were incubated from the outset in solutions containing Ara-C and CdR and initial uptake velocities measured. First we looked at the uptake velocity of a fixed concentration of Ara-C in presence of various concentrations of CdR, and then the effect of a fixed concentration of CdR on the uptake of different concentrations of Ara-C. Lineweaver–Burk and Dixon plots (Figs. 7, 8) demonstrated that the transport of Ara-C was inhibited in a competitive manner by CdR: the K_i = 600 μM and V_{max} = 110–170 n-mole/min/10^9 cells at 19°.

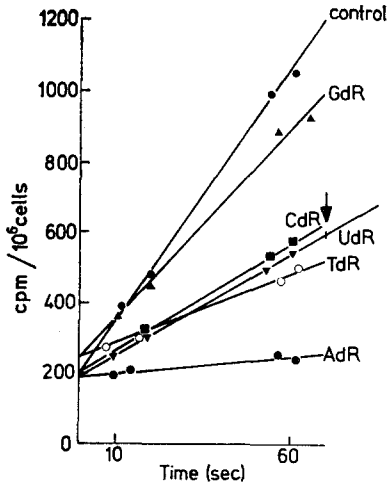


Fig. 6. Relationship between time and Ara-C uptake (400 μM) by Yoshida ascites sarcoma cells at 19° and the influence of 1 mM deoxynucleosides on the uptake. (GdR stands for deoxyguanosine, UdR for deoxyuridine, TdR for deoxythymidine.

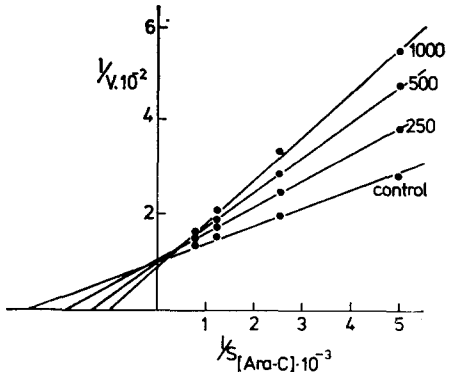


Fig. 7. Lineweaver–Burk plot for Ara-C uptake at various CdR concentrations (μM). S = Ara-C concentration, μM; v = initial uptake velocity of Ara-C, n-mole/min/10⁹ cells at 19°.

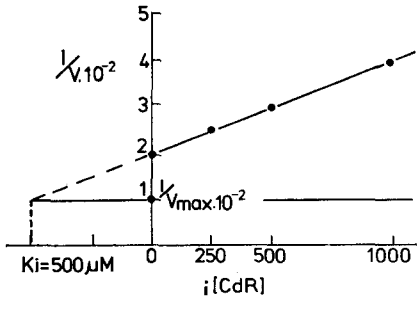


Fig. 8. Dixon plot: 1/v against i. i = CdR concentration, μM; v = initial uptake velocity of Ara-C, n-mole/min/10⁹ cells at 19°.

In ancillary experiments, Ara-C was shown to inhibit competitively the uptake of CdR. The K_m for CdR was 600 μM, V_{max} 180–200 n-mole/min/10^9 cells at 19° and the K_i for Ara-C 400 μM. Using a concentration of 16 mM CdR there was a linear correlation between rate of uptake and concentration of Ara-C, indicating passive diffusion (Fig. 4).

Uptake of Ara-C by L_{1210} and L_{5178Y} cells

The existence of two saturable uptake processes for Ara-C was not confined to the Yoshida cell: a similar mechanism obtained in the case of L1210 and L5178Y cells. In both these cell strains, unlike the Yoshida, the initial uptake velocity was linear at 37° for at least 10 min. To determine the initial uptake velocity we measured the ct/min at 1 min and 10 min and determined the slope through these points. Plots of initial uptake velocities against Ara-C concentration for these two cell strains are shown in Fig. 9. For both cell types

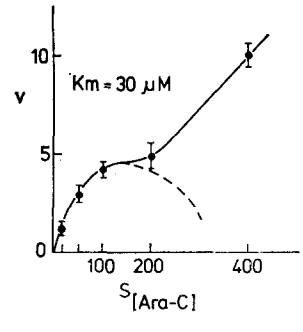


Fig. 9. Relationship between S and v in L1210 and L5178Y cells. The curve is a composite of several separate experiments within the range of 20–400 μM. Differences between replicate determinations in one experiment did not exceed 10%, while overall differences from experiment to experiment were not greater than 15%. The dotted line represents the theoretical curve of the process with the lowest K_m. S = Ara-C concentration, μM; v = initial uptake velocity of Arac-C, n-mole/min/10^9 cells at 37°.

the K_m for the first process was 30 μM and V_{max} 5–6 n-mole/min/10^9 cells at 37°. Although in each cell type, the second process was saturable at concentrations approaching 12 mM Ara-C, there was a measure of irreproducibility in the data making it difficult to assign numerical values to K_m and V_{max}. Again, with both the L1210 and L5178Y cell lines, there was an initial rapid association of the drug with the cells.

DISCUSSION

Ara-C is not phosphorylated or deaminated by Yoshida cells (A. B. Robins—personal com-

munication), which no doubt explains the insensitivity of this tumour to the drug. Hence, in this cell strain the accumulation of tritium was the result of Ara-C transport across the plasma membrane, unmodified by metabolism. This is not the case in the L1210 and L5178Y cells, where the time course is suggestive of a more complicated uptake mechanism; as the uptake curve levels off, net accumulation of tritium is the resultant of the rates of influx and efflux and additionally, of phosphorylation and deamination.

The existence of a dose-related intercept at $t = 0$ is suggestive of a rapid absorption of the drug to the surface of the cells. This has been observed previously for Ara-C [4] and for methotrexate [19, 20].

From the data shown in Figs. 4, 9, it may be concluded tentatively that two facilitated diffusion processes, each possessing different kinetic parameters, are responsible for Ara-C uptake in the cell lines studied. The contribution of passive diffusion to the overall rate of uptake at concentrations below 1 mM is negligible.

When theoretical uptake curves are plotted using the kinetic constants measured for these two facilitated diffusion processes, the sum of the theoretical curves does not show the point of inflection apparent in the observed overall uptake curve. The point of inflection can be achieved only (theoretically) if the uptake curve for the first process reaches a maximum and then declines (Fig. 9). This situation is characteristic of that observed when certain enzymes are inhibited by excess substrate [21] and has been observed by Scholtissek [22] in relation to the inhibition by adenosine of its own transport into chick embryo fibroblasts. For an alternative explanation, we considered the possibility that efflux during the washing procedure at concentrations above 800 μM might result in lowered net uptake of Ara-C. As shown in Fig. 3, Ara-C transport could be stopped with ice-cold PBS as long as the concentration was below 800 μM. At Ara-C concentrations above 800 μM, the drug presumably continued to enter Yoshida cells, although at a much reduced rate, during the washing procedure. Similarly, for intracellular Ara-C in excess of 800 μM, efflux would become significant as the extracellular Ara-C concentration was reduced during the washing procedure. To alleviate this problem, the incubation time was standardised at 2 min (even though uptake was linear over at least 5 min). As a result, the intracellular Ara-C concentration never approached 800 μM, thus minimising Ara-C efflux during the washing procedure. By standardising the washing procedure, the amount of efflux possible would be proportional to the concentration. Hence the slope of the second part of the curve in Fig. 4 would be unchanged; furthermore it is steeper than that of passive diffusion. We concluded that we are observing the combined effects of two facilitated diffusion processes associated with passive diffusion.

To obtain kinetic data relevant to the clinical situation, it is necessary to investigate ranges of drug concentrations which are found *in vivo*; *i.e.*, as low as technically possible. We fully agree with Goldman [23] that in order to do uptake studies the kinetics should be established over a broad range. A small deviation near the intercept of a S/v (substrate/initial uptake velocity) against S plot could indicate the existence of a second process at a much lower concentration range than that used in the original experiment.

The inhibition of Ara-C influx by CdR and vice versa shows that the K_m/K_i ratios were both unity, indicating that both compounds possessed the same affinity for the common transport system. We incubated cells with Ara-C and CdR for the same time in order to study influx and not influx plus efflux. By pretreating cells with, for instance, deoxyadenosine (AdR), we found a slight enhancement of Ara-C uptake. An explanation could be that the intracellularly-accumulated AdR competes with Ara-C for efflux, which results in an apparant increase in Ara-C influx. This could explain the enhancement of uridine uptake by adenosine, found by Lemkin and Hare [24].

Although not conclusive, the consensus in the literature seems to be that purine and pyrimidine oxy- and deoxy-ribonucleosides are transported by facilitated diffusion on the same carrier [16, 18, 25–27]. From our work and from the literature, two consequences emerge, one methodological and one clinical. Where isotopically-labelled thymidine is used to follow DNA synthesis in presence of Ara-C, erroneous values may arise due to the competition of Ara-C and thymidine for transport [18, 28]; the reduced uptake by tumour cells of a drug resulting *in vivo* from the competition between a nucleoside analogue and a nucleoside may be of clinical significance. At present no information is available concerning the levels of nucleosides in human plasma and particularly in plasma of patients suffering from neoplastic disease who have received a successful course of radio- and/or chemotherapy. In this situation, elevated levels of nucleosides may be present locally as a result of extensive tumour cell autolysis. This could modify a subsequent response of the tumour to Ara-C.

REFERENCES

1. J. S. Evans, E. A. Musser, G. D. Mengel, K. R. Forsblad and J. H. Hunter, Antitumor activity of I-β-D-Arabinofuranosylcytosine Hydrochloride. *Proc. Soc. exp. Biol Med. (N.Y.)* **106,** 350 (1961).
2. M. Y. Chu and G. A. Fischer, A proposed mechanism of action of I-β-D-Arabinofuranosylcytosine as an inhibitor of the growth of leukaemic cells. *Biochem. Pharmacol.* **11,** 423 (1962).
3. I. Wodinsky and C. J. Kensler, Activity of cytosine arabinoside (NSC-63878) in a spectrum of rodent tumors. *Cancer Chemother. Rep.* **47,** 65 (1965).
4. M. Y. Chu and G. A. Fischer, Comparative studies of leukaemic cells sensitive and resistant to cytosine arabinoside. *Biochem. Pharmacol.* **14,** 333 (1965).
5. A. W. Schrecker and J. J. Urshel, Metabolism of I-β-D-Arabinofuranosylcytosine in leukaemia L1210: studies with intact cells. *Cancer Res.* **28,** 793 (1968).
6. J. P. Durham and D. H. Ives, Deoxycytidine kinase I. Distribution in normal and neoplastic tissues and interrelationships of deoxycytidine and I-β-D-Arabinofuranosylcytosine phosphorylation. *Molec. Pharmacol.* **5,** 358 (1969).
7. R. L. Momparler, Kinetic and template studies with cytosine arabinoside 5′-triphosphate and mammalian DNA polymerase. *Molec. Pharmacol.* **8,** 362 (1972).
8. F. L. Graham and G. F. Whitmore, Studies in mouse L-cells on the incorporation of I-β-D-Arabinofuranosylcytosine into DNA and on inhibition of DNA polymerase by I-β-D-Arabinofuranosylcytosine 5′-triphosphate. *Cancer Res.* **30,** 2636 (1970).
9. D. Kessel, T. C. Hall and D. Rosenthal, Uptake and phosphorylation of cytosine arabinoside by normal and leukaemic human blood cells *in vitro*. *Cancer Res.* **29,** 459 (1969).
10. A. W. Schrecker, Metabolism of I-β-D-Arabinofuranosylcytosine in leukaemia L1210: nucleoside and nucleotide kinases in cell-free extracts. *Cancer Res.* **30,** 632 (1970).
11. K. Uchida and W. Kreis, Studies on drug resistance—I. Distribution of I-β-D-Arabinofuranosylcytosine, cytidine and deoxycytidine in mice bearing Ara-C-sensitive and -resistant P815 neoplasms. *Biochem. Pharmacol.* **18,** 1115 (1969).
12. D. H. W. Ho, Distribution of kinase and deaminase of I-β-D-Arabinofuranosylcytosine in tissues of man and mouse. *Cancer Res.* **33,** 2816 (1973).
13. D. H. W. Ho and E. Frei III, Clinical pharmacology of I-β-D-Arabinofuranosylcytosine. *Clin. Pharmacol. Ther.* **12,** 945 (1972).
14. R. L. Momparler, A. Labitan and M. Rossi, Enzymatic estimation and metabolism of I-β-D-Arabinofuranosylcytosine in man. *Cancer Res.* **32,** 408 (1972).
15. R. L. Momparler, M. Y. Chu and G. A. Fischer, Studies on a new mechanism of resistance of L5178Y murine leukemia cells to cytosine arabinoside. *Biochim. Biophys. Acta (Amst.)* **161,** 481 (1968).
16. J. M. Oliver and A. R. P. Paterson, Nucleoside transport I. A mediated process in human erythrocytes. *Canad. J. Biochem.* **49,** 262 (1971).
17. D. Kessel and S. B. Shurin, Transport of two non-metabolized nucleosides, deoxycytidine and cytosine arabinoside, in a subline of the L1210 murine leukaemia. *Biochim. Biophys. Acta. (Amst.)* **163,** 179 (1968).
18. T. L. Steck, Y. Nakata and J. P. Bader, The uptake of nucleosides by cells in culture. I. Inhibition by heterologous nucleosides. *Biochim. Biophys. Acta. (Amst.)* **190,** 237 (1969).
19. R. E. Handschumacher and G. L. Fischer, III. Methods for determination of permeability of cells to drugs. *Meth. Med. Res.* **10,** 269 (1964).
20. N. S. Lichtenstein, V. T. Oliverio and I. D. Goldman, Characteristics of folic acid transport in the L1210 leukemia cells. *Biochim. Biophys. Acta* **193,** 456 (1969).
21. M. Dixon and E. C. Webb, In *Enzymes.* p. 75. Longmans & Green, London (1964).
22. C. Scholtissek, Studies on the uptake of nucleic acid precursors into cells in tissue culture. *Biochim. Biophys. Acta (Amst.)* **158,** 435 (1968).

23. I. D. GOLDMAN, Uptake of drugs and resistance. In *Drug Resistance and Selectivity*. Edited by E. MIHICH, p. 299. Academic Press, New York (1973).
24. J. A. LEMKIN and J. D. HARE, Nucleoside transport in normal and polyoma transformed cells: kinetic differences following adenosine and serum or insulin stimulation. *Biochim. Biophys. Acta (Amst.)* **318,** 113 (1973).
25. R. A. TAUBE and R. D. BERLIN, Membrane transport of nucleosides in rabbit polymorphonuclear leukocytes. *Biochim. Biophys. Acta (Amst.)* **255,** 6 (1972).
26. M. T. HAKALA and L. N. KENNY, Common mechanism for the passage of purine and pyrimidine nucleosides through the plasma membrane of sarcoma 180 cells (S-180). *Fed. Proc.* **31,** 457 (1972).
27. T. S. LIEU, R. A. HUDSON, R. K. BROWN and B. C. WHITE, Transport of pyrimidine nucleosides across human erythrocyte membranes. *Biochim. Biophys. Acta (Amst.)* **241,** 884 (1971).
28. P. G. W. PLAGEMANN, Nucleoside transport by Novikoff rat hepatoma cells growing in suspension culture. Specificity and mechanism of transport reactions and relationship to nucleoside incorporation into nucleic acids. *Biochim. Biophys. Acta (Amst.)* **233,** 688 (1971).

Europ. J. Cancer Vol. 11, pp. 381–382. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer — *May, 1975*

M. Börzsönyi and M. Csik: Induction of malignant lymphomas in Swiss mice by N-nitroso compounds formed *in vivo*.

F. J. O'Neill: Control of nuclear division in SV40 and adenovirus type 12 transformed mouse 3T3 cells.

O. M. Jensen: Lung cancer and smoking in Danish women.

H. R. Strausser and J. L. Humes: Prostaglandin synthesis inhibition: Effect on bone changes and sarcoma tumor induction in BALB/c mice.

J. Lotem and L. Sachs: Induction of specific changes in the surface membrane of myeloid leukemic cells by steroid hormones.

M. Lieber, J. Mazzetta, W. Nelson-Rees, M. Kaplan and G. Todaro: Establishment of a continuous tumor cell line (PANC-1) from a human carcinoma of the exocrine pancreas.

R. C. Rees, J. Bray, R. A. Robins and R. W. Baldwin: Subpopulations of multiparous rat lymph node cells cytotoxic for rat tumour cells and capable of suppressing cytotoxicity *in vitro*.

E. F. Plow and T. S. Edgington: Isolation and characterization of a homogeneous isomeric species of carcinoembryonic antigen: CEA-S.

M. Siddiqi and P. T. Iype: Studies on the uptake of 2-deoxy-D-glucose in normal and malignant rat epithelial liver cells in culture.

P. Rogerson, W. Jarrett and L. Mackey: Epidemiological studies on feline leukaemia virus infection. I. A serological survey in urban cats.

S. Kimura, V. L. Flannery, B. Levy and P. A. Schaffer: Oncogenic transformation of primary hamster cells by herpes simplex virus type 2 (HSV–2) and an HSV–2 temperature-sensitive mutant.

Y. Hinuma, M. Suzuki and T. Sairenji: Epstein–Barr virus-induced cap formation in human lymphoblastoid cells.

K. Nelson, S. B. Pollack and K. E. Hellström: Specific anti-tumor responses by cultured immune spleen cells. I. *In vitro* culture method and initial characterization of factors which block immune cell-mediated cytotoxicity *in vitro*.

W. D. Winters and J. R. Rich: Human meningioma antigens.

A. W. Boddie, Jr., E. C. Holmes, J. A. Roth and D. L. Morton: Inhibition of human leukocyte migration in agarose by KCl extracts of carcinoma of the lung.

G. Giraldo, E. Beth, F. M. Kourilsky, W. Henle, G. Henle, V. Miké, J. M. Huraux, H. K. Andersen, M. R. Gharbi, S. K. Kyalwazi and A. Puissant: Antibody patterns to herpesviruses in Kaposi's sarcoma: Serological association of European Kaposi's sarcoma with cytomegalovirus.

H. Okabe, R. V. Gilden and M. Hatanaka: Murine sarcoma virus related nucleic acid sequences in a non-transforming virus derived from an interspecies pseudotype sarcoma virus.

British Journal of Cancer *May, 1975*

L. B. Epstein and R. A. Knight: Studies on mouse moloney virus induced tumours: I. The detection of p30 as a cytotoxic target on murine moloney leukaemic spleen cells, and on an *in vitro* moloney sarcoma line by antibody mediated cytotoxicity.

L. B. Epstein and R. A. Knight: Studies on mouse moloney virus induced tumours: II. Detection of p30 in the serum of mice with moloney leukaemia by *in vitro* blocking of complement-dependent antibody mediated cytotoxicity.

T. Neveu, D. Staebler, G. Chavanel and P. Burtin: Study of the antigenic cross reactivity between carcinoembryonic antigen and "non specific cross reacting antigens" (NCA and NCA 2).

J. B. Shelton, C. W. Potter and I. Carr: Cellular immunity to myelin protein in man and in an animal model system as measured by the macrophage migration inhibition test.

O. Y. Pletyushkina, J. M. Vasiliev and I. M. Gelfand: Neoplastic fibroblasts sensitive to the growth inhibition by homologous cells but insensitive to the inhibition by parent normal cells.

J. F. Smyth and K. R. Harrap: Adenosine deaminase activity in leukaemia: A lymphoid characteristic with diagnostic and therapeutic potential.

D. A. Cooper, V. Petts, E Luckhurst, J. C. Biggs and R. Penny: T- and B-cell population in blood and lymph node in lymphoproliferative disease.

J. Breborowicz, G. C. Easty, M. Birbeck, D. Robertson, R. Nery and A. M. Neville: The monolayer and organ culture of human colorectal carcinomas and the associated "normal" colonic mucosa and their production of carcinoembryonic antigens.

P. A. M. Shipman, V. Littlewood, A. C. Riches and G. H. Thomas: Differences in proliferative activity of rat and human prostate in culture.

A. F. Bagshawe, D. M. Gacengi, C. H. Cameron, J. Dorman and D. S. Dane: Hepatitis Bs antigen and liver cancer. A population based study in Kenya.

A. Flaks and D. B. Clayson. *Short Communication*: The influence of ammonium chloride on the induction of bladder tumours by 4-ethylsulphonylnaphthalene-1-sulphonamide.

J. Read: *Short Communication.* The activation of dimethylnitrosamine (DMN) by yeast extracts.

P. M. Naha: *Short Communication.* Unique protein in the nucleus of a cell line transformed by the carcinogen methylnitrosourea.

S. Muldal: *Letter to the Editor.* Possibility of pre-selection of patients for prognosis in acute myeloid leukaemia.

I. D. H. Todd: *Book Review.* Cancer of the lung.

M. J. Peckham: *Book Review.* Bronchial carcinoma.

A. J. Bateman: *Book Review.* Genetics and the animal cell.

Europ. J. Cancer Vol. 11, pp. 383–388. Pergamon Press 1975. Printed in Great Britain

The Effect of (±) 1,2-bis(3,5-dioxopiperazin-1-yl) Propane (ICRF 159) on Liver Metastases from a Hamster Lymphoma

ANNE ATHERTON

Cancer Chemotherapy Department, Imperial Cancer Research Fund, P.O. Box 123, *Lincoln's Inn Fields, London WC2A* 3*PX, Great Britain*

Abstract—*The effect of* (±)1,2,bis(3,5-*dioxopiperazin*-1-*yl*) *propane* (*ICRF* 159) *on liver metastases from a subcutaneously implanted hamster lymphoma was studied. This agent significantly decreased the incidence of secondaries as determined by liver weight and the presence of tumour nodules at the liver surface, at doses which did not change the growth of the primary implant. On histological examination, the peripheral zones of untreated tumours showed sheets of cells between which were erythrocytes and large sinuses, while the peripheries of ICRF* 159 *treated tumours contained a well endothelialized vasculature.*

INTRODUCTION

THE GROWTH of s.c. implanted Lewis lung carcinoma (3LL) and the presence of spontaneously occurring lung metastases from this tumour in mice are inhibited by (±)1,2-bis-(3,5-dioxopiperazin-1-yl)propane (ICRF 159) [1]. The inhibition of secondary growth, however, is obtained at doses lower than those which will inhibit the growth of the primary tumour, suggesting that ICRF 159 is a drug unusual in having a specific antimetastatic action in addition to a powerful antimitotic action [1]. The Lewis lung carcinoma is a representative tumour model of blood-borne metastases which can be used for screening substances for antimetastatic action. It is also the only metastasizing tumour on which ICRF 159 has been extensively tested. To ascertain whether ICRF 159 behaved similarly in preventing metastases in a different species, type of tumour, and site of secondary growth, it was tested against a hamster lymphoma (ML) giving rise to secondaries in the liver [2].

Accepted 28 February 1975.

This tumour is a lymphoblastic lymphosarcoma, which arose spontaneously. When it is implanted s.c. as a tumour mash, secondary deposits appear regularly after two weeks in the axillary nodes of the ipsilateral side, in the liver, and occasionally in the kidneys and lungs. Hamsters bearing this tumour were treated (i) with varying doses of ICRF 159 and (ii) for different lengths of time during the growth of the tumour. The effectiveness of ICRF 159 in preventing the appearance of liver metastases was evaluated.

It has been suggested that one possible mechanism for the mode of action of ICRF 159 is that very few malignant cells are released into the bloodstream due to a normalization of the blood vessel structure at the invading margin of the primary tumour [3]. To see if treatment with ICRF 159 affected the peripheral vasculature of the lymphoma as it did that of the Lewis lung carcinoma, all tumours were removed for histology. In addition, the permeability of treated and untreated tumour vessel walls was observed by labelling with Pelikan ink [4].

The distribution of metastatic deposits after growth of the tumour in the cheek pouch and

after single i.v. injections of cells was also studied.

MATERIAL AND METHODS

Implantation and treatment of subcutaneous tumours

Twelve groups of 10 Syrian female hamsters weighing approximately 150 g were given s.c. implants (0·1 g.) of finely minced lymphoma (ML) into the flank on day 0. In some experiments, 6 mg benzylpenicillin and 10 mg streptomycin in 0·1 ml isotonic saline was added to 1·0 g of tumour mash to inhibit bacterial contamination. Treated hamsters received an i.p. injection of ICRF 159 suspended in 0·2 ml carboxymethyl-cellulose (CMC) (0·5% in 0·9% sodium chloride solution). Control hamsters received 0·2 ml CMC only. In preliminary experiments it was found that doses greater than 30 mg/kg ICRF 159 were effective in reducing primary tumour growth. Thus, to determine the effect of ICRF 159 on liver metastases, hamsters were given a once daily i.p. injection of 7·5, 15, or 30 mg/kg on days 1–8 or 1–11. To determine the shortest time for which treatment was effective, 4 groups of 10 hamsters were treated with 15 mg/kg from days 1–4, 1–8, 1–12 and 1–16. A control group of 10 hamsters received CMC only from day 1–16.

Implantation and treatment of cheek pouch tumours

Hamsters were anaesthetised by i.p. administration of sodium pentabarbital (60 mg/kg). One-tenth ml of finely minced tumour mash was inserted between the epithelial and connective tissue layers of the everted hamster cheek pouch. Four groups of 10 female Syrian hamsters were implanted with tumour on day 0, and treated on days 1–11 with an i.p. peritoneal injection of 15 mg/kg ICRF 159 or CMC, as were those hamsters bearing s.c. tumour implants.

Excision of primary tumour and evaluation of metastases

Between 16 and 19 days after implantation, the hamsters were sacrificed and the primary tumour carefully separated from the surrounding tissue (Tables 1–3). The livers were removed also and any other sites of metastatic involvement noted. The primary tumours were weighed. The extent of secondary growth in the liver was evaluated by two criteria: (i) the liver weight, and (ii) by counting any discrete nodules appearing on the surface (see Results). The increase in liver weight was judged to be due to metastatic deposits firstly because histological examination revealed varying degrees of lymphoma cell infiltration and secondly, because transplantation of such livers resulted in the growth of tumours at the site of implantation. Microscopic examination of livers with varying numbers of nodules counted macroscopically at the surface, suggested that these were in proportion to the nodules found within the liver tissue. There was no macroscopic or histological change in the size or structure of 10 female hamster livers after ICRF 159 (30 mg/kg) or CMC treatment for 21 days.

In vivo *blood vessel labelling*

Twenty hamsters were inoculated s.c. with ML, and 10 were treated with ICRF 159 (15 mg/kg) for days 1–11, while 10 control animals received CMC only. On day 14, they each received 0·75 ml of a colloidal suspension of "Pelikan" biological ink (C11/1431a) into a femoral vein one hour before sacrifice. The original Pelikan ink was diluted 1:4 with 1% gelatin in sterile saline [5]. After sacrifice, the tumours were carefully removed and fixed in neutral buffered formalin.

Intravenous injection of tumour cell suspension

Suspensions containing many discrete tumour cells were made by agitation of tumour fragments with TC199 medium. After filtration through sterile cotton wool, the cells were counted and the percentage viability determined by the uptake of Trypan Blue. Cells were injected into the right femoral vein in a volume of 0·2 ml on day 0. On day 14, the animals were sacrificed and examined for the distribution of secondary deposits.

To determined the effect of ICRF 159 treatment on the growth of "metastases" following i.v. injection of lymphoma (ML) cells, 10 Syrian female hamsters were given 4×10^6 live tumour cells in 0·2 ml of suspension (66% viability) and subsequently treated with either 15 mg/kg ICRF 159 or CMC from days 1–11. On day 14 they were sacrificed and examined for secondaries.

RESULTS

Inhibition of liver metastases by ICRF 159

Dose response to ICRF 159. Macroscopic and microscopic examination of the livers revealed

metastatic involvement either as a diffuse infiltration of lymphoma cells, resulting in an increase in liver weight, or as discrete pale nodules at the liver surface with a minimal weight increase. Occasionally nodules were seen on the surface of an enlarged liver. Consequently, metastases were evaluated either by liver weight or by counting the nodules (Methods). It is possible that the addition of crystamycin to the original tumour mash had some effect in causing secondary deposits to grow in a rather more diffuse form (Tables 1–3). Although 0·1 ml of tumour mash was implanted in each animal, a characteristic of this tumour was that the growth rate varied with each experiment. It must be stressed that within one batch of animals, implanted with the same tumour mash, each tumour grew similarly and the size of the tumour at sacrifice bore no relationship to the number of animals showing metastatic involvement, which by 19 days, was usually 100%.

The results of three experiments involving 120 hamsters implanted with tumour in the flank are summarized in Table 1. For all different regimes of ICRF 159 there was no significant difference in the weights of the primary tumours. In experiment 1, treatment from day 1–8 produced no significant effect on liver metastases in either form. An increase in mean tumour weight was observed at a dose of 7·5 mg/kg, but was not reproducible and did not affect metastases of either type. Treatment from days 1–11 and sacrifice at day 16 resulted in a significant decrease in mean liver weight at doses of 15 and 30 mg/kg, while the number of discrete nodules was decreased significantly only at 30 mg/kg. In experiment 3, the same treatment, but with sacrifice at day 19 showed no decrease in liver weight over the controls, but liver nodules were decreased significantly at 15 and 30 mg/kg.

Secondary deposits in the liver from tumours growing in the cheek pouch were inhibited by the same doses as those growing in the flank. Tumour bearing animals treated from days 1–11 and sacrificed on day 17–18 had significantly less metastatic involvement with no effect on the primary tumour (Table 2).

Minimum period required to inhibit metastases. When hamsters were treated with 15 mg/kg ICRF 159 for different lengths of time (days 1–4, 1–8, 1–12 and 1–16) during the growth of s.c. implants the size of the tumours on sacrifice at day 18 was not significantly decreased. Metastasis was only inhibited in those animals which had been treated for 1–12 or 1–16 days (Table 3). This suggests that the minimum time required for effective treatment of the hamster lymphoma at low doses of ICRF 159 lies between 8 and 12 days after implantation.

Histological examinations of primary tumours. Control and treated tumours having no significant differences in weight were compared histologically. Control tumours showed a larger zone of central necrosis, while tumours treated with dose regimes which inhibit metastases showed a structural change at the periphery comparable with that demonstrated in 3LL tumours [4]. The advancing edge of control tumours showed tightly packed sheets of cells with poorly formed blood vessels, sinusoids, and frequent areas of haemorrhage between the cell layers. In contrast, the edge of treated tumours contained many well-formed blood vessels among loose layers of cells (Fig. 1a, 1b).

Injection of Pelikan ink however, failed to

Table 2. Dose response to ICRF 159 of hamster lymphoma (ML) implanted in the cheek pouch

Four groups of 10 hamsters were implanted in the cheek pouch with 0·1 ml of tumour mash on day 0. They were treated on the days indicated with an i.p. injection of 15 mg/kg ICRF 159 in 0·2 ml CMC. Control animals received CMC only. The hamsters were sacrificed, and after removal of the primary tumour, the livers were evaluated for metastases.

Expt. No.	ICRF 159 (mg/kg)	Days of treatment	Day of killing	Mean tumour wt. (g)	Mean liver wt. (g)	Crystamycin
1	15	1–11	18	T 0·38 / C 0·48 } NS	T 10·0 / C 15·5 } $P = < 0{\cdot}02$	0·1 ml/ml tumour mash
2	15	1–11	17	T 2·18 / C 1·91 } NS	T 10·49 / C 16·60 } $P = < 0{\cdot}001$	Nil

P = significance value (Student's t-test).

Table 1. *The response of hamster lymphoma (ML) (implanted s.c.) to ICRF* 159

Twelve groups of 10 hamsters were implanted s.c. with 0·1 ml of tumour mash on day 0. They were treated on the days indicated with an i.p. injection of 7·5, 15, or 30 mg/kg ICRF 159 in 0·2 ml CMC. Control animals were given CMC only. The hamsters were sacrificed, and after removal of the primary tumour, the livers were evaluated for metastases.

Dose of ICRF 159 (mg/kg body weight)	Days of treatment	Sacrificed day	Mean tumour weight (g)	Mean liver weight (g)	Mean no. discrete liver nodules	Crystamycin
7·5	1–8	16	T 10·8 C 6·88 ($P < 0·02$)	T 9·99 C 11·19 NS	T 42·10 C 33·11 NS	NIL
15	1–8	16	T 9·86 C 6·88 NS	T 9·36 C 11·19 NS	T 13·10 C 33·11 NS	NIL
30	1–8	16	T 7·91 C 6·88 NS	T 9·47 C 11·19 NS	T 11·30 C 33·11 NS	NIL
7·5	1–11	16	T 3·21 C 2·72 NS	T 8·34 C 9·71 NS	T 23·95 C 29·60 NS	0·1 ml/ml tumour mash
15	1–11	16	T 4·04 C 2·72 NS	T 7·0 C 9·71 ($P < 0·05$)	T 15·21 C 29·60 NS	0·1 ml/ml tumour mash
30	1–11	16	T 2·38 C 2·72 NS	T 6·20 C 9·71 ($P < 0·02$)	T 6·23 C 29·60 ($P < 0·05$)	0·1 ml/ml tumour mash
7·5	1–11	19	T 4·57 C 4·19 NS	T 9·29 C 7·22 NS	T 22·16 C 18·80 NS	NIL
15	1–11	19	T 4·81 C 4·19 NS	T 7·58 C 7·22 NS	T 0·87 C 18·80 ($P < 0·05$)	NIL
30	1–11	19	T 4·33 C 4·19 NS	T 7·39 C 7·22 NS	T 1·00 C 18·80 ($P < 0·02$)	NIL

P = significance value (Student's t-test).

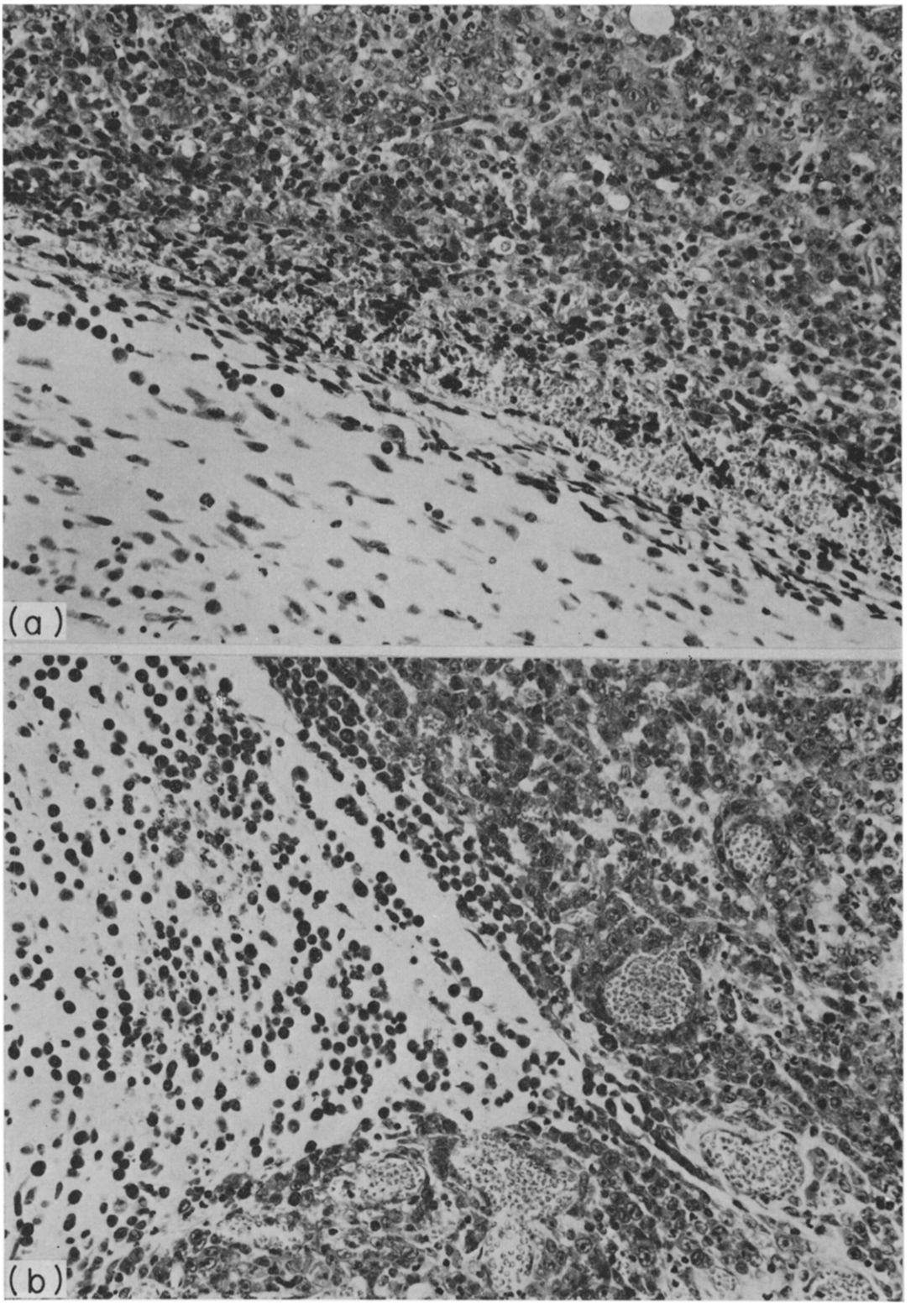


Fig. 1(a). *Section of a primary ML tumour* 14 *days after implantation, treated daily with CMC. The cells are in tight sheets, between which are many loose erythrocytes and a few large sinuses.* (*H and E* × 225)

Fig. 1(b). *Section of a primary ML tumour* 14 *days after implantation, treated daily with ICRF* 159. *There are many well-formed blood vessels at the periphery with endothelial cells clearly visible.* (*H and E* × 225)

(to face p. 386)

Table 3.

Five groups of 10 hamsters were implanted s.c. with 0·1 ml of tumour mash containing crystamycin on day 0. They were treated on the days indicated with 15 mg/kg ICRF 159, and for the days remaining up to day 16 with CMC. Controls received CMC for 16 days. They were sacrificed on day 18 and as nodules were absent, the livers were weighed to evaluate metastases.

Days of treatment	Mean tumour weight (g)	Mean liver weight (g)
1–4	T 3·27, C 3·22 } NS	T 10·38, C 10·11 } NS
1–8	T 3·82, C 3·22 } NS	T 9·87, C 10·11 } NS
1–12	T 2·72, C 3·22 } NS	T 6·04, C 10·11 } $P < 0·02$
1–16	T 2·69, C 3·22 } NS	T 6·43, C 10·11 } $P < 0·05$

P = significance value (Student's t-test).

demonstrate any obvious difference in vessel wall permeability to carbon particles between treated and untreated tumours.

"Metastases" arising from a single i.v. injection of tumour cell suspension. After a single bolus injection of ML cells into a femoral vein and sacrifice at 14 days, the hamsters were found to have "metastases" only in the livers. The extent of this involvement was not significantly affected by treatment with 15 mg/kg ICRF 159 for days 1–11.

DISCUSSION

The s.c. implanted hamster lymphoma ML habitually metastasizes to the liver. However, the large variation in tumour and liver weight between each experiment indicates that this tumour, although useful as a model for a tumour metastasising to the liver, may be unsatisfactory for routine use in screening chemotherapeutic agents. Only 50% of the hamsters show any macroscopic evidence of liver secondaries 14 days after implantation, but this increases to over 90% by 18–19 days. Death ensues at 20–21 days. The antimetastatic agent ICRF 159, known to be effective in preventing lung metastases in mice [1] is also active in decreasing liver metastases arising from both s.c. and cheek pouch lymphomas. It is however, ineffective against "metastases" arising from an i.v. injection. The antimetastatic effect is, like that seen in 3LL [1], observed at doses which do not decrease the primary growth. It is necessary to use larger doses of ICRF 159 (15–30 mg/kg) than those used on the 3LL tumour in mice to obtain an antimetastatic effect in hamsters. It is also necessary to continue treatment until at least the 11th day of tumour growth and this agrees with previous work in which treatment from days 1–7 had no effect on liver secondaries [6].

The changes in tumour blood vessel structure in the lymphoma were very similar to those observed in the 3LL tumour, suggesting that in both tumours, ICRF 159 might decrease the number of circulating cells by normalizing the structure of the tumour vessel walls. However, in contrast to 3LL tumours, injection of Pelikan ink into the ML failed to demonstrate any change in tumour vessel permeability produced by ICRF 159. The 3LL control tumours may show pooling, rather than extravasation, of carbon in the sinuses [4], which would indicate blood flow rather than permeability differences. These facts do not exclude a common action of the drug on both tumours which may be due to the amount of blood flow through the tumour, rather than any effect on tumour vessel permeability.

CONCLUSION

(±)1,2-Bis(3,5-dioxopiperazin-1-yl)propane (ICRF 159) inhibits liver metastases in hamsters bearing a metastasizing lymphoma (ML), at doses which do not affect the growth of the primary implant. The peripheries of control tumours were haemorrhagic with poorly formed blood vessels, while the same areas in treated tumours had discrete and well-formed blood vessels. These observations suggest that the antimetastatic effect of ICRF 159 is mediated via its action on the development of the tumour vasculature.

REFERENCES

1. A. J. Salsbury, K. Burrage and K. Hellmann, Inhibition of metastatic spread by ICRF 159: selective deletion of a malignant characteristic. *Brit. med. J.* **4,** 344 (1970).
2. R. L. Carter and R. K. Gershon, Studies on Homotransplantable lymphomas in hamsters—I. Histologic Responses in Lymphoid Tissues and their relationship to Metastasis. *Amer. J. Path.* **49,** 637 (1966).
3. S. E. James and A. J. Salsbury, Effect of (±)1,2-bis(3,5-dioxopiperazin-1-yl) propane on tumour blood vessels and its relationship to the antimetastatic effect in Lewis lung carcinoma. *Cancer Res.* **34,** 839 (1974).
4. A. W. Le Serve and K. Hellmann, Metastases and the normalization of tumour blood vessels by ICRF 159: a new type of drug action. *Brit. med. J.* **1,** 597 (1972).
5. N. T. A. Oswald and D. B. Cater, Effect of endotoxin from *Serratia Marcescens* on the permeability of vessels in hepatomas and carragenin granulomas of rats. *Brit. J. exp. Path.* **50,** 84 (1969).
6. R. L. Carter, Unpublished data.

Europ. J. Cancer Vol 11, pp. 389–396. Pergamon Press 1975. Printed in Great Britain

The Prognosis Following the Surgical Excision of Canine Mammary Neoplasms*

D. E. BOSTOCK

Department of Clinical Veterinary Medicine, School of Veterinary Medicine, Madingley Road, Cambridge, Great Britain

Abstract—*Three hundred and twenty bitches from which mammary neoplasms had been excised were followed up until death, or for at least 2 years post surgery. The median survival time of animals with histologically benign tumours was at least 114 weeks, whilst for dogs with carcinomas it was 70 weeks. Only 43% of dogs with carcinoma eventually died as a result of the tumour but a more accurate prognosis was possible by subdividing these tumours into their different morphological types. Animals with solid and anaplastic carcinomas had a significantly poorer survival time than those with papillary or tubular carcinomas. Other valuable prognostic aids included histological evidence of infiltration of the surrounding tissues by tumour cells in the case of tubular and solid carcinomas, and the volume of the tumour at excision.*

Canine mammary tumours may be of value as models to study the effect of various types of post surgical therapy, and particularly immunotherapy. The observation that most tumours which are destined to kill the host do so within one year of surgery indicates that evaluation of results will be possible much sooner than could be expected from human clinical trials.

INTRODUCTION

THERE is an increasing awareness of the value of spontaneous neoplasms in the dog and cat as models for the study of human cancers. One of the most important of these is carcinoma of the breast.

In the bitch, mammary tumours account for between 25 and 50% of all neoplasms [1–3], making them the largest single group, but in spite of their frequent occurrence, their biological behaviour, especially following surgical excision, is not well documented. Cotchin [4] found that, although 43·9% of bitches had histologically malignant tumours, only 9·8% of all mammary tumours proved themselves to be behaviourally malignant by metastasizing, while Howell *et al.* [5] described eventual metastasis from 6 of 22 (27%) of histologically malignant tumours of the mammary gland. In contrast, Schneider and his co-workers [3] found that 58% of bitches with surgically excised, histologically malignant neoplasms died, or were destroyed, as a result of the tumour. Fidler *et al.* [2] found that 48% of dogs treated surgically for this type of lesion succumbed to the tumour, while in the experience of Else [6], more than 50% of animals treated for mammary malignancy were dead within three months of diagnosis.

A number of workers have studied the post surgical survival time of dogs which had mammary tumours excised, but few have attempted to correlate the survival time with the histological appearance of the tumour. Misdorp *et al.* [7, 8] in their informative papers on this topic, showed, in retrospective studies, that it was possible to link post surgical survival time to the histological appearance in those animals with histologically malignant tumours which subsequently proved themselves to be

Accepted 31 January 1975.

*This work was supported by grants from the World Health Organisation and the Jowett fund and forms part of cancer research in the dog supported by the Cancer Research Campaign and the Medical Research Council.

behaviourally malignant by metastasising. This is a highly selected group however, since at the time of operation the eventual outcome cannot be foreseen. Fowler *et al.* [9] also found differences in survival time between dogs with different histological types of mammary carcinoma.

Canine mammary tumours may provide a useful model for the human disease, especially in the field of post surgical therapeutic trials, e.g. immunotherapy, chemotherapy or hormonal treatment, but before the result of such treatment can be evaluated it is essential that the effect of surgical interference alone be well understood. The aim of this paper is, therefore, to provide further information on the life expectancy of bitches from which spontaneously arising mammary tumours have been excised, but in which no other therapy was performed.

MATERIAL AND METHODS

Bitches from which mammary neoplasms had been excised and examined histologically at the Department of Clinical Veterinary Medicine, University of Cambridge, were followed up until death, or for at least 2 years after surgery. The source of cases and the follow-up method employed have been described previously [10]. Surgical interference consisted of complete excision of the tumour, together with a large area of surrounding, apparently normal, tissue but radical excision of adjacent mammary glands and regional lymph nodes was not performed unless they were obviously involved in the neoplastic process. Animals which showed clinical, histological, or radiological evidence of metastasis to regional lymph nodes or lungs at the time of surgery were not included in the subsequent follow-up studies although the possibility that small metastases were already present in lymph nodes left *in situ* cannot be excluded.

Immediately after removal, the tumour was fixed in 10% buffered formalin, followed by routine processing to produce 4 μm paraffin sections, which were then stained with Harris's haematoxylin and eosin. Blocks were taken from between 2 and 4 areas of each tumour in order to obtain as complete a picture as possible of the lesion, since, as discussed below, the histological appearance of a canine mammary tumour can vary considerably in different areas.

Each tumour was classified, according to the schema proposed by Misdorp *et al.* [7, 8] before the eventual survival time and cause of death was known, and no attempt was made to select cases for follow-up on the basis of the histological appearance of the tumour.

All tumours which contained epithelial elements, myoepithelial tissue and cartilage or bone were classified as mixed mammary tumours. Histologically benign tumours which contained both well-differentiated epithelial elements, and myoepithelial tissue, were classified as complex adenomas whilst tumours with a histologically malignant epithelial component were classified as carcinomas. These were subdivided into 4 categories on the basis of their histological appearance, these being papillary, tubular, solid and anaplastic, each category being further classified as complex or simple depending upon the presence or absence of myoepithelial tissue in addition to the epithelial element.

When attempting to subdivide carcinomas into the major subgroups described by Misdorp and his colleagues, some difficulty was experienced with the many tumours which contained both solid, and tubular or papillary tissue, either in adjacent areas or, sometimes, in the same field. In these cases the tumour was placed in the category of the most abundant type of tissue. The anaplastic carcinomas represented a relatively homogeneous group, being highly invasive, simple, and consisting of small foci of poorly differentiated, often polygonal, epithelial cells, arranged as solid clumps, irregularly shaped, flattened tubules with a slit like lumen, or as individual, infiltrating cells. A diagnostic feature was the presence of an abundant fibrous reaction around the neoplastic cells. Each carcinoma group, apart from the anaplastic tumours which were all invasive, was categorised as well circumscribed or invasive on the basis of the histological appearance of the junction between the tumour and the surrounding normal tissue. Figure 1 shows a well-defined solid and Fig. 2 a well-defined tubular carcinoma whilst Figs. 3 and 4 illustrate an invasive solid carcinoma and an anaplastic carcinoma respectively.

Some dogs had more than one mammary tumour removed simultaneously, and in these cases the animal was included only in the category of the most histologically malignant of its tumours.

When analysing survival times, animals which died as a result of surgical complications, and in which there was no evidence of tumour on post mortem examination, were not included. Some animals were still alive at the time of writing and in statistical studies (Wilcoxon Rank Sum Test) their survival times were taken as ties of the highest rank.

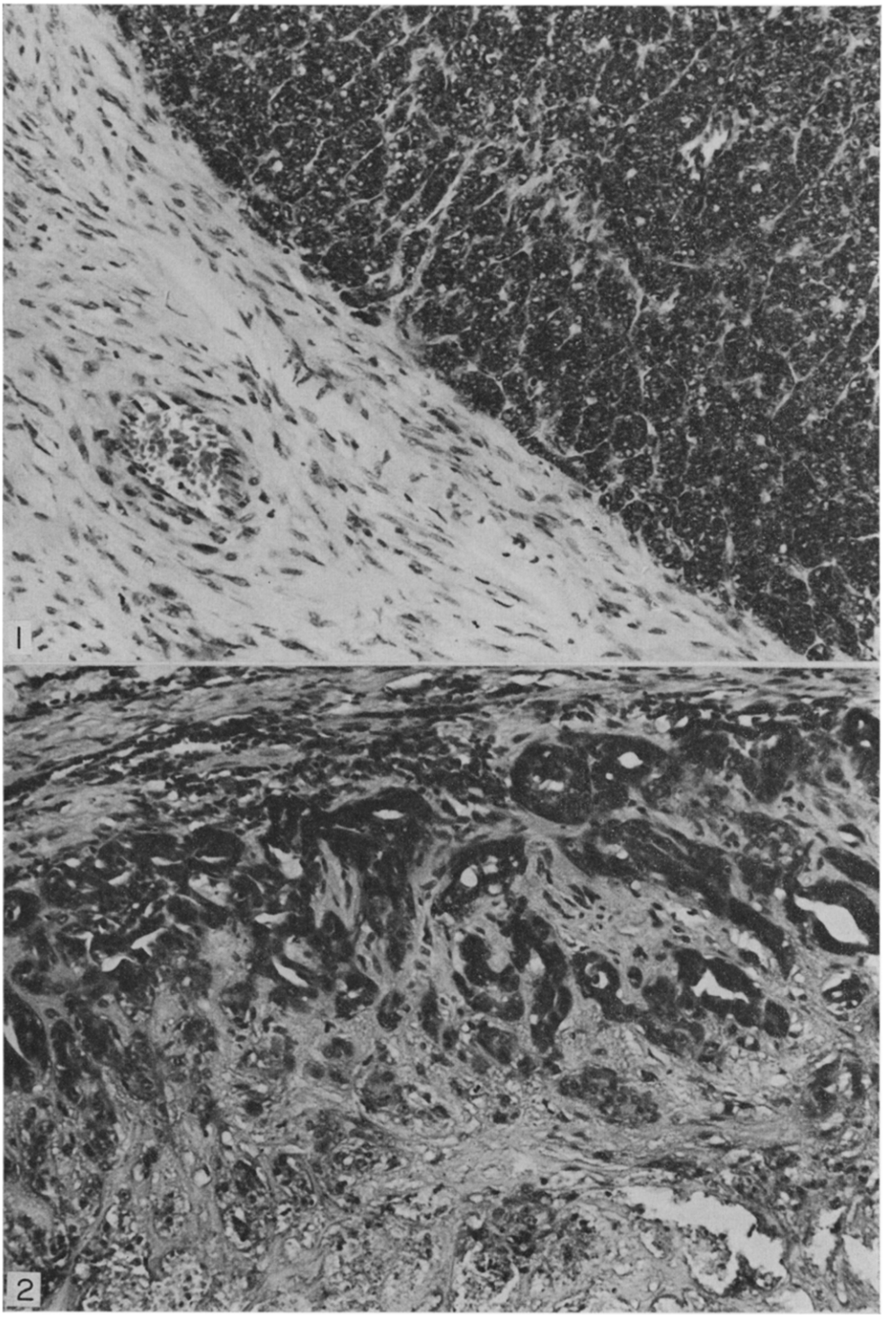


Fig. 1. *Well-defined solid mammary carcinoma.*

Fig. 2. *Well-defined tubular adenocarcinoma. Most of the tumour is necrotic and viable tissue is confined to a thin rim at the periphery.*

(to face p. 390)

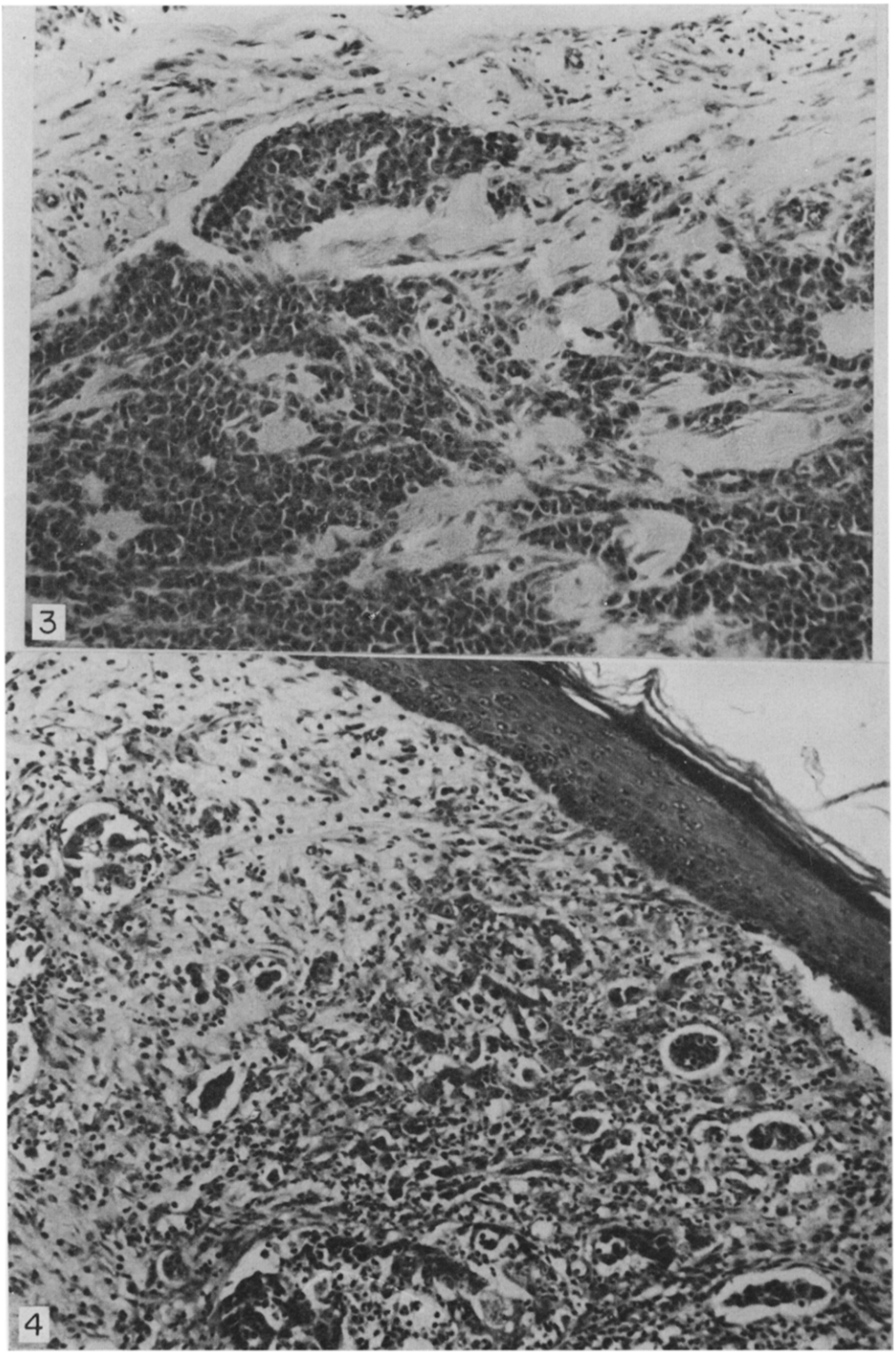


Fig. 3. Invasive, solid carcinoma.

Fig. 4. Anaplastic carcinoma showing intense infiltration of the dermal connective tissues. Vascular invasion is also conspicuous in this field.

Very few dogs were allowed to die naturally and where, in the text or tables, death is referred to, this usually implies euthanasia at the request of the owner. An animal was considered to have died as a result of the tumour when euthanasia was requested because of inoperable local recurrence, making it painful for the animal to walk, or because of severe respiratory distress as a result of pulmonary metastases. Although these animals would have survived for slightly longer without euthanasia, the increase in survival time would probably be of the order of 1–2 weeks or less.

Two animals which were destroyed because of intercurrent disease had small, mobile, recurrent tumours, without evidence of metastasis on post mortem examination, and these were not included as tumour deaths.

A number of dogs with the other types of mammary tumour described by Misdorp and his colleagues, including simple adenomas, spindle cell carcinomas, carcino sarcomas and simple sarcomas were followed up but the results have not been included here because of the small number of cases in each group.

RESULTS

Three hundred and twenty dogs have been followed up, and of these 89 (28%) are still alive. The median survival times of animals from which the commonest morphological types of mammary tumour were excised, with the total and tumour death rate, are presented in Tables 1 and 2, and the cumulative percentage of animals dying in the successive 20 week post surgical periods, from all causes and as a direct result of the tumour, is shown for each tumour category in Figs. 5–10.

There was no significant difference, using the Wilcoxon Rank Sum Test, between the survival time of animals with complex adenomas or mixed mammary tumours. Both these types showed a similar biological behaviour, although, as can be seen from Table 1, 6% of mixed mammary tumours did eventually lead to the death of the host.

The survival time of animals from which carcinomas had been removed was significantly poorer ($P < 0{\cdot}01$) than for dogs with complex adenomas or mixed mammary tumours.

Further subdivision of the carcinomas into their main histological categories revealed a marked decrease in median survival time from papillary to anaplastic, with a similar increase in the percentage of dogs dying from the tumour in the first post surgical year (Table 2). Thus, 15% of dogs with papillary adenocarcinoma, but 73% of animals with anaplastic carcinoma died because of the tumour in this time, whilst at the end of the follow-up period the median survival time for dogs with papillary adenocarcinoma was 121 weeks and that for anaplastic carcinoma only 11 weeks.

Animals with tubular and solid carcinoma showed intermediate post surgical death rates and median survival times. The difference between survival times of dogs with papillary or tubular adenocarcinoma was not significant, when the survival data were analysed by Life Table Methods [11], but dogs with solid carcinoma had a significantly poorer survival time than either the papillary or tubular type ($P < 0{\cdot}0005$) and a significantly better survival time than for animals with anaplastic carcinoma ($P < 0{\cdot}001$). Using these methods the ratio of risk of death from the tumour, taking the risk of death from papillary adenocarcinoma as unity, were as follows:— Tubular adenocarcinoma 1·7, solid carcinoma 4 and anaplastic carcinoma 8·5.

An important finding, which is best illustrated in the graphs (Figs. 5–10) is that, while deaths from all causes progressed in an almost linear fashion with time, tumour deaths showed a definite plateau, so that most animals which

Table 1. *The tumour type, age at onset, median survival time, total and tumour death rate in bitches with surgically excised mammary neoplasms*

Tumour type	Number of dogs	Mean age (years) (± S.E.)	No. of dogs dead (%) After 1 yr.	No. of dogs dead (%) After 2 yr.	No. dead from tumour (%) After 1 yr.	No. dead from tumour (%) After 2 yr.	Median survival time in weeks
Complex Adenomas	45	9·5 (0·62)	6(13)	14(30)	0	0	125
Mixed mammary Tumours	48	9·4 (0·54)	8(17)	18(37)	3(6)	3(6)	114
Carcinomas	227	9·7 (0·19)	115(51)	164(72)	88(39)	97(43)	70

Table 2. The median survival time, total and tumour death rate in bitches with surgically excised mammary carcinomas. The carcinomas have been subdivided on a histological basis

Tumour types	Number of dogs	Mean age (years) (± S.E.)	No. of dogs dead (%) After 1 yr.	No. of dogs dead (%) After 2 yr.	No. dead from tumour (%) After 1 yr.	No. dead from tumour (%) After 2 yr.	Median survival time in weeks
Papillary Adenocarcinoma	34	9·1 (0·48)	9(26)	17(50)	5(15)	7(21)	121
Tubular Adenocarcinoma	88	9·2 (0·36)	29(35)	53(60)	21(24)	28(32)	90
Solid Carcinoma	64	9·8 (0·31)	36(56)	45(71)	32(50)	34(53)	44
Anaplastic Carcinoma	41	11·0 (0·43)	36(88)	40(98)	30(73)	31(76)	11

were destined to die from their tumour had done so within 1 year of surgery.

Using the Wilcoxon Rank Sum Test, no significant difference was found within each carcinoma category, between survival times of animals with complex or simple tumours. Because the large numbers of deaths from other causes may have obscured small differences in tumour death rate, the combined survival times for all complex carcinomas were compared to those for all simple carcinomas, using Life Table Analysis, but again, no significant difference was detected. In the case of papillary carcinoma more animals eventually died from simple than complex carcinomas, but for solid carcinomas the position was reversed (Table 3). None of these differences was significant using the Chi-square test, however.

When the difference in survival times between dogs with invasive or well defined tumours was examined, for both tubular and solid carcinoma, dogs with histologically invasive tumours had a significantly poorer survival time ($P < 0{\cdot}05$) than those with a well defined tumour. No difference was found between dogs with well defined or invasive papillary adenocarcinoma but this may have been due to the small number of animals with invasive tumours in this category.

As well as having a shorter survival time, a greater percentage of animals with invasive tumours died as a result of the neoplastic

Table 3. The relationship between median survival time, tumour death rate and histological appearance in bitches with surgically excised mammary carcinomas

Tumour type	No. of animals (% of total)	No. dead from tumour (%)	Significance of difference (Chi-square)	Median survival time	Significance of difference (Wilcoxon rank sum test)
Papillary adeno-carcinoma					
Invasive	6(18)	3(50)		65	None
Well-defined	28(82)	4(14)	None	128	
Complex	9(26)	1(11)		90	
Simple	25(74)	6(24)	None	134	None
Tubular adeno-carcinoma					
Invasive	26(29)	12(46)		38	$P = 0{\cdot}02$
Well defined	62(71)	16(26)	None	110	
Complex	63(72)	20(35)		84	
Simple	25(28)	8(32)	None	64	None
Solid carcinoma					
Invasive	35(55)	26(74)	$P < 0{\cdot}001$	26	$P < 0{\cdot}05$
Well defined	29(45)	8(27)		82	
Complex	7(11)	6(86)		20	
Simple	57(89)	28(49)	None	48	None

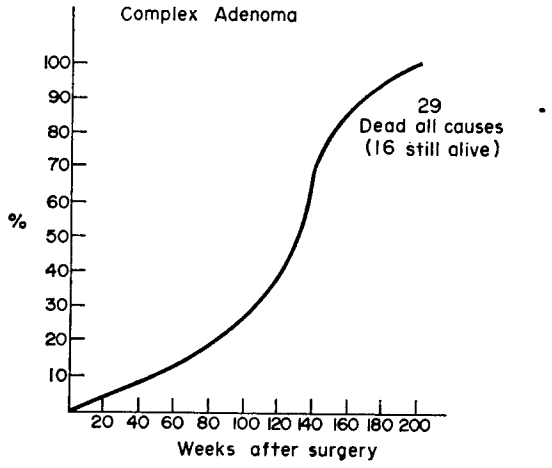


Fig. 5. *Percentage deaths from all causes against time after surgery for dogs with complex adenoma.*

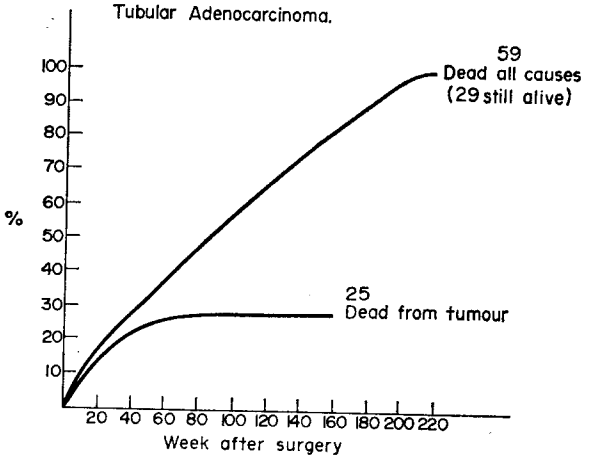


Fig. 8. *Percentage deaths from all causes, and tumour deaths, against time after surgery for dogs with tubular adenocarcinoma.*

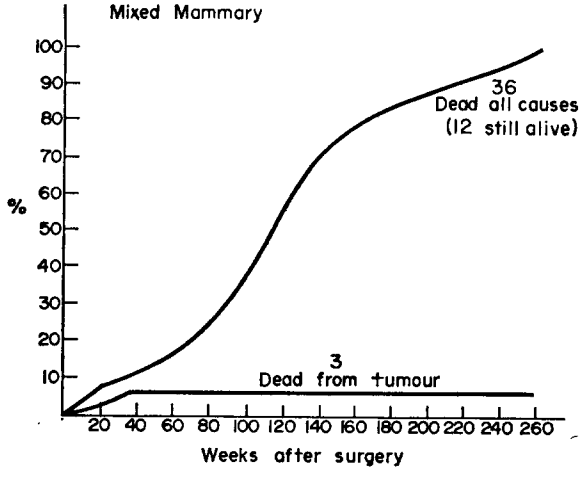


Fig. 6. *Percentage deaths from all causes, and tumour deaths, against time after surgery for dogs with mixed mammary tumour.*

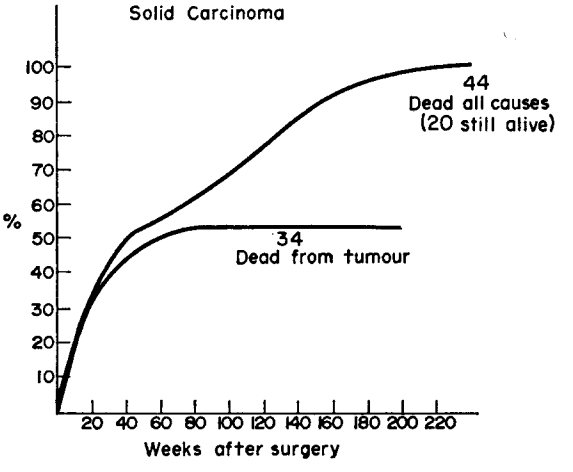


Fig. 9. *Percentage deaths from all causes, and tumour deaths, against time after surgery for dogs with solid carcinoma.*

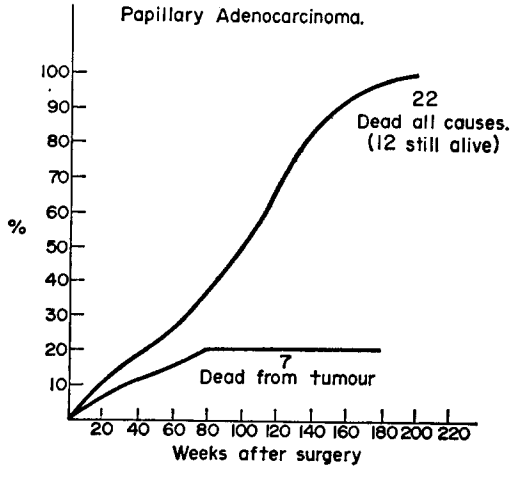


Fig. 7. *Percentage deaths from all causes, and tumour deaths, against time after surgery for dogs with papillary adenocarcinoma.*

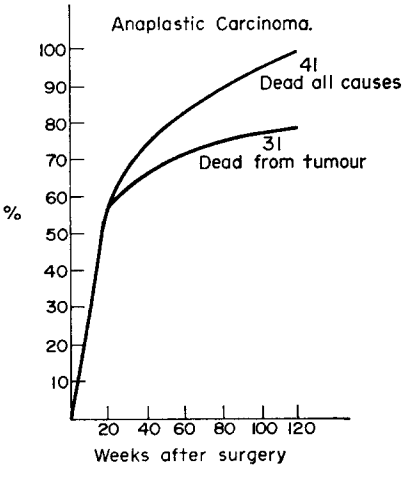


Fig. 10. *Percentage deaths from all causes, and tumour deaths, against time after surgery for dogs with anaplastic carcinoma.*

process than did those with well defined lesions (Table 3). These differences in tumour death rate were not significant in the case of papillary or tubular carcinoma ($P > 0{\cdot}05$), but were significant for solid carcinomas ($P < 0{\cdot}001$) using the Chi-square test.

Other factors considered to be important as prognostic aids included clinical observations of the volume of the tumour at excision, and the length of the delay between its observation and surgical removal. When comparing groups of dogs in which these parameters were markedly different (Table 4), a significant reduction in the survival time for animals with tubular or solid carcinomas which were more than 100 cm^3 in volume at excision was noted ($P < 0{\cdot}05$). In the case of tubular carcinomas this appeared to be the most valuable single prognostic feature, as 79% of dogs with the larger tumours eventually died from them. About twice as many animals with the larger tubular or solid carcinomas died as a direct result of the neoplasm, compared to those with smaller tumours, these differences being significant ($P < 0{\cdot}001$ for tubular and $P = 0{\cdot}05$ for solid carcinomas) using the Chi-square test.

There was no significant difference between survival times of animals in which the delay between observation and surgery was less than, or more than, 20 weeks and likewise no difference between the number of animals dying from the tumour in these categories.

From Table 2 it can be seen that there was an increase in the age of onset of carcinomas with increase in malignancy. Using Student's t test there was no significant difference in age of onset between animals with papillary, tubular or solid carcinomas, but dogs with anaplastic carcinomas were significantly ($P < 0{\cdot}05$) older than any other group when the tumour was first noted. Using the Life Table Methods previously described, deaths from other causes, excluding cancer were significantly higher in dogs from which anaplastic carcinoma had been removed than in any other carcinoma group, this finding probably being due to the increased age of onset of anaplastic carcinomas.

Table 4. The relationship between tumour volume at removal, delay between detection and removal, and the survival of bitches with surgically excised mammary carcinomas

Tumour category	No. of animals (% of total)	No. dead from tumour (%)	Significance of difference (Chi-square)	Median survival time	Significance of difference (Wilcoxon Rank sum test)
Papillary carcinoma					
< 100 cm^3	12 (60)	2 (17)	None	149	None
> 100 cm^3	8 (40)	2 (25)		108	
< 20 wks. delay	10 (45)	0 (0)	None	104	None
> 20 wks. delay	12 (55)	4 (33)		120	
Tubular carcinoma					
< 100 cm^3	38 (67)	9 (24)	$P < 0{\cdot}001$	112	$P < 0{\cdot}05$
> 100 cm^3	19 (33)	15 (79)		50	
< 20 wks. delay	28 (45)	9 (32)	None	106	None
> 20 wks. delay	34 (55)	10 (29)		112	
Solid carcinoma					
< 100 cm^3	25 (63)	8 (32)	$P < 0{\cdot}05$	112	$P < 00{\cdot}5$
> 100 cm^3	15 (37)	10 (67)		33	
< 20 wks. delay	25 (63)	12 (48)	None	65	None
> 20 wks. delay	15 (37)	7 (47)		33	
Anaplastic carcinoma					
< 100 cm^3	18 (75)	13 (72)	None	20	None
> 100 cm^3	6 (25)	5 (83)		10	
< 20 wks. delay	18 (87)	13 (72)	None	17	Group too small for statistical analysis
> 20 wks. delay	3 (13)	2 (67)		84	

Many workers, including Fidler and Brodey [2] and Cotchin [4] have noted the predisposition of the inguinal mammary glands to the development of tumours. This is again illustrated in Table 5, where it can be seen that although the first 4 glands had a roughly equal distribution of tumours, the incidence was 2·5–3 times greater in the posterior glands.

Table 5. *The distribution of tumours in canine mammary glands*

Gland No.	% All tumours
1	16
2	14
3	13
4	16
5	41

There was no evidence, in the present series, of any one breed of dog having a greater incidence of mammary neoplasia than would be expected from its prevalence in the general canine population.

DISCUSSION

As a number of authors, including Cotchin [4] and Misdorp and his colleagues [7] have noted, there is a relatively large number of canine mammary tumours which are difficult to categorise as obviously benign or malignant using the usual histological criteria. Thus, according to the latter authors, estimates of histological malignancy in different series of canine mammary tumours have varied from 26 to 74% of the total. Although some of these differences could have arisen due to unintentional selection of the more clinically malignant tumours, it appears that, in the past, different histological criteria of malignancy have been employed by various workers. If results from more than one centre are to be compared meaningfully, it is essential that the same criteria are used in each, and it is obviously in the borderline cases that differences in classification could arise. In this work, as suggested by Misdorp *et al.* [7, 8] some tumours which showed no evidence of infiltration of the surrounding connective tissues or vascular system, and even, in a few cases, were apparently encapsulated, were classified as carcinomas. In these cases the diagnosis of malignancy was based upon one or more of the following criteria: The presence of large zones of necrosis within the tumour mass, obvious mitotic activity, cellular anaplasia or extensive squamous metaplasia.

Using these criteria to classify all the canine mammary tumours received in this Department over a 5 year period, 51% were found to be histologically malignant, this figure agreeing with that of Fidler and Brodey [2].

As can be seen from Table 3, the majority of well-defined carcinomas were, in fact, behaviourally benign, the different histological types all behaving in a similar fashion, but no histological criterion was identified which could distinguish the well-defined tumour which was destined to kill its host, from that which was not.

There was no evidence that delay between detection and surgery significantly altered the survival time. This may have been due to the fact that advice is sought quickly for rapidly growing tumours, whilst more slowly growing lesions tend to be neglected until they become very large, and therefore carry a worse prognosis. These factors would tend to obscure any increase in survival time which would probably occur if the more slowly growing lesions were removed promptly.

Three mixed mammary tumours led directly to the death of the host. One tumour, which recurred without metastasis, was predominantly a well-defined, complex solid carcinoma which contained a number of small zones of cartilage towards its centre. One tumour produced multiple metastatic epithelial tumours of a poorly differentiated nature, whilst in the third the epithelial elements had undergone obvious squamous metaplasia. In the latter case, the recurrent tumour and pulmonary metastases which subsequently developed had the histological appearance of squamous cell carcinomas.

From Table 1 it can be seen that more than half the dogs with mammary carcinomas can be cured by surgery alone, so that further therapy is not indicated. Following the surgical excision of some tumours, however, the post surgical tumour death rate is high and it is important to identify these cases in order to institute further therapy. This could include chemotherapy, hormonal treatment or immunotherapy, the latter now being of considerable interest in the treatment of human neoplasms. Owen and Bostock [12] have recently shown that active, non-specific immunotherapy using B.C.G. can delay the onset of metastases from canine osteosarcoma following amputation of the affected limb, and it is possible that a similar phenomenon may apply to post surgical immunotherapy of mammary neoplasms. As Mathé and his colleagues [13] have pointed out, it is important that, at the commencement of immunotherapy, relatively few cancer cells are present in the host. In the context of

mammary carcinoma this is achieved immediately after complete surgical excision of the tumour, in those animals without clinical or radiographic evidence of metastasis.

In order to evaluate the effect of post surgical immunotherapy as quickly and accurately as possible, those types of tumour which have a very high probability of recurrence or metastasis should be selected for treatment. These include all anaplastic carcinomas (Fig. 4), invasive solid carcinomas (Fig. 3) and all solid and tubular carcinomas which measured more than 100 cm^3 in volume at surgery. Tumours which are of less value in evaluation of therapy, because of the difficulty of predicting accurately which tumours would metastasise without further treatment, include all well-defined carcinomas (Figs. 1, 2) and the smaller, invasive tubular and papillary carcinomas.

Since the canine tumour which is destined to destroy its host usually does so within 1 year of surgery, the effect of post surgical immunotherapy of mammary carcinoma could be evaluated much more quickly in the dog than in man, where post surgical survival times of 5 years are common.

Acknowledgements—I wish to thank all those colleagues in practice who submitted material for this work, which could not have been completed without their active cooperation. I would also like to thank Drs. A. R. Jennings, W. Misdorp and L. N. Owen for their advice and encouragement, Mr. D. G. Clayton for his invaluable assistance with the statistics and Mr. K. Hunt, Miss M. Dye, Miss P. Money and Miss R. A. Dickerson for technical assistance.

REFERENCES

1. A. C. Anderson, Parameters of mammary gland tumours in aging Beagles. *J. Amer. vet. med. Ass.* **147,** 1653 (1965).
2. I. J. Fidler, D. A. Abt and R. S. Brodey, The biological behaviour of canine mammary neoplasms. *J. Amer. vet. med. Ass.* **151,** 1311 (1967).
3. R. Schneider, C. R. Dorn and D. O. N. Taylor, Factors influencing canine mammary cancer development and post surgical survival. *J. nat. Cancer Inst.* **43,** 1249 (1969).
4. E. Cotchin, Mammary neoplasms of the bitch. *J. comp. Path.* **68,** 1 (1958).
5. J. McC. Howell, J. Ishmael, J. Tandy and I. B. Hughes, A 6 year survey of tumours of dogs and cats removed surgically in private practice. *J. small Anim. Pract.* **11,** 793 (1970).
6. R. W. Else, Letter to the editor. *J. small Anim. Pract.* **15,** 417 (1974).
7. W. Misdorp, E. Cotchin, J. F. Hampe, A. G. Jabara and J. Von Sandersleben, Canine malignant mammary tumours. II. Adenocarcinomas, solid carcinomas and spindle cell carcinomas. *Vet. Path.* **9,** 447 (1972).
8. W. Misdorp, E. Cotchin, J. F. Hampe, A. G. Jabara and J. Von Sandersleben, Canine malignant mammary tumours. III. Special types of carcinomas, malignant mixed tumours. *Vet. Path.* **10,** 241 (1973).
9. E. H. Fowler, G. P. Wilson and A. Koestner, Biologic behaviour of canine mammary neoplasms based on a histogenetic classification. *Vet. Path.* **11,** 212 (1974).
10. D. E. Bostock, The prognosis in cats bearing squamous cell carcinoma. *J. small Anim. Pract.* **13,** 119 (1972).
11. M. Hills, *Statistics for Comparative Studies.* Chapter 5. p. 148. Chapman & Hall, London (1974).
12. L. N. Owen and D. E. Bostock, Effects of intravenous B.C.G. in normal dogs with spontaneous osteosarcoma. *Europ. J. Cancer* **10,** 775 (1974).
13. G. Mathé, J. L. Amiel, L. Schwarzenberg, M. Schneider, A. Cotton, J. R. Schlumberger, M. Hayat and F. DeVassal, Active immunotherapy for acute lymphoblastic leukaemia. *Lancet* **1,** 697 (1969).

Europ. J. Cancer Vol. 11, pp. 397–402. Pergamon Press 1975. Printed in Great Britain

Further Studies on the Radioresistance of Highly Purified Mouse and Mammary Tumor Virus*

C. GORKA† and J. MOURIQUAND

Laboratoire de Biologie Cellulaire, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble, B.P. 85 Centre de tri-38041 Grenoble Cedex, France, and Laboratoire d'Histologie, Faculté de Médecine, Domaine de la Merci, 38700 La Tronche, France

Abstract—*The effects of high doses of γ-rays, ranging from 1080 to 3960 kR, on cell free extracts from mammary tumors and highly purified MTV are compared.*

Mammary tumor occurrence as well as latency in BALB/c inoculated mice are not affected by the high doses used.

Dilutions from 10^{-2} to 10^{-5} of irradiated samples are not followed by a decrease of biological activity, even at the dilution of 10^{-5} which is characterized by a drop in the mammary tumor-inducing capacity of the non-irradiated control specimen.

The incubation with ribonuclease crystalline (RNase)‡, desoxyribonuclease (DNase)‡ and pronase B grade (pronase) § does not affect the biological activity of irradiated samples.

Ultrastructural examination of irradiated virus suspensions shows very rare remnants of MTV compared to the typical aspect of the non-irradiated virus.

INTRODUCTION

RADIATION-induced effects on different types of single stranded nucleic acid viruses have been evaluated by several authors, using X- and γ-rays. Tobacco Mosaic virus, Φ × 174 and R17 bacteriophages were used mainly as basic models for these studies [1–3].

Recently, the same techniques were applied to oncornaviruses, and the dose response curves thus obtained were exponential. In some cases different functions of a same virus (adsorption, hemagglutination, infectivity) could be dissociated because of their different radiosensibility.

The mammary tumor virus (MTV) is characterized by a high resistance to ionizing radiation [4]. Samples of viral suspensions submitted to high doses of γ-rays (4000 kR) still induce mammary tumors in mice. Infectivity is preserved in spite of the high doses of γ-rays used. This peculiar behavior of MTV is unique. Other oncornaviruses such as Rous sarcoma virus (RSV) and Gross leukemia virus (GLV) display, under similar conditions, a loss of infectivity: the D_{10} dose which reduces viral infectivity to one-tenth of its original value is 600 kR for RSV and GLV [5] and 1200 kR for Friend leukemia virus [6].

Previous studies on MTV with X-rays or γ-rays used infected milk or cell free extracts from mammary tumors. The samples were irradiated unfrozen [7] or frozen in liquid nitrogen [4] or lyophilized [8]. The very high radioresistance observed in our previous studies may be due to some subcellular component,

Accepted 14 January 1975.

*This work was supported by a grant attributed by the "Caisse Nationale d'Assurance Maladie des Travailleurs Salaries" from the "Institut National de la Santé et de la Recherche Médicale."

†Attachée de Recherche de l'Institut National de la Santé et de la Recherche Médicale.

‡Nutritional Biochemical Corp., Cleveland, Ohio.

§Calbiochem, Los Angeles, California.

present in milk or cell free extracts which could interfere with γ-rays. The purpose of the present study was to question such a hypothetical radioprotective factor by using highly purified MTV suspensions. The virus preparations clarified on density gradients were suspended in a saline buffer and exposed to several doses of γ irradiation. Serial dilutions of the samples were tested for tumor-inducing capacity. Several enzymatic treatments also were used on irradiated specimens prior to bioassays: Pronase, DNase, RNase were thus tested.

MATERIAL AND METHODS

1. *The virus: isolation and purification of virus particles*

Mammary tumor viruses were extracted from spontaneous mammary tumors of the PS mice [9]. Four pools of tumors were used.

Tumors were excised and pooled in a freezer at $-50°C$, in the following suspending medium: 15% calf serum, 75% modified medium 199, 10% dimethyl-sulfoxide (DMSO). The storage period did not exceed one month.

(*a*) *The tumor extract.* Ten grams of tumors were mixed with 100 ml of buffer. The buffer used consisted of 0·61% NaCl + 0·04% bovine serum albumin + 0·02M McIlvaine phosphate-citrate buffer (pH 7).

Cells and debris were removed from the homogenate by two runs at 3000 *g* for 5 min, and 10,000 *g* for 15 min, respectively. To the resulting supernatant, 1/100 volume of 0·1M sodium EDTA, pH 7 was added. The centrifugation at 10,000 *g* for 15 min was repeated and the clarified supernatant was spun at 150,000 *g* for 60 min in a Spinco rotor type 40. Pellets were suspended in the same buffer to which 30% calf serum was added: the dilution was 20 ml/1 g tumor.

(b) *Highly purified virus.* The method described by Hageman *et al.* [10] has been modified for the preparation of virus suspensions. The cell-free extracts pellets were suspended in 10 ml of buffer and layered on a discontinuous Ficoll D_2O + 7% sucrose gradient. This discontinuous density gradient was performed by layering 0·4 ml of 4 Ficoll solutions with densities ranging from 1·12 to 1·16; the densities required were obtained by adding to sucrose D_2O solution, 10% H_2O, 5·4% W/V Ficoll, 9·4% Ficoll and 20% Ficoll. This gradient was performed immediately before use. After centrifugation at 175,000 *g* for 105 min in a Spinco SW 39 L rotor, the gradients were collected by puncturing the bottom of the tubes. The virus zone which was found to be situated between the densities 1·15 and 1·16, was diluted to 10 ml with buffer and centrifuged at 10,000 *g* for 15 min and 150,000 *g* for 60 min.

The pellet was suspended in a small volume of buffer, layered on top of a 9–20% Radioselectan* gradient and centrifuged at 69,000 *g* for 20 min in a Spinco SW 39 L rotor.

A light-scattering zone observed in the middle of the tube was collected, diluted with buffer and spun at 144,000 *g* for 60 min. The pellet thus obtained was suspended in the buffer using the same dilutions as for the tumor extracts.

The purity of the virus pellets, in several instances, was controlled under electron microscopic examination.

2. *Irradiation procedure*

Extracts and virus suspensions were poured into quartz ampullae and frozen in liquid nitrogen. The frozen specimens were placed in a pile and submitted under direct effect conditions to γ ray radiation (1·5 MeV): 6000 rad/min. The following doses were used: 1080 kR, 2160 kR and 3960 kR.

The samples remained in a frozen state during the irradiation procedure.

3. *Enzymatic treatments*

The effect of several enzymes was studied on 2160 kR irradiated virus samples. The enzymes assays were carried out in 0·02M phosphate-citrate buffer containing 0·1M NaCl. After incubation the solution was kept at +4°C until the mice were inoculated.

(a) *Ribonuclease and desoxyribonuclease.* Irradiated virus samples were incubated with RNase or DNase (10 μg/ml) for 20 min. at 37°C [11].

(b) *Pronase.* Irradiated virus samples were incubated with pronase (1 mg/ml) for one hour at 37°C.

4. *Tumor inducing capacity tests*

Four hundred and five three-week-old mice of the BALB/c strain were inoculated i.p. with 0·25 ml of virus suspension per mouse.

Irradiated suspensions were injected at dilutions ranging from 10^{-2} to 10^{-5} (equivalent to 120 and 0·12 mg respectively of fresh tumor tissue per mouse).

The irradiated specimens submitted to enzymatic treatments belonged to the same virus sample. They were inoculated at a dilution of 10^{-3} corresponding to 12 mg of fresh tumor tissue per mouse.

*Société d'Exploitation des Produits Pharmaceutiques 47, rue Carpeaux—59. Roubaix (France) (Free sample).

The control groups consisted of mice injected either with the same non-irradiated cell free extracts and purified virus preparations, or with BALB/c embryo cell-free extracts irradiated at the same doses. The same treatments (freezing, thawing, diluting) were applied to the control specimen as well as to the irradiated one.

The mice were forced-bred and kept under observation until occurring of tumors. The animals dead before the age of four months were excluded from our data (53 out of 458). The cause of this early mortality is mostly due to some endogenous virus such as mouse hepatitis.

5. *Electron microscopy*

Ultrastructural examinations of irradiated virus suspensions were performed with a few drops of the sample which was tested for tumor-inducing capacity. Irradiated virus samples were fixed for 1 or 2 hr with 0·005% glutaraldehyde solution (final dilution) or in osmium tetroxyde vapors. One drop of suspension was placed on a grid coated with a carbon formwar film. After one min, excess material was removed from the grid by touching its edge with a filter paper. One drop of 2% phosphotungstic acid (PTA) adjusted to pH 7 was placed on the grid, then drawn off within one min with a filter paper and the grid air-dried.

RESULTS

Since the amount of MTV present in tumors is highly variable, results obtained from irradiated virus suspensions are always compared to those from a non-irradiated one prepared with the same pool of tumors. This situation accounts for the limited amount of viral suspensions available for each of the groups tested.

The non-irradiated samples always receive freezing and thawing treatment similar to the irradiated one.

1. *Biological activity of cell-free extracts and of virus suspensions after irradiation*

The biological activity of cell-free extracts and highly purified virus suspensions irradiated with doses ranging from 1080 to 3960 kR is compared to that of the control samples (Table 1).

No statistically significant decrease of biological activity is observed either with cell-free extracts ($\chi^2 = 0{\cdot}01$, d.f. 1) or highly purified virus solutions ($\chi^2 = 0{\cdot}05$, d.f. 1). The average time of the mammary tumor's occurring is not modified except for samples irradiated with 1080 kR: at this dose mammary tumors occur earlier, 186 days for irradiated cell-free extracts ($t = 2{\cdot}18$, d.f. 20) and 203 days for irradiated purified virus ($t = 2{\cdot}93$, d.f. 26) compared to 247 days and 258 days respectively for the control groups.

Table 1. *Biological activity of mammary tumor cell-free extracts and highly purified virus samples irradiated at a dilution of* 10^{-3} *compared to control groups respectively*

Doses (kR)	Virus suspensions: Mammary tumor cell-free extracts	Virus suspensions: Highly purified virus
0*	9/14*	15/23
	(258)†	(247)
1080	13/15	13/20
	(186)	(203)
2160	9/18	15/21
	(191)	(217)
3960	9/13	8/14
	(216)	(249)

*Mammary tumor incidence: number of mice with tumor/number of mice.
†Average age of tumor occurrence in days.

There is no statistically significant difference in biological activity between the cell-free extracts and highly purified virus samples after irradiation ($\chi^2 = 0{\cdot}04$, d.f. 1).

These tumors induced in BALB/c inoculated mice are similar to spontaneous breast tumors of the PS mice. These adenocarcinomas from the mammary glands contain numerous type A and B virus particles (12).

2. *Effect of serial dilutions on biological activity of irradiated virus samples*

To check whether or not a few remaining intact particles could be responsible for the biological activity of the irradiated samples, the effect of serial dilutions was studied and compared to the same dilutions of the non-irradiated specimens. A suspension of purified virus was submitted to 2160 kR at a dilution of 10^{-2}; serial dilutions from 10^{-2} to 10^{-5} were then performed with the irradiated suspension as well as with the control one, and their infectivity was compared.

The results are given in Table 2. At dilution of 10^{-5} the non-irradiated virus samples show a statistically significant decrease of tumor incidence ($\chi^2 = 8{\cdot}68$, d.f. 3). No decrease of tumor incidence is observed in the non-irradiated control samples at dilutions of 10^{-2},

10^{-3} and 10^{-4}. No statistically significant difference in tumor incidence is noticed following these dilutions for the 2160 kR irradiated samples, even after a dilution of 10^{-5} (χ^2 = 2·22, d.f. 3). There is no difference either between control samples at dilutions of 10^{-2}, 10^{-3} and 10^{-4} and the irradiated samples at dilution of 10^{-5} (χ^2 = 0·79, d.f. 1).

Table 2. Biological activity of 2160 kR irradiated purified virus sample at serial dilutions ranging from 10^{-2} to 10^{-5} compared to biological activity of non-irradiated sample at the same dilutions

	Non-irradiated samples		2160 kR irradiated samples	
Dilutions	Number of mice with tumors	Total number of mice	Number of mice with tumors	Total number of mice
10^{-2}	14/21		5/6	
10^{-3}	15/23		6/6	
10^{-4}	16/27		4/6	
10^{-5}	3/14		4/8	

3. *Effects of enzymatic treatments on 2160 kR irradiated virus samples*

In order to obtain further information regarding the biological activity observed after irradiation, the purified virus suspension used for the above-mentioned experiments was submitted to the action of several enzymes. Pronase, RNase or DNase treatment have no statistically significant effect on tumor-inducing activity of irradiated virus samples (χ^2 = 3·54, d.f. 1 for RNase). The tumor occurrence is not delayed (Table 3).

Table 3. Biological activity of 2160 kR irradiated virus samples at a dilution of 10^{-3} submitted or not to enzymes treatments

	2160 kR irradiated virus suspension			
		Samples treated with enzymes		
	Untreated samples	RNase	DNase	Pronase
Total number of mice	15	13	10	6
Number of mice with tumors	13	6	8	4
Average age of tumor occurrence (days)	233	236	209	170

4. *Ultrastructural studies*

Ultrastructural observations were performed on negatively-stained virus suspensions.

Non-irradiated virus particles examined by the standard negative staining method are spherical and covered with spikes. The size of this virus ranges between 80 and 120 nm. Most of the particles are spherical, some of them show elongated forms (Fig. 1, 2). In irradiated virus samples no intact particles can be found, only very rare membranous structures covered with spikes suggest disrupted virus particles (Fig. 3).

To appreciate the purity of our virus preparations, ultrathin sections of virus pellets, obtained using our method, were observed. No other types of virus-like particles, no contamination with other cytoplasmic fragments was found. The B particles were round, and their structure was well preserved (Fig. 4).

DISCUSSION

The results recorded here are in agreement with our previous data obtained with crude extracts of mammary tumors [4, 12]. Exposure of highly purified frozen virus samples to doses up to 3960 kR failed to produce any noticeable decrease in infectivity as compared to irradiated control samples.

Radioprotective components might be present in the cell-free extracts prepared according to the Gross's technique used in our preliminary experiments, as mentioned by Latarjet in similar experimental conditions [5]. This author has suggested that chemical protection provided by a high concentration of organic inert substances protects extracts irradiated in a liquid state at 4°C, from indirect effects. The radioresistance observed in our previous studies, with crude extracts of mammary tumors might be explained by a similar mechanism. The present results with highly purified virus preparations show that a protective effect of cellular organic components seems unlikely: there is no statistically significant difference in biological activity between cell-free extracts and highly purified virus samples; the average time of mammary tumors occurring is not different.

An increase of infectivity after irradiation has been reported by Moore with samples of milk submitted to 80 kR [8], and by Latarjet with Friend virus and Rous sarcoma virus [13, 14]. A significant increase of infectivity was observed in our preliminary results for samples submitted to 1200 and 1950 kR [12]. In the present experiments, a statistically

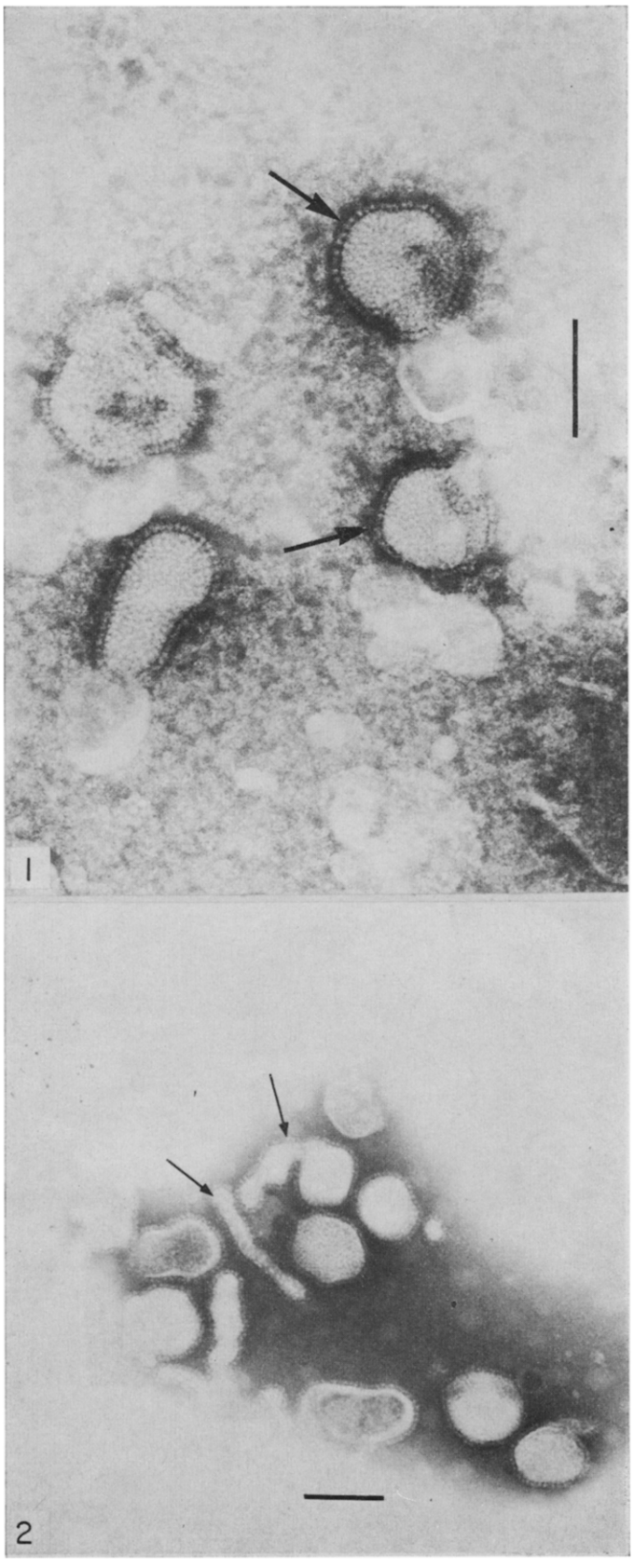


Fig. 1. *Negative contrast micrograph showing non-irradiated virus particles: virus are spherical and covered with spikes (arrows); scale line = 100 nm.*

Fig. 2. *Negative contrast micrograph of non-irradiated virus particles; some of them show elongated forms (arrows); scale line = 100 nm.*

(to face p. 400)

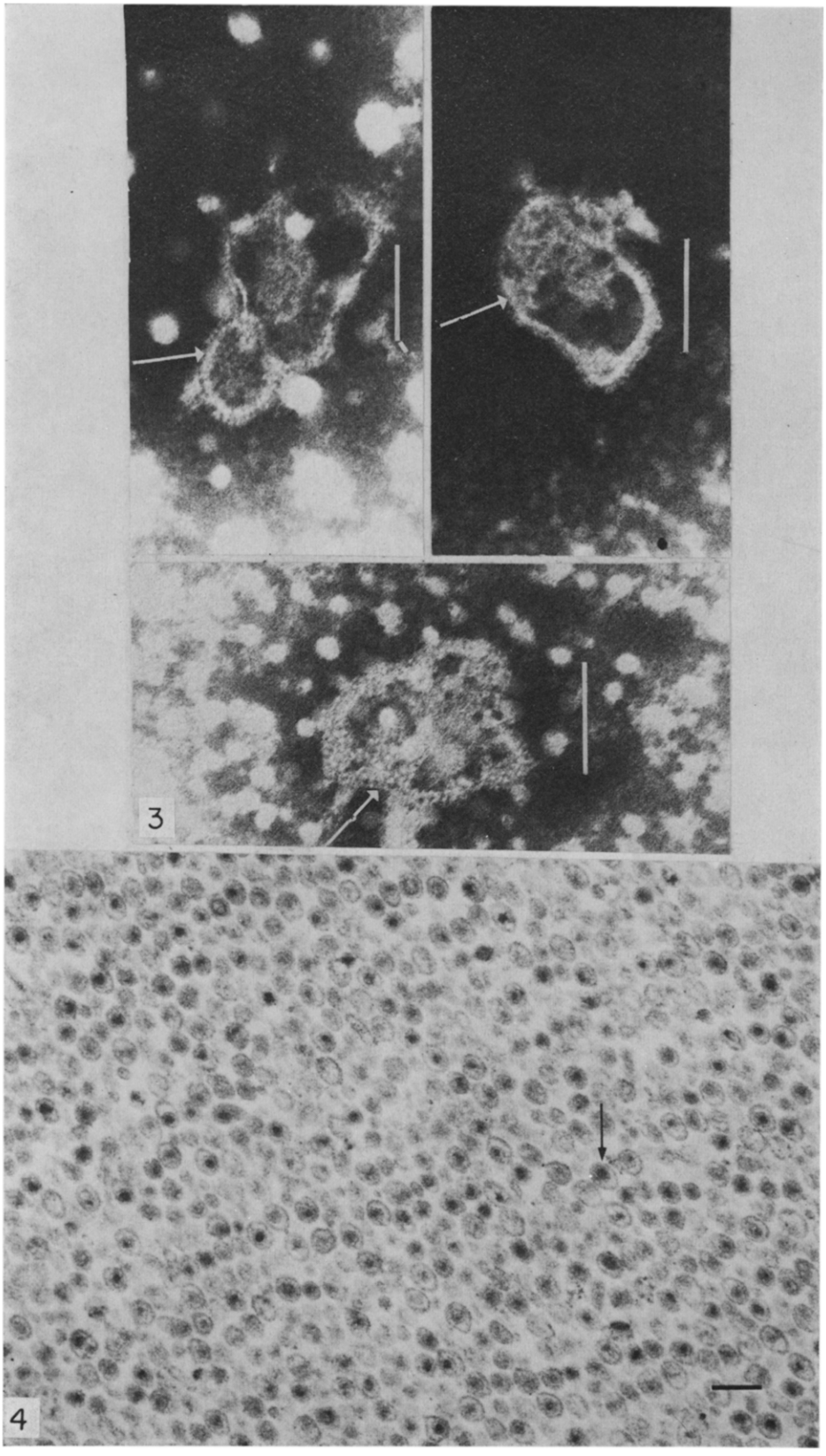


Fig. 3. Negative contrast micrographs showing irradiated virus particles suspensions: membranous structures covered with spikes suggesting remnants of disrupted virus particles (arrows); scale line = 100 nm.

Fig. 4. Thin section of a virus pellet. Numerous particles are present. They are round and well preserved (arrow); scale line = 100 nm.

significant decrease of latency of mammary tumors occurring is observed with 1080 kR.

One could suggest that some intact virus particles are left after irradiation, and are involved in the mammary tumor-inducing activity of the irradiated samples; in that case serial dilutions of the irradiated virus suspensions should result in a decrease or a disappearance of the tumor-inducing activity in the more diluted samples. In order to investigate this point, serial dilutions up to 10^{-5} were applied to the unirradiated virus suspension as a control and to a virus suspension submitted to 2160 kR. A significant decrease of tumor-inducing activity was observed at 10^{-5} with the control unirradiated sample. At the same dilution (Table 3) the irradiated specimen shows no decrease of mammary tumor incidence. These results show that there is no sign of virus inactivation whatsoever. This may indicate that intact virus particles are not involved in the infectivity observed [15].

Ultrastructural observations have been undertaken, not as a quantitative evaluation but in order to appreciate the morphology of the virus after irradiation. Negatively-stained suspensions were observed by electron microscopy. The fragility of virus particles submitted to physical treatment such as freezing and thawing has been established in our previous studies [16]. It is not surprising that when irradiated with doses of γ-rays up to 4000 kR, the MTV is strikingly damaged: intact particles are no longer seen, only a few viral envelopes covered with spikes and disrupted virus particles are recognizable, nucleoids are not observed by ultrastructural studies.

One may suggest that virus particles submitted to irradiation remain intact but they are too fragile to preserve their morphology during fixation or during the procedure of negative staining.

Irradiated samples were sent to Doctor Hilgers [17] and tested against rabbit anti-MTV serum with the micro-Outcherlony test of Wadsworth and Crowle as applied to MTV by Fink *et al.* [18]. Extracts of mammary tumors of GR mice, and frozen-thawed virus suspensions were used as a control; no precipitating lines, characteristic of the virus, were observed, suggesting that the structural proteins responsible for the viral antigenicity were damaged.

A possibility is that a component present in the irradiated virus suspension activates an endogenous MTV, known to be present in BALB/c mouse strain [19, 20] although this does not seem very likely.

The structure of several oncornavirus has been studied by treating the entire virus particles with various enzymes [21]. Further studies have involved the disruption of the virus by Tween-ether or Desoxycholate and testing the sensitivity of MTV components with different enzymes, such as Pronase, ribonuclease, phospholipase by immunological and ultrastructural techniques [22]; tests of the tumor-inducing capacity have not been applied to these experiments. Similar procedures have been used with AMV by Bolognesi *et al.* [23]. An attempt was made to obtain more information relating to the bioactivity mechanism which had been observed using the highly irradiated virus suspensions; the effects of ribonuclease, desoxyribonuclease and pronase were tested on such preparations. Using these experimental procedures the infectivity is not affected by any of these enzymes treatments. Such results might suggest that the tumor-inducing principle is still surrounded by an intact membrane, as it is resistant to pronase. However, our results can also favor the hypothesis of an active virus fragment, involved in mammary tumor induction. Whatever this factor might be, it is linked with MTV as no mammary tumor was observed in the control group inoculated with irradiated normal tissue cell-free extracts from the BALB/c mice.

Acknowledgement—For substantial technical assistance, we thank Miss Marie-Ange Mermet, Mrs. Colette Penisson, Mrs. Annie Gunther and Mr. Canevet (Centre d'Etudes Nucléaires de Grenoble) for irradiation procedure.

NOTE:

Since this paper was submitted for publication, virus suspensions irradiated at several doses of γ-rays (1080 kR, 2160 kR, 2880 kR, 3960 kR) were used at dilutions up to 10^{-6} to inoculate 414 BALB/c mice. One hundred and eighty-eight mice were inoculated with non-irradiated samples. The tumor-inducing capacity of these suspensions compared to those of the control specimens agrees with the results reported here.

Higher doses of γ-rays, up to 8640 kR, and more diluted samples were used. Five hundred and thirty-eight animals are under observation.

REFERENCES

1. W. Ginoza and A. Norman, Radiosensitive molecular weight of tobacco mosaic virus nucleic acid. *Nature (Lond.)* **179,** 520 (1957).
2. S. W. Englander, A. Buzzell and M. A. Lauffer, The relationship between inactivation of Tobacco Mosaic virus by X-rays and breakage of nucleic acid. *Biochim. biophys. Acta* **40,** 385 (1960).
3. W. Ginoza, Radiosensitive molecular weight of single stranded nucleic acids. *Nature (Lond.)* **199,** 453 (1963).
4. J. Mouriquand, C. Mouriquand, P. Mistry and C. Gorka, Radiorésistance du virus de la tumeur mammaire murine soumis à une irradiation γ. *C.R. Acad. Sci.* **274,** 2259 (1972).
5. R. Latarjet, Inactivation du virus leucémogène de Gross par les radiations u.v., X et γ. *Int. J. Cancer* **6,** 31 (1970).
6. R. Latarjet and L. Chamaillard, Inactivation du virus de Friend par les rayons X et ultraviolets. *Bull. Cancer* **49,** 382 (1962).
7. S. N. Ardashnikov and I. G. Spasskaia, Employment of biophysical method for determining the nature of the so called milk factor. *J. Microbiol. Immunobiol. USSR,* **9,** 44 (1949).
8. D. H. Moore, E. C. Pollard and C. D. Haagensen, Further correlations of physical and biological properties of mouse mammary tumor agent. *Fed. Proc.* **21,** 942 (1962).
9. J. Mouriquand and C. Mouriquand, Tumeurs mammaires et leucémies de la souche PS; considérations étiologiques. *Path. et Biol.* **13,** 630 (1965).
10. J. Calafat and P. Hageman, Some remarks on the morphology of virus particles of the B type and their isolation from mammary tumors. *Virology* **36,** 1308 (1968).
11. L. Veprek, D. Beard, A. J. Langlois, R. Ishizaki and J. W. Beard, Transmission of Avian Myeloblastosis by BAI strain A virus ribonucleic acid. *J. nat. Cancer Inst.* **46,** 713 (1971).
12. C. Gorka, P. Mistry and J. Mouriquand, Effet de hautes doses d'irradiation γ sur le virus de la tumeur mammaire murine. In *Fundamental Research on Mammary Tumors.* 7th International Symposium on Mammary Tumors. p. 163. (Saint Pierre de Chartreuse, France, 12–14 Juin 1972) (1972).
13. R. Latarjet, R. Cramer, A. Golde and L. Montagnier, Irradiation of oncogenic viruses: dissociation of viral functions. In *Carcinogenesis: A Broad Critique,* p. 678. Williams & Wilkins, Baltimore (1967).
14. A. Golde and R. Latarjet, Dissociation, par irradiation, des fonctions oncogène et infectieuse du virus de Rous, souche Schmidt–Ruppin. *C.R. Acad. Sci.* Paris, **262,** 420 (1966).
15. T. H. Maugh, RNA viruses: the age of innocence ends. *Science* **183,** 1181 (1974).
16. C. Viala-Gorka, J. Mouriquand and C. Mouriquand, Morphology and bioactivity of the mouse mammary tumor virus: effects of freeze-thawing and addition of dimethyl sulfoxide to the suspending medium. *J. Microscopie* **14,** 7 (1972).
17. J. Hilgers, Unpublished data.
18. M. A. Fink, W. F. Feller and L. R. Sibal, Methods for detection of antibody to the mammary tumor virus. *J. nat. Cancer Inst.* **41,** 1395 (1968).
19. P. Bentvelzen, J. H. Daams, P. Hageman, J. Calafat and A. Timmermans, Interaction between viral and genetic factors in the origin of mammary tumors in mice. *J. nat. Cancer Inst.* **48,** 1089 (1972).
20. P. Hageman, J. Calafat and J. H. Daams, The mouse mammary tumor viruses. In *RNA Viruses and Host Genome in Oncogenesis.* (Edited by P. Emmelot and P. Bentvelzen) 6, p. 283. North Holland, Amsterdam, London (1972).
21. J. Calafat, P. Hageman, The structure of the mammary tumor virus. *Virology* **38,** 364 (1969).
22. J. H. Daams, P. Hageman, J. Calafat and P. Bentvelzen, Antigenic structure of murine mammary tumor viruses. *Europ. J. Cancer* **9,** 567 (1973).
23. D. P. Bolognesi, H. Gelderblom, H. Bauer, K. Mölling and G. Hüper, Polypeptides of avian RNA tumor viruses. V. Analysis of the virus core. *Virology* **47,** 567 (1972).

Europ. J. Cancer Vol. 11, pp. 403–412. Pergamon Press 1975. Printed in Great Britain

Morphological, Histochemical and Ultrastructural Observations of Diethylstilbestrol-induced Kidney Tumors in the Syrian Golden Hamster*

ANTONIO LLOMBART-BOSCH† and AMANDO PEYDRO
Dept. of Pathology, Servicio de Cancerología Experimental, Facultad de Medicina, Valencia, Spain

Abstract—*A new approach, employing histochemical and electron-microscopic techniques was used in an attempt to obtain a more complete knowledge of estrogen-induced kidney tumor in the golden hamster (G.H.). Three estrogen derivatives, diethylstilbestrol (DES), diethylstilbestrol monoglucoronide (DESGA), and dimethyl ether (DESDME), were used on non-castrated male hamsters. DES had the highest incidence of tumor induction (69% of the animals) which is associated with other tumors (epididymal and hepatic adenomas); DESDME induced tumors in 31%, and non-neoplastic changes could be observed in the DESGA-intoxicated group.*

Histologically, three main types of structures were seen which might be present in the same tumor: solid-blastemal, more tubular-epithelial and papillary-cystic areas. These kidneys also had a pluricentric tubular dysplasia and an interstitial cell hyperplasia. A histochemical study of the embryonic metanephron and the interstitial cells of newborn G.H. kidney, when compared to the DES-induced kidney tumor and the dysplastic pretumoral foci, showed marked similarities between the enzymes present. The comparative ultrastructural analysis of the embryonic developing kidney and the tumor itself yields further evidence of an interstitial origin of the neoplasia, which has a poorly differentiated and ciliated cell structure.

The estrogen-induced G.H. kidney tumor may well begin in the interstitial cells of the residual metanephric tissue in the kidney stroma.

INTRODUCTION

WE HAVE described previously the morphology of the kidney tumors induced by estrogen in young male hamsters [1, 2], and others have described the general characteristics of these tumors [3, 4]. However, their structure and histogenesis remain a matter of debate.

According to many investigators, these tumors are of a carcinomatous nature and probably arise from the convoluted tubule of the nephron, [2, 5–8]. Dontenwill and Eder [9] have postulated an interstitial (blastemal) cell origin, and Kirkman and Robbins [10] have also described the existence of tumor cells with the appearance of connective tissue as an interstitial hyperplasia in estrogen-treated hamster kidneys. These cells contain sudanophilic lipoids, are alkaline phosphatase positive, and adopt an epithelial structure from which these cilia emerge.

Primitive tumor cells show several types of an epithelial nature (cubic prismatic or papillary, ciliated or non-ciliated) and a blastemal (spindle, mesenchymal) one. The two types of structure are continuous or close to each other

Accepted 13 December 1974.

*This investigation was performed under the auspices of the AECC (Madrid) and Ministerio de Educación y Ciencia, with the technical assistance of Antonio Sanchez and Chelo Joly.

†Present address: Professor of Pathology, Facultad de Medicina de Murcia, Spain.

[2, 6]. Histochemistry studies of tumor cells [6, 9, 11], support the postulate of a renal interstitial cell origin.

The ultrastructure of these tumors was described earlier by Mannweiler and Bernhard [12], who considered it as being of exclusively epithelial origin. A highly differentiated ciliated border is present in most of the tumor cells, although it was not observed in the kidneys of the control hamster. Recent electron-microscopic studies in this laboratory of primary tumors [13] have confirmed the presence of a ciliated border in both the proximal and distal tubules of tumorous G.H. kidneys as well in the tumorous cells.

DES and two derivatives, a physiological one, DESGA, and a non-physical one, DESME, were chronically administered to male hamsters with the purpose of obtaining tumors and comparing hepatic β-glucuronidase and O-demethylase activities. The results obtained have been discussed by Lacomba and Gabaldon [14]. The animals used in the actual experiments were the same ones in which the renal tumors had been induced by the chronic administration of the substances mentioned. The relevant biological and biochemical data have already been published [14]. The present communication deals with the morphological, histochemical and electron-microscopic study of primary renal tumors. It represents a new approach to G.H. hormone-dependent tumors, since they are regarded as hormone-dependent nephroblastomas, a fact which might open up new possibilities in the biology of kidney tumors in mammals.

We have also performed a histochemical and electron-microscopic study of the developing metanephros in 11 and 16-day-old fetuses (post coitum) and in newborn G.H.

MATERIAL AND METHODS

Animals and substances

As previously described [14], 180 male Syrian G.H. were used for the induction of renal tumors by the administration of DES, DESDME and DESGA. Morphological studies of both kidneys after autopsy, following the injection of DES (in 55 animals) DESDME (in 57) and DESGA (in 55).

Five kidneys of G.H. fetuses on the 11th day of gestation, and 5 more on day 16, were obtained through caesarean section. Moreover, 5 newborn G.H. (6 hr after delivery) were killed and all kidneys dissected for histochemical and electron-microscopic examination.

Tumor transplants

Some references appear (see Discussion, *infra*): about two lines of stilbestrol-induced and transplanted G.H. kidney tumors (one hormone-dependent and the other hormone-independent); both tumor lines were obtained in our laboratory from an originally estrogen-induced tumor [1] and since then carried out by s.c. transplants [15] on DES-bearing G.H. for the hormone-dependent tumor and normal male for hormone-independent one.

Morphological and histochemical studies

For optical microscopy, the material was fixed in formalin (10% neutral) and Bouin's fluid. Semiserial sections were performed on paraffin-embedded kidneys and stained with hematoxylin-eosin, Gomori silver stain for reticular fibres, and trichromic solution of Masson and Periodic-acid-Schiff (PAS). One kidney of each animal was processed for histochemical studies. Serial sections from the frozen (dry ice) kidney, with or without tumor, were cut in a cryostat (IEC) at −20°C, and incubated in the appropriate media so as to demonstrate the following enzymes: alkalin and acid phosphatase; fructose 1–6 diphosphatase; ATP-ase; lactate and malate dehydrogenase; succinate dehydrogenase; isocitrate dehydrogenase; NADH and NADPH tetrazolium reductase; glucose-6-phosphatase; non-specific sterase; β-glucuronidase; β-hydroxybutyrate dehydrogenase; glucose-6-phosphate-dehydrogenase; monoamino oxidase; Sudan III for neutral lipids and PAS for glycogen associated with α-amylase and Dimedon. Nitro-blue tetrazolium was used as the hydrogen acceptor. The techniques used have been described previously [16]. Enzymatic activity was evaluated as follows: none (−); low activity (+); moderate activity (++); and intense activity (+++).

Electron microscopy

Ten embryonic kidneys, 10 kidneys of newborn hamsters, 15 kidneys bearing tumors in the DES injected group, 10 in the DESDME group and 10 in the DESGA group, were fragmented into small pieces (1 mm^3) and fixed in glutaraldehyde and osmium tetroxide (OsO_4). The tissues were then rapidly dehydrated in a graded solution of ethanol-acetone, immersed in propylene oxide and embedded in Durcupan ACM (Fluka A.G.). Thin sections were cut with a Reichert ultramicrotome and stained with uranil acetate or lead hydroxide. Electron

microscopes (Zeiss M-9 and Jeol JEM-100B) were used for the study of the samples.

RESULTS

1. *Microscopic confirmation of renal tumors*

Table 1 gives the real incidence (microscopically confirmed) of kidney tumors induced by the three estrogen-derivatives (DES, DESDME and DESGA).

The DES-treated G.H. have the highest index of renal tumors (69%) which were detected early within the 7th or 10th month of the experiments. There was also evidence of other tumors (epididymal and hepatic adenomas). The incidence of tumors in the DESDME-injected group was not only lower (31%) but also began to appear later, namely, between the 10th and 12th months. No extrarenal tumors were found. DESGA does not appear to induce kidney tumors in the G.H. None of the 55 animals examined histologically showed any tumorous proliferation in the kidneys.

Estrogen-induced renal tumors are multicentrical in distribution. In our series, the incidence of pluricentric tumoral foci was as frequent in DES-treated G.H. (45%), as in those treated with DESDME (44%). But in only 20% of the DES-treated G.H. were more than 3 histologically confirmed tumors found in the same animal.

2. *Histological characterization of kidney tumors*

A total of 99 primary tumors were studied histologically. Microscopically, three main types of structure were differentiated in these tumors:

(I) The most common structure is the *solid blastemal*, which consists of cords or branches of anastomotic cells of varying thickness, and in whose growth some normal tubules of glomerules may be involved. Pseudo-rosette-like structures can be seen around a central core of necrosis (Fig. 1). At times, the tumor cells also adopt a perivascular radiated distribution with a spindle-shaped configuration. There exist areas of progressive or direct transition between the solid basophilic cells and the more blastemal mesenchymal spindle-shaped ones. Occasionally, pseudo-glomerular, tubular or alveolar images adopting a more or less definite cubical epithelial shape (Fig. 5) coexist with aforementioned structures (Fig. 2 and 3).

(II) The *solid-tubular* structure is the second most common. At times, small alveolar structures, lined with a cubical epithelium and resembling nephron tubules are present. A definite membrane is not always detectable, and a certain kind of continuity exists between epithelial cells and the blastemal-mesenchymal ones (Fig. 3 and 4).

(III) The *papillary-cystic* structure of the tumor is the least frequent. These cystic spaces are lined with irregular cubical or prismatic cells exhibiting a large amount of cilia (Fig. 4). The cyst may be filled with amorphous basophilic PAS negative material (Fig 6).

These three histological structures may appear at random in the same kidney tumor.

3. *Tubular dysplasia and interstitial cell hyperplasia as earlier neoplastic lesions*

Two different proliferating changes might be considered as earlier neoplastic lesions in the

Table 1. *Microscopically confirmed incidence of tumors in the Golden Hamster treated with DES, DESDME and DESGA*

Treatment period (months)	DES		DESDME		DESGA	
	Kidney	Others	Kidney	Others	Kidney	Others
4–7	9/4 (44%)		2/0 (0%)			
7–8	14/10 (71%)		2/0 (0%)			
8–9	22/17 (77%)		3/0 (0%)			
9–10	10/7 (70%)	10/3*† (30%)	5/0 (0%)			
10–11			32/11 (34%)			
11–12			13/7 (53%)			
		*one hepatoma †two epididymal adenomas				
Total	55/38 (69%)	55/3 (5%)	57/18 (31%)		55/0 (0%)	

N.B.: 0·2 ml of a 11·2 mM suspension of the substance in the vehicle was administered every other day, 3 times a week.

Table 2. Tubular dysplasia and interstitial cell hyperplasia associated or not with renal tumor in estrogen-treated hamster

Type of lesion	DES	DESDME	DESGA
Tubular cell dysplasia	55/27	57/11	55/10
Interstitial cell hyperplasia	55/10	57/6	55/0
Total number of cases with dysplasia or hyperplasia	55/37 (67%)	57/17 (29%)	55/10 (18%)

intoxicated G.H. kidney. First there are tubular alterations with cellular dysplasia; secondly, an interstitial cell hyperplasia forms small clusters of cells which proliferate between tubular structures or in perivascular areas.

Their incidence has been determined, as shown in Table 2, in DES, DESDME and DESGA-injected hamsters. The frequency of tubular dysplasia is higher than that of interstitial cell hyperplasia; both have a multicentric disposition, numerical incidence being more significant in G.H. kidneys with tumors. The presence of no abnormality other than tubular cell dysplasia in the DESGA-intoxicated G.H. should be stressed together with the fact that no tumor was found in this group.

Tubular dysplasias. These consist of a cystic transformation with increased cell height and the presence of cilia. This increase of cell volume is associated with a high cytoplasmic basophilia and nuclear changes, some degree of mitosis also being present. Tubular basals may break, and a certain degree of continuity can be found between tubular cells and the interstitial anastomotic cords of the tumors. As will be described later, the histochemical characterization of tubular and interstitial cell hyperplasia is the same. Indeed, both have a histochemistry similar to that of the established neoplastic cells.

Interstitial hyperplasia. This is less frequent than tubular dysplasia, but may play an important role in DES-induced renal tumors in the G.H. Fibroblastic-like cells are sometimes found together with lymphocytes and plasma cells, in which case, an inflammatory origin cannot be excluded.

Furthermore, small groups of fibroblast-like cells may be detected in the interstitial spaces of the tubular nephrone, where no inflammatory cells are present. The nuclei are elongated, and cytoplasmic vacuoles are not infrequent, being filled, as in the case of certain renal tumor cells, with lipids.

4. *Histochemical studies of tumorous and premalignant lesions in the kidneys*

The histochemistry of the tumoral fields (solid-blastemal, solid-tubular and cystic-papillary) reveals specific characteristics resembling somewhat the enzymatic activity of the normal convoluted tubule (Table 3). Moreover, interstitial cell hyperplasia and tubular dysplasia show the same enzymatic activity as tumorous tissue.

Table 3. Histochemistry of DES and DESDME-induced kidney tumors in the golden hamster

Enzymatic activity*	Renal tumor	Tubular structures
Alkaline phosphatase	+ + +	+ +
ATP-ase (pH 9·4)	+ + +	+
Fructose 1-6 diphosphatase	+ + +	+
Glucose-6-phosphatase	−	+ + +
Isocitrate DH	+ + +	+ +
Succinate DH	+	+ +
Malate DH	+	+ +
Lactate DH	+	+ +
NADH diaphorase	+ + +	+ + +
NADPH diaphorase	+ + +	+ + +
β-glucuronidase	+	+ +
Acid phosphatase	+	+ +
Non-specific sterase	+	+ +
β-hydroxybutyrate DH	−	+ +
Glucose-6-phosphate DH	−	+
6 phosphogluconate DH	−	+
Monoamino oxidase	−	+
Glutamate DH	−	+
Lipids (Sudan III)	+ +	−
P.A.S.	−	−

*Number of cases investigated = 25 primary tumors.

Both kinds of tumor cells (epithelial and blastemal) display analogous histochemical behavior, but undifferentiated blastemal areas contain a great deal of lipids that are usually present in the solid, tubular or cystic aggregates of epithelial tissue.

The tumorous cells possess a strong enzymatic activity in alkaline phosphatase, ATP-ase and fructose 1–6 diphosphatase, which is higher than that observed in the normal convoluted tubule. Isocitrate dehydrogenase activity was higher in tumoral cells than in normal kidneys. All these activities may be considered as specific marker enzymes for DES-induced G.H. kidney tumors. Cytoplasmic localization of other

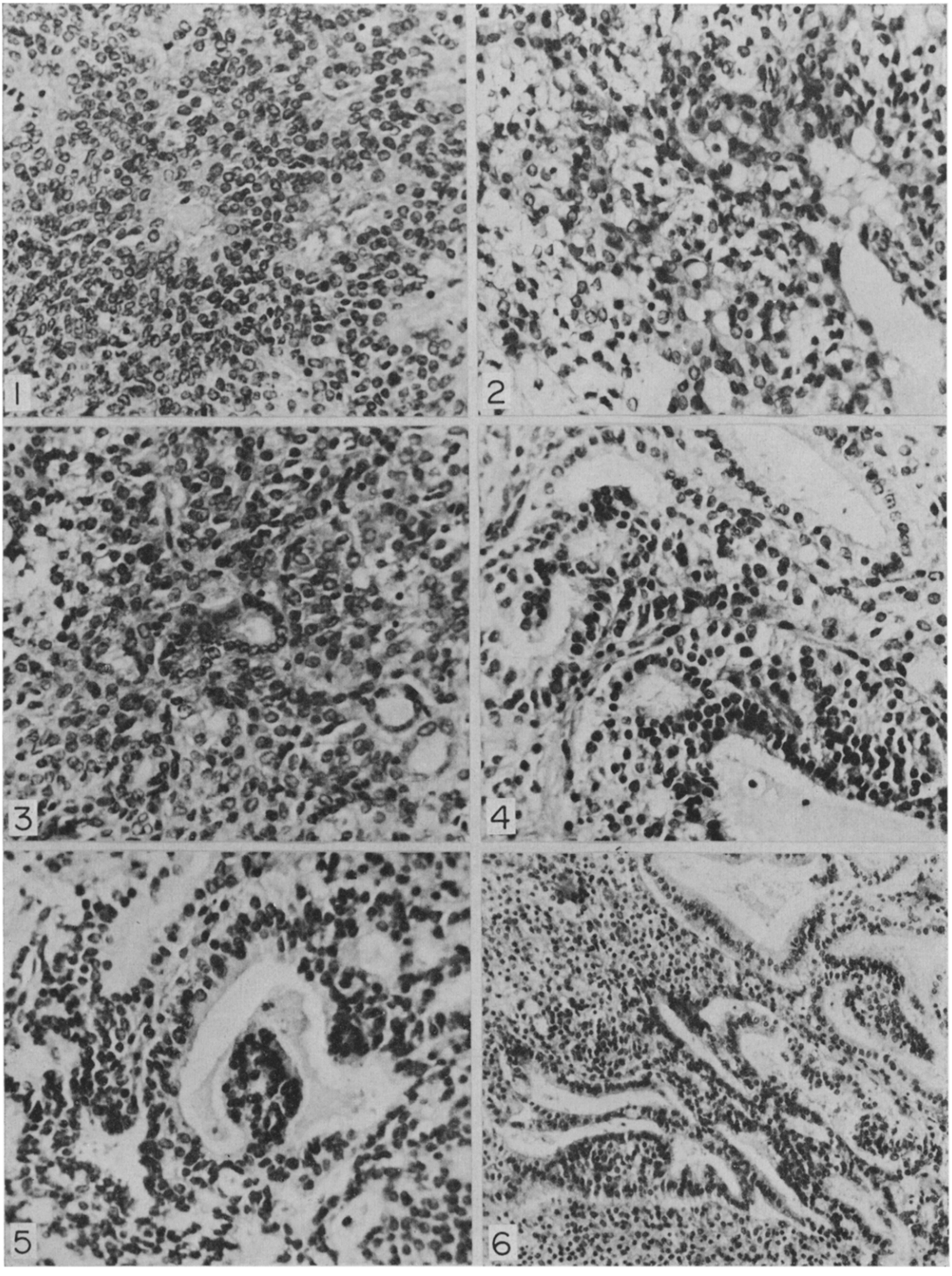


Fig. 1. *Histological appearance of a solid-blastemic area of a kidney tumor. Tumoral cells are close to one another and pseudo-rosette-like structures are present.* (× 40, *H.E.*).

Fig. 2. *Solid-blastemic type tumor with very rare tubular structures and progressive formation of signet-ring, lipid-rich cells.* (× 40, *H.E.*).

Fig. 3. *Progressive transformation of solid-blastemic areas into tubular-alveolar structures.* (× 40, *H.E.*).

Fig. 4. *Alveolar structures resembling nephron tubules lined with cylindrical and undifferentiated blastemic cells,* (× 40, *H.E.*).

Fig. 5. *Pseudo-glomerular images within solid strands of epithelial cylindrical and undifferentiated blastemic cells.* (× 40, *H.E.*).

Fig. 6. *Papillary-cystic tumor structure in close association with the predominantly blastemic variety filling the interstices.* (× 10, *H.E.*).

(to face p. 406)

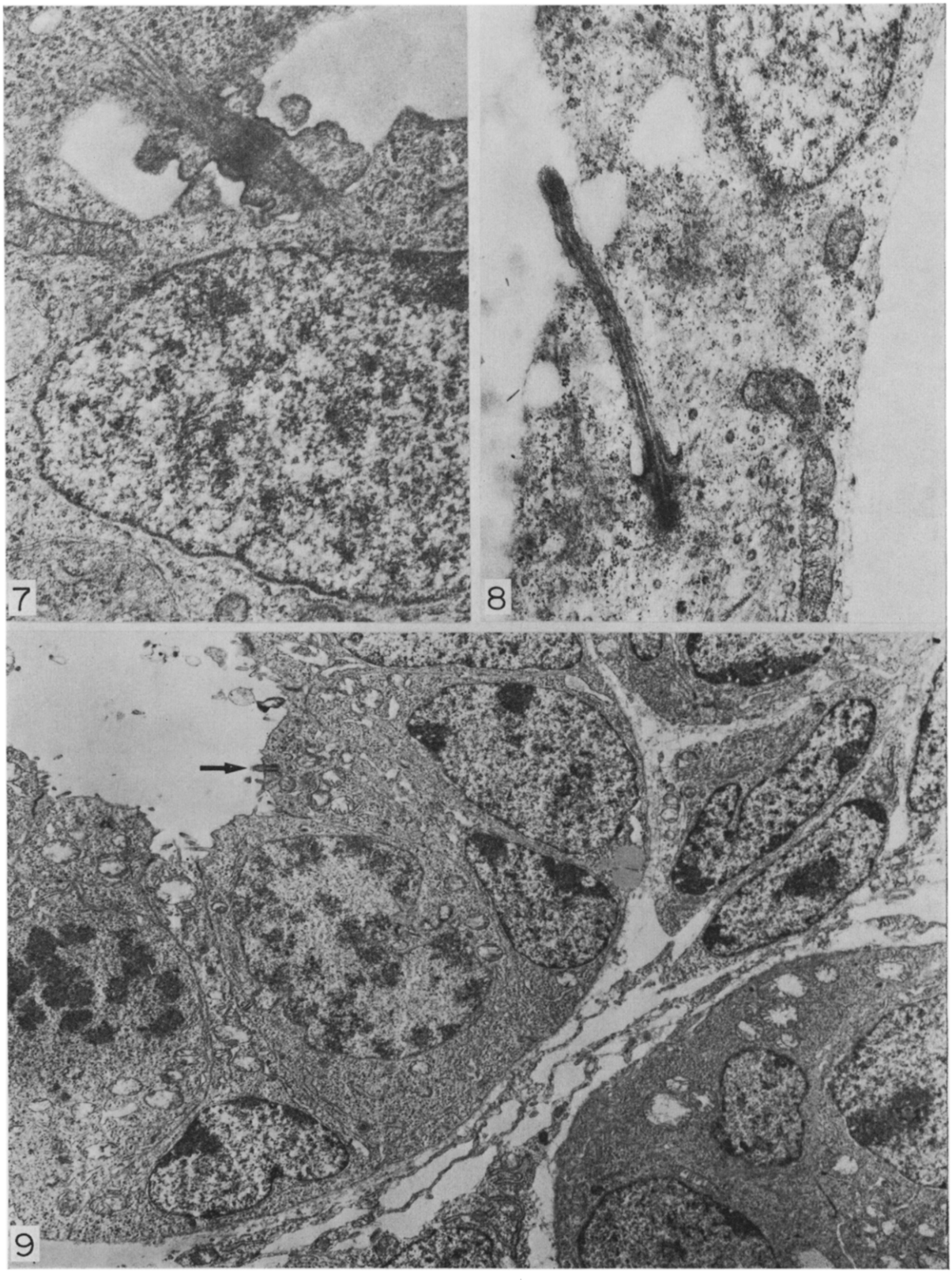
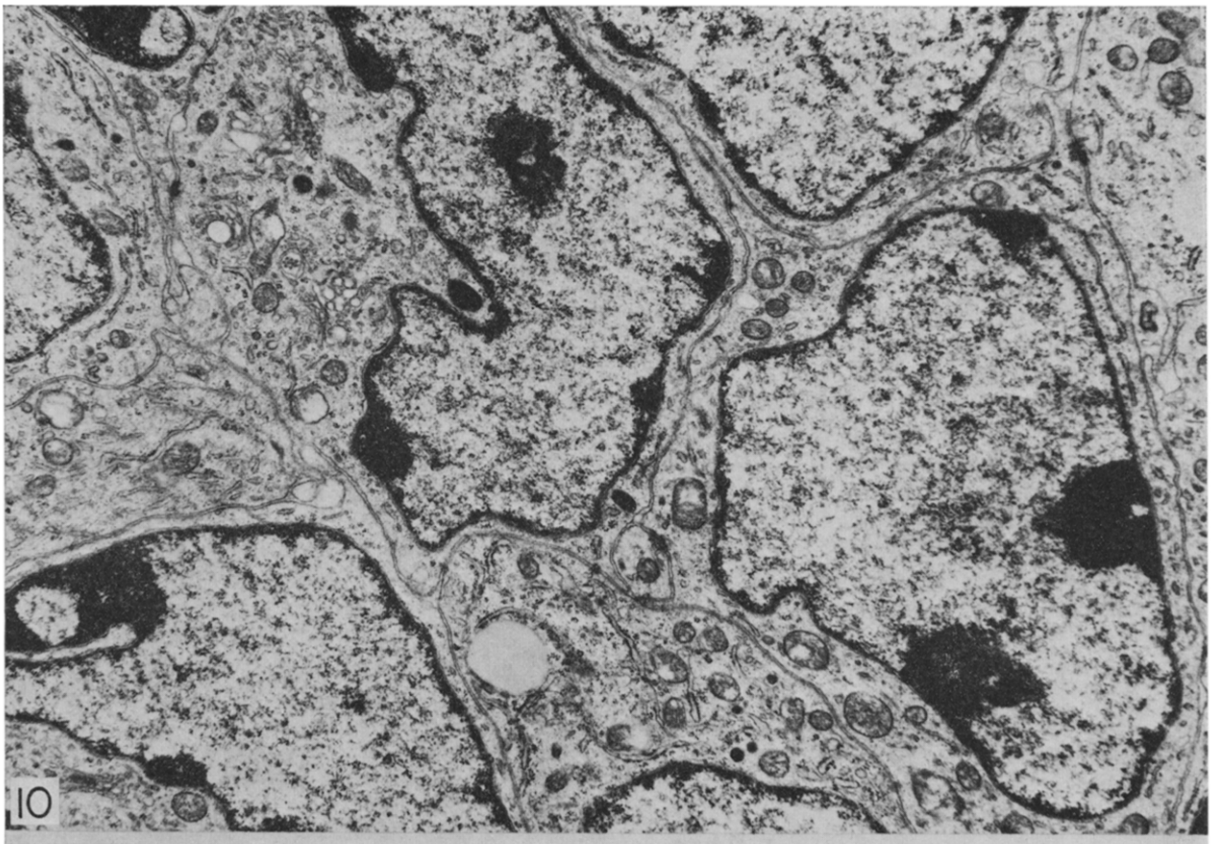


Fig. 7. Desmosome between blastemic metanephrogenic cells at the 11th day of embryonic development. (× 20,000).

Fig. 8. High resolution of cilia in a mesenchymal metanephrogenic cell. (× 16,000).

Fig. 9. G.H. fetal kidney at the 16th day of evolution. Tubules of epithelial metanephrogenic cells and surrounding blastemic and more undifferentiated area in close contiguity ; cilia inside the lumen (arrow). (× 6000).



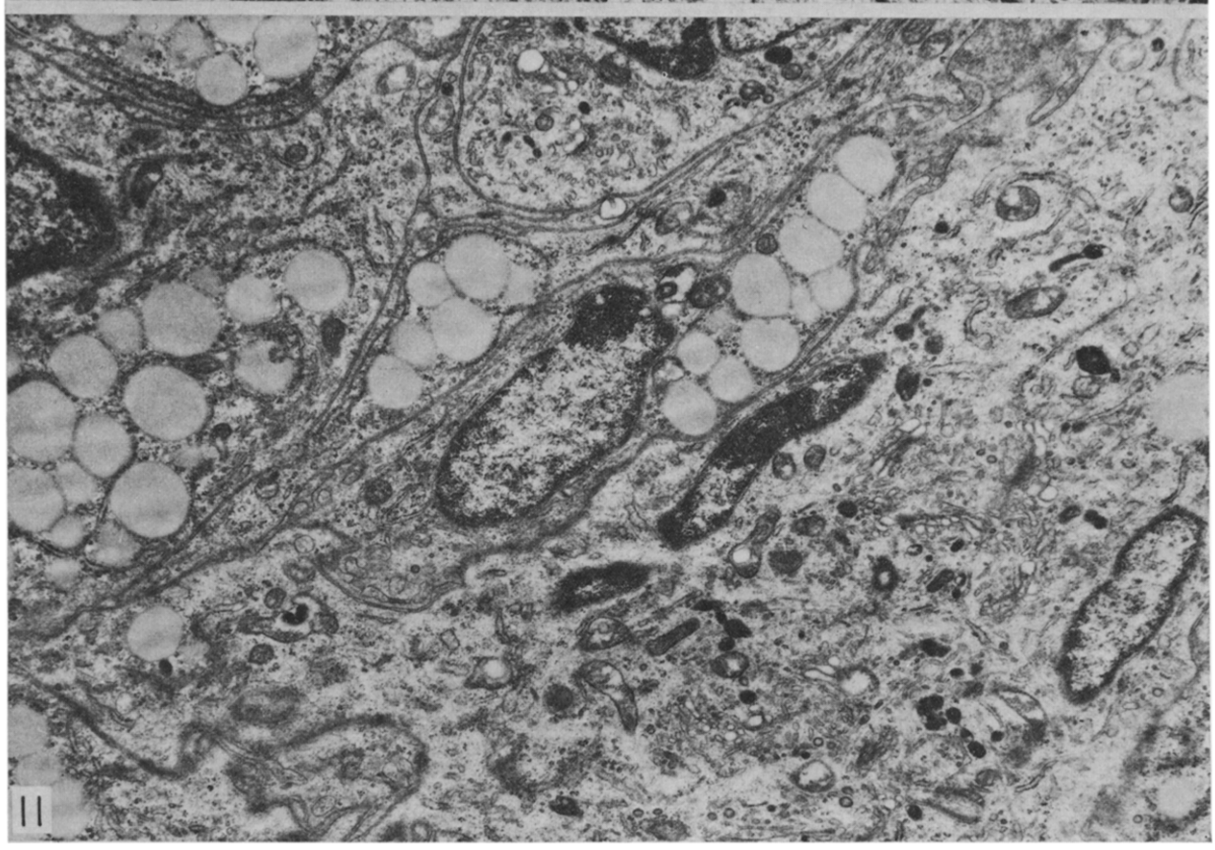


Fig. 10. *Compact groups of polygonal cells of a solid-blastemic area of a kidney tumor. Tumoral cells are close to one another. Presence of abundant condensations of the plasma membranes suggest immature intercellular junctions.* (× 5000).

Fig. 11. *Undifferentiated mesenchymal tumor cells with a large number of lipid droplets in the cytoplasm.* (× 4000).

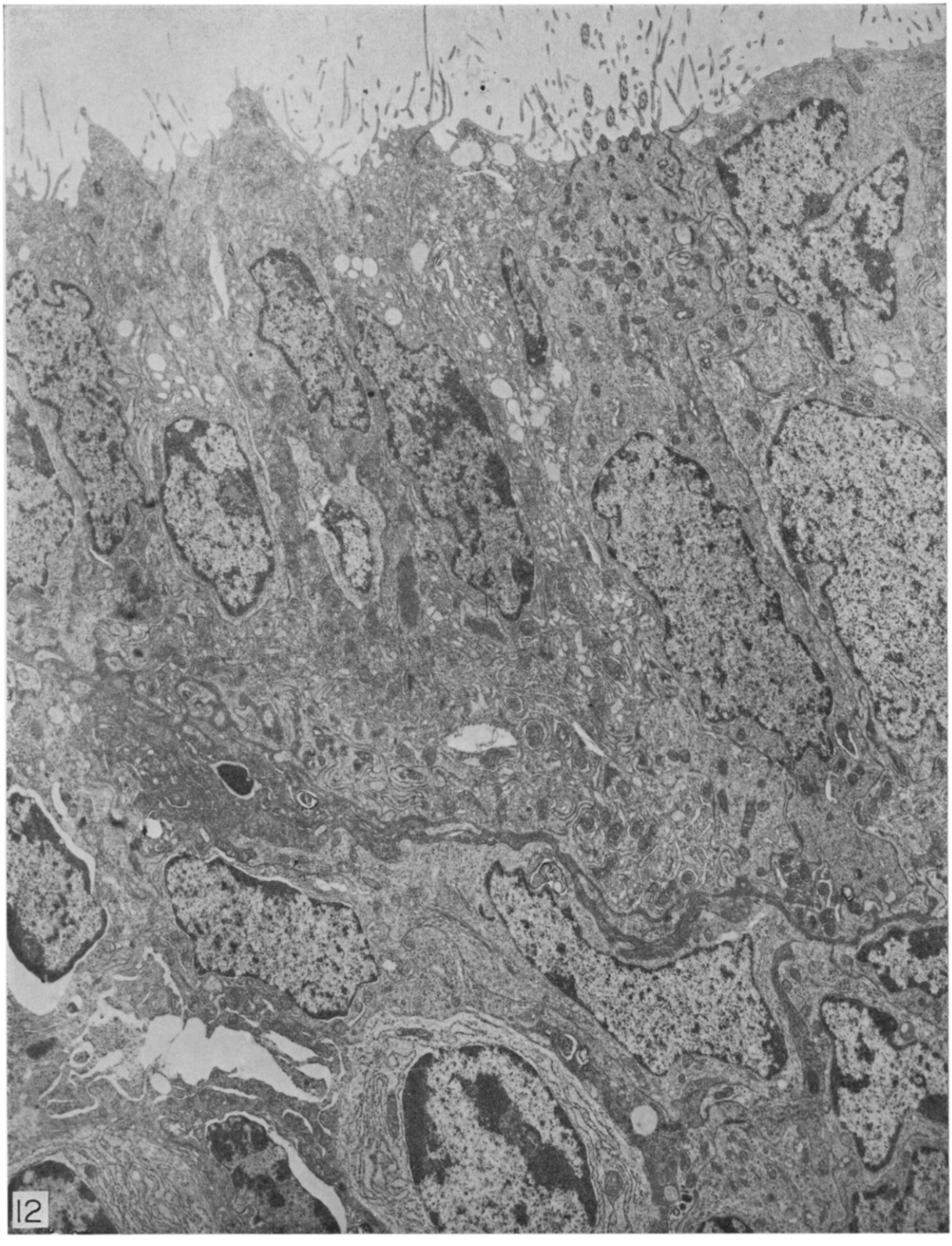


Fig. 12. *Area of cylindrical-ciliated and well-differentiated epithelial cells in close continuity with the more mesenchymal structures. Irregular basal profiles separate both of them.* (× 1825).

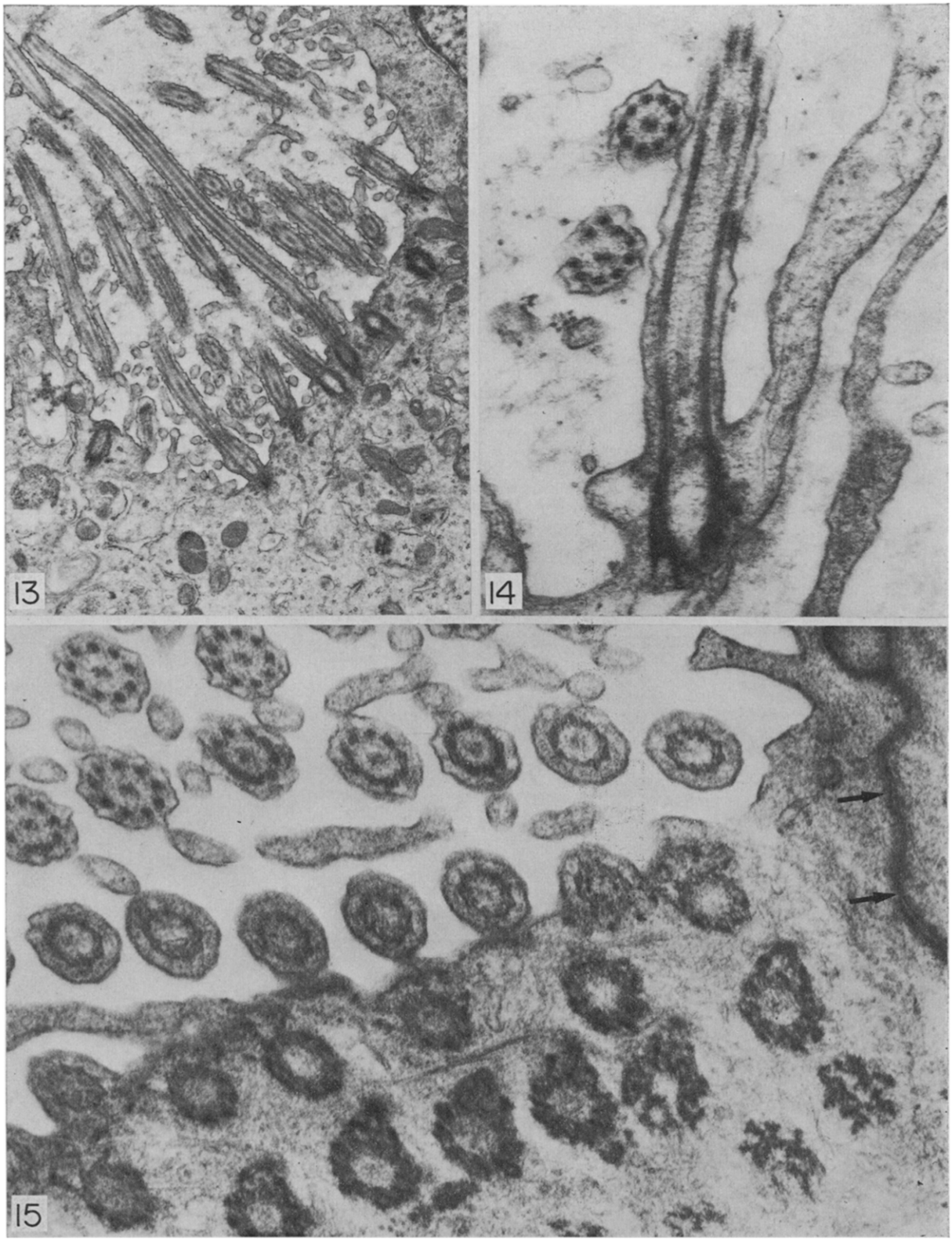


Fig. 13. *Heavily ciliated neoplastic cell with microvilli and cilia emerging from apical surface.* (× 6000).

Fig. 14. *Detail of cilia in continuity with the basal body in a tumorous cell.* (× 18,000).

Fig. 15. *Section at different levels of the cilia, emerging from a tumoral epithelial cell, with a completely normal differentiation of the outer and inner cores of the tubules. At the top (arrows), presence of junction complexes.* (× 18,000).

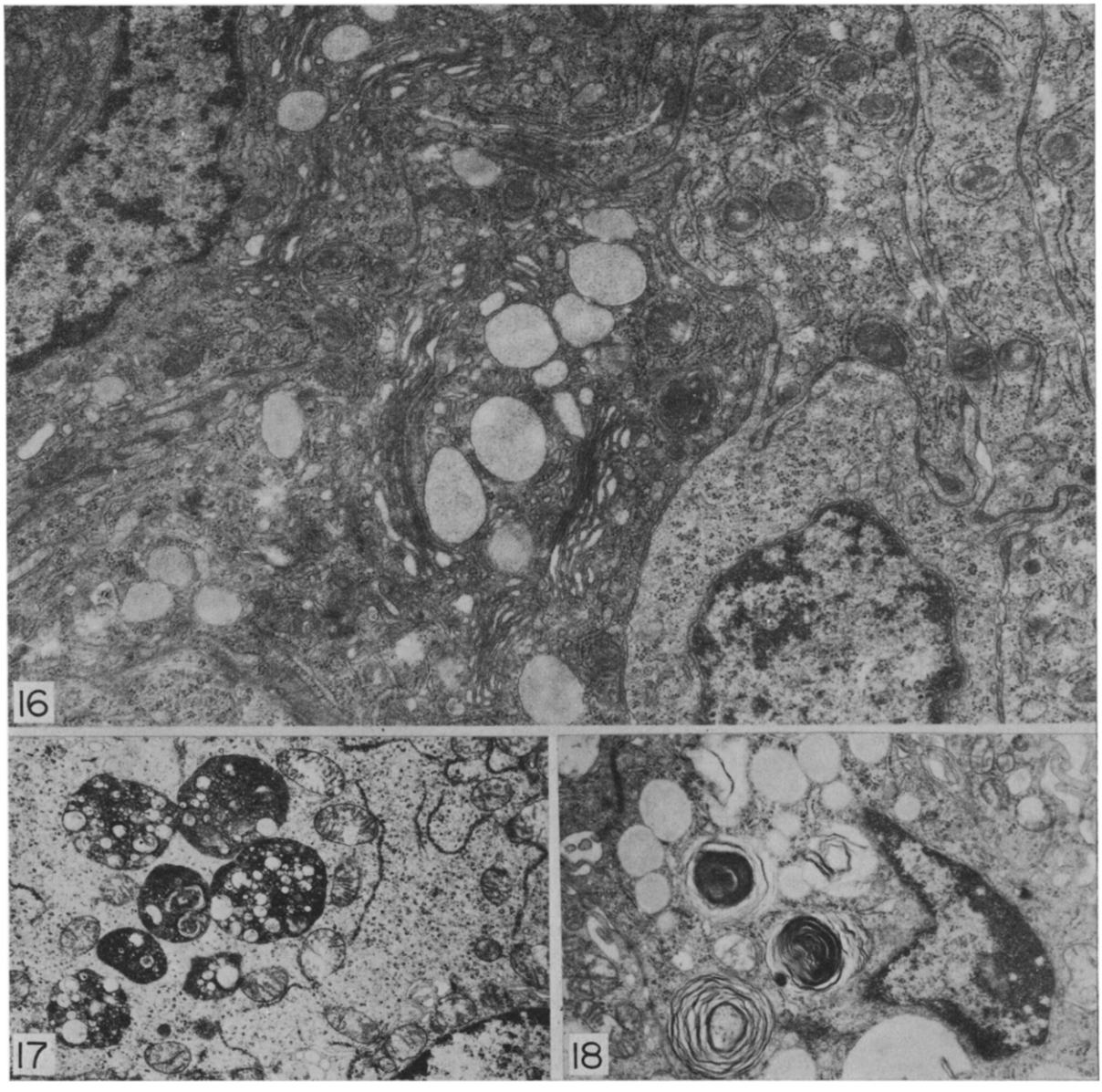


Fig. 16. *Epithelial secretory area with fingerlike infoldings of the membrane in neighbouring cells. Close contact between RER and mitochondria. Well-developed Golgi in continuity with secretory material.* (× 6000).

Fig. 17 *and* 18. *Phagosome bodies* (*Fig.* 17, × 6000) *and myelin-like figures* (*Fig.* 18, × 6000) *inside the cytoplasm of a mesenchymal tumorous cell.*

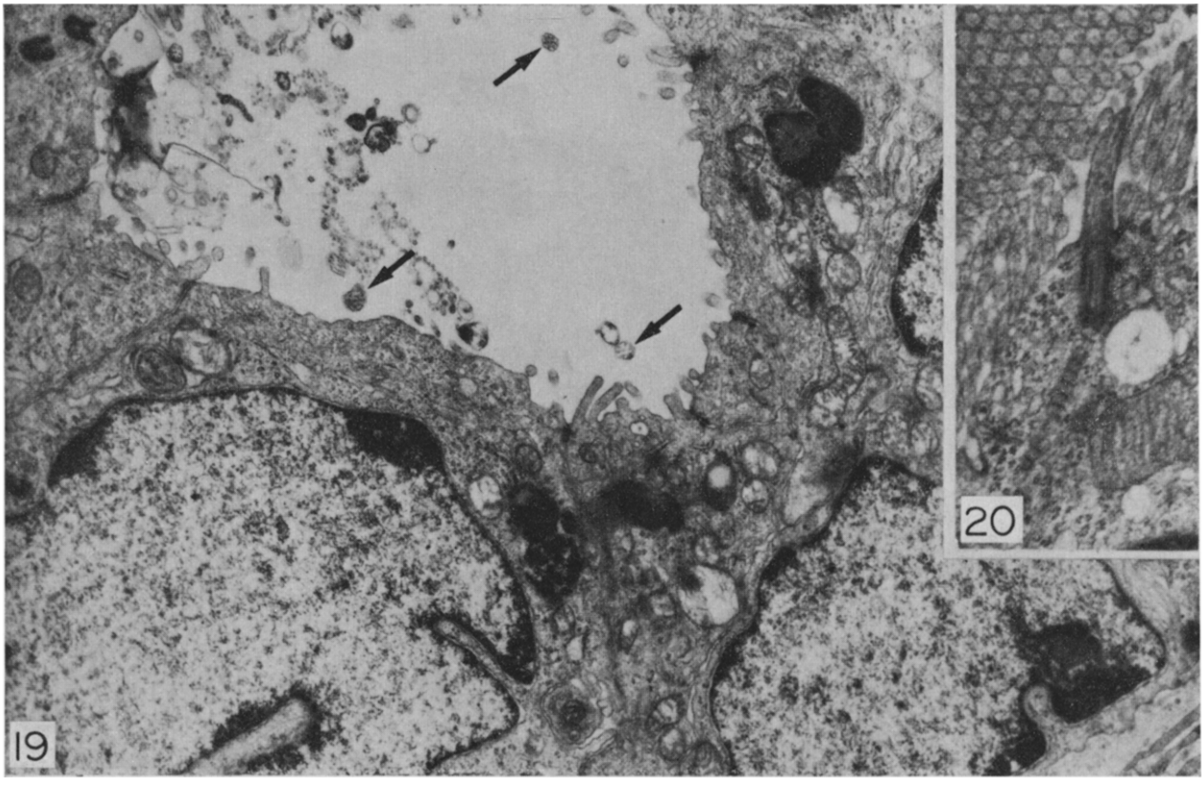


Fig. 19 *and* 20. *Cell dysplasia in a distal convoluted tubule with marked irregularity of the nucleus and presence of numerous cilia inside the lumen (arrows)* (*Fig.* 19, ×4000). *Presence of a fully developed cilia in a proximal convoluted tubule* (*Fig.* 20, ×6000).

dehydrogenases (lactate, malate, succinate) and diaphorase (NADH and NADPH tetrazolium reductase) display histochemical behaviour similar to that found in normal tubular structures.

A total absence of activity in glucose-6-phosphatase and β-hydroxybutyrate dehydrogenase was also apparent in all types of tumorous cells, although these enzymes are almost invariably present in the proximal and distal tubes of the nephron, respectively. Staining for acid phosphatase, β-glucuronidase and non-specific sterase activities decreases in neoplastic cells compared to tubular structures.

5. *Histochemical studies in embryonic kidney of the G.H.*

Table 4 summarizes the results of the histochemical studies in developing metanephros (in 11-day-old embryos and in the newborn hamster) whose activities were compared to those of the kidney tumor.

Interstitial blastemal matanephrogenic cells of the G.H. show high activity for alkaline phosphatase and isocitrate dehydrogenase. A low activity for diaphorase and fructose 1–6 diphosphatase is also evident.

The blastemal caps of metanephros seen at the tubular ends of the ureteral branches have activity only for NADH diaphorase and isocitrate dehydrogenase, but no alkaline phosphatase is visible.

The enzymatic activity of proximal convoluted tubules in 16-day-old fetal kidneys and in the newborn hamster show a marked resemblance to that seen in the tumorous cells.

6. *Ultrastructural study of normal developing metanephros*

The earliest metanephral blastemal cells in the kidney at the 11th day of embryonic life are formed by branching units of immature cells with an abundant amorphous interstitial substance. They show a high nucleo-cytoplasmic ratio, sparse organelles, with some free ribosomes and a poorly developed RER. The nuclei are round or oval, with sparse chromatin. There are no specific differentiations such as cilia or desmosomes, but close contacts exist between these cytoplasmic projections by means of very immature intercellular junctions (Fig. 7).

The blastemal caps of metanephrogenic cells are grouped at the blind end of the ureteral branches which are rich in glycogen. These cells have an epithelial character, being closely packed and possessing desmosomes. No basal membranes exist, and the cells are continuous with the blastemal interstitial metanephros. The cytoplasm contains abundant free ribosomes, sparse RER and a poorly developed Golgi. Lipids are present in the form of small droplets inside the cytoplasmic matrix, and some of them have a centriole with cilia.

In the more mature embryonic kidney at the 16th day of intrauterine life, metanephrogenic epithelial cells adopt an elongated contour, and close contacts with highly developed desmosome complexes exist between them. Moreover, the blastemal caps are transformed into tubules. It seems that the tubular lumen is formed through activated lysosomes which show an autophagic character. In this case, abundant necrobiotic cytoplasmic processes are seen in the central lumen of the newly-formed tubule. The more developed tubular structures, which resemble the proximal convoluted tubule of the adult kidney, have microvilli as well as cilia with basal bodies and ciliary rootlets (Fig. 9).

Furthermore, ciliary structures exist in the

Table 4. *Some histochemical enzymatic activities of kidney tumors compared with embryonic* (11*th and* 16*th day*), *newborn and adult normal male kidneys of the golden hamster*

	Renal tumor	Embryonic kidney		Newborn kidney		Adult kidney
	All cells	Blastemic caps	Interst. cells	Tubular cells	Interst. cells	Tubular cells
Alkaline phosphatase	+ + +	−	+ + +	+ + +	+ +	+ +
ATP-ase (pH 9·4)	+ + +	−	−	+ + +	−	+
Fructose 1-6 diphosphatase	+ + +	−	+ + +	+ + +	+ +*	+
Glucose-6-phosphatase	−	−	−	+ + +	−	+ + +
NADPH diaphorase	+ + +	+	+	+ + +	+	+ + +
Isocitrate DH	+ + +	+	+	+ + +	+ +	+ +
P.A.S.	−	−	−	+†	−	−

*Isolated cells.
†Isolated cells of the collecting tubules

mesenchymal-looking metanephrogenic cells surrounding these new tubular structures (Fig. 8).

The ultrastructure of these cells shows a more highly developed RER and clusters of free ribosomes; each of them has a basal body and cilia. The cytoplasmic processes are in close contact with each other, and highly developed desmosomes complexes exist. Present structures should not be confused with the vascular cells since no contacts exist between them.

7. *Ultrastructural study of the tumor*

Up to three types of structures may be present, depending on the degree of tumorous organization: blastemal, tubular and papillary areas. These structures may be found in the same tumor and closely continuous to one another.

The cells are arranged in ill-defined groups of cords, bands, tubes or cysts, in which two predominant types of cells (blastemal and epithelial) are irregularly distributed. Certain blastemal and almost all epithelial cells bear a highly differentiated, ciliated border in close association with microvilli. Some also display a secretory activity with well-developed Golgi complexes and RER.

Blastemal areas are formed by compact groups of polygonal or elongated cells in close contact (Fig. 10). No desmosomes were evident among them, but occasional condensations of the plasma membranes suggest immature intercellular junctions. The nuclei were elongated or round, occasionally with a prominent peripheral condensation of chromatin. There was a poorly developed cytoplasm and sparse, circular or elongated mitochondria with few cristae. Rare profiles of ergastoplasm and numerous ribosomes were found. A large number of lipid droplets was present in almost all blastemal cells, and a few bundles of microfilaments were also visible (Fig. 10 and 11). Among the immature blastemal cells, other cells resembling immature smooth muscle were rarely found.

Some blastemal cells, like the epithelial ones, possessed cilia with basal bodies and axial filamentous matrixes. Large phagosome bodies were observed occasionally, and some myelin-like figures appeared in the cytoplasm (Fig. 17 and 18). Vascularization in the tumor was slight, and no relation was established between the endothelial and perithelial cells of the capillaries and the neoplastic cells.

The blastemic differentiation into tubular structure shows close continuity between each other, the cells adopting a more cubical or prismatic shape. Intercellular junctions are more evident. Free cellular surface is associated with tufts of microvilli and cilia in compact pockets (Fig. 13 and 15). The other cytoplasmic characteristics remain as undifferentiated as those already described, the only difference being a greater degree of RER and the presence of a Golgi apparatus. Some nuclei are more elongated with irregular patterned chromatin and infoldings of the membranes.

The more differentiated tubular or papillary epithelial areas are found in close continuity with the blastematous ones. Usually no basals exist, but at times highly irregular profile condensations separate epithelial and blastemal cells (Fig. 12).

The ultrastructure of prismatic or polygonal epithelial cells shows a highly differentiated cytoplasm. The nuclei are usually spherical or elongated with infoldings of the membrane. The chromatin is thinly dispersed throughout the nucleoplasm, with some densely clumped peripheral granules; there is a prominent nucleolus. These cells are apparently stratified. The free cell borders organize microvilli and cilia. In fact, an extremely well-differentiated ciliated border exists in the more apical areas of the cells. Sometimes, ciliary projections are found in deep cytoplasmic invaginations of a neighbouring cell. The association of ciliated tufts with microvilli and brush border is common within the same epithelial cell. Clusters of finger-like digitations are also present in adjoining cell borders.

Intercellular junctions between epithelial cells are particularly prominent, and shape a kind of junction complex in the cell border next to the lumen. The cytoplasm reveals abundant mitochondria, especially around the basal body and the ciliary matrix. Ribosomal particles in clusters are particularly abundant, and a RER is sometimes found in close contact with the mitochondria. Secretory vacuoles are present. The Golgi apparatus possesses dilated lamellae and prominent saccules (Fig. 16).

The cilia is the most striking feature of the estrogen-induced G.H. kidney tumor. Epithelial and blastemic cells possess a large quantity of these structures which are identical to those observed in other human beings and animals. Cross-sections revealed the nine outer doublets and the two inner cores of tubules (9+2); but in some cases 8+2, 7+2 and 10+2 cores of filaments were encountered. Occasional stereocilia were found with only the nine outer doublets. The filaments were in continuity with the second centriole of the basal body and possessed striated rootlets (Fig. 13, 14 and 15).

Isolated ciliated renal tubular cells (proximal

and distal) were also present in DES, DESDME and DESGA intoxicated kidneys with or without neoplasias.

8. *Ultrastructural aspects of the tubules in the kidney with tumors*

Some tubular and glomerular structures are sequestered by the neoplastic tissue and encircled by several layers of tumoral cells; but these components are usually separated from the surrounding infiltrating neoplasia by an intact basal membrane. The latter, in some cases, seems to be thickened. In more advanced states the basal membrane vanishes.

The electron microscopy of what has already been described as tubular cell dysplasia shows a more marked resemblance to the malignant infiltrating cells of the tumor (Fig. 19). Nuclei are large, elongated or round, with some infoldings of the membrane; and the chromatin has a peripherical distribution. Microvilli in the apical cytoplasmic area are also less numerous than in the normal proximal tubular cells. Regularly, one or more cilia with a basal body and ciliary rootlets are also present (Fig. 19 and 20). The cytoplasm is dense with abundant, irregularly distributed mitochondria showing vacuolated matrix with a sparse number of cristae. There is also a high incidence of lipids and lysosomes.

DISCUSSION

Chronic administration of DES induces kidney tumors in 69% of male G.H. The present results agree with previous observations [2, 3, 5, 9]. The incidence is higher between the 6th and 10th months of the experiment; and 12 months' uninterrupted intoxication with the substance also induces a 5% incidence of other tumorous growths (two epididymal adenomas and one hepatoma). DESGA, physiological derivative of DES, has not produced renal tumors in male G.H. when administered chronically but, microscopically, there has been evidence of dysplastic changes in tubules. Interstitial cell hyperplasia and tubular cell dysplasia have been considered by different authors as early neoplastic changes in the DES-intoxicated male G.H. [1, 5, 8, 9].

The non-physiological DES-derivate, DESDME, also induces kidney tumors in the male G.H. after 10–12 months' administration. The incidence is lower than in the DES group (51%). No extra renal tumors could be detected in the DESDME-intoxicated group, but a moderate number of pre-malignant renal changes (tubular and interstitial) were observed. Our own findings now confirm the hypothesis adduced by Lacomba and Gabaldon [14].

Histologically, the following three types of structures may be detected in the same tumor: blastemal, solid-tubular and papillary-cystic epithelial. The latter two appear in close association with areas of blastemic cells and the more mesenchymatous, spindle-shaped ones. A progressive involution into lipid-rich signet-ring cells is also evident. These structures resemble those already described by most who have investigated the histology of DES-induced G.H. kidney tumors and transplants [1, 2, 5, 7, 9. 10, 17–22].

No experimental or human kidney neoplasia of an epithelial nature shows a close histological similarity to the DES-induced G.H. renal tumor [23–25], but the high lipid content of the tumorous cells somewhat resembles the human hypernephroma [2, 26–27].

An epithelial-tubular origin has been postulated by several authors [1, 2, 6–8, 18–20] but so far, arguments at optical level also support an interstitial cell origin [9, 10, 17, 22]. Our observations are a further confirmation of the difficulties involved in determining by mere optical means a tubular or interstitial cell origin for the tumor. There are obvious arguments in favor of the two possibilities, probably because DES in G.H. kidney induces not only a hyperplastic response of tubular epithelial cells (in the proximal and distal convoluted tubules) but also a hyperplasia of the remaining cells located in the interstitial areas of the kidney.

Interstitial cell-hyperplasia must be considered the initial origin of this neoplasia, as it is found only in the DES and DESGA groups closely associated with the tumor. The histochemical and ultrastructural studies also support this hypothesis.

Furthermore, the histochemistry of these tumors invariably displays, independent of the histological structure, a common enzymatic activity resembling that of the normal proximal convoluted tubule. A high activity is found in the following systems: alkaline phosphatase, fructose 1–6 diphosphatase, ATP-ase, isocitrate DH, succinate DH and NADH-NADPH diaphorases, but analogous enzyme activity also exists in the nodules of hyperplastic interstitial cells detected in pre-neoplastic areas of hormone-stimulated kidneys. An activity absent in these tumoral cells is that for glucose-6-phosphatase which is constant in the proximal convoluted tubule. We did not find the PAS-positive glycogen in the

tumorous cells reported by Arcadi [28]. A comparative histochemical analysis of the developing metanephros, the metanephrogenic cells of the interstices in new-born G.H., and the DES-induced neoplastic kidney tumors, shows similarities between the enzymes present. Most of these findings agree with other observations on the present tumors [6, 9, 11]. Dodge and Kirkman [29] and Dodge [30] have recently demonstrated changes in the isoenzyme patterns of lactate DH and glucose-6-phosphate DH of a stilbestrol-induced and dependent renal carcinoma differing from that of an autonomous hormone-independent variant derived from it. These differences suggest the possibility of using isoenzyme analysis in distinguishing hormone-dependent from hormone-independent renal tumors.

To date, only Manweiler and Bernhard [12] have performed an ultrastuctural study of the DES-induced G.H. renal tumor. Our own findings are in agreement with those of these authors, as we, too, have observed a high ciliary content in almost all tumorous cells, which is the most striking feature of the neoplasia. Existence of cilia in this renal tumor was first mentioned by Kirkman in 1950, who also described them in detail by optical means some years later [6]. The results of the present study also confirm our earlier findings of cilia in the kidney proximal tubular epithelium of the G.H. intoxicated with DES and in the tumorous cells [13].

The presence of isolated cilia is common in the proximal convoluted tubule of most kidneys, both mammal and human, [31–33] and has no correlation with any pathological condition as was first thought [34–36]. These ciliary structures would be the first evidence of an epithelial tubular origin of the present neoplasia if they were only visible in the more epithelially differentiated tubular, papillary or cystic structures, as Kirkman [6] and Mannweiler and Bernhard [12] indicate. Nonetheless, as we have seen, ciliated tufts are also present in the blastemal cells, and are visible too, under the electron microscope, in normal metanephrogenic cells in the G.H., the rat and in man [37, 38]. Furthermore, new ultrastructural studies, presently in progress in our laboratory on hormone-dependent transplants of a stilbestrol-induced kidney tumor show the presence of ciliated cells in transplants.

A marked resemblance exists between these tumorous cells and the normal blastemal metanephros of the developing animal. Interstitial ciliated cells are also seen in the normal adult human kidney [31]. Furthermore, Osvaldo and Latta [39] give evidence for the ultrastructure of the interstitial cells of the rat kidney, which have cilia and show other features that differentiate them from the normal fibroblasts, a characteristic which we have also observed in kidneys of newborn G.H.

Moreover, an evident structural similarity exists between this hormone-dependent renal neoplasia, and metanephric tissue and nephroblastomas, when observed by electronic-microscope [12, 37]. In human Wilms' tumor, Balsavar *et al.* [40] indicate both the existence of a stromatic cell of mesenchymal appearance and more epithelially differentiated areas, with the presence of junctional complexes and an abundant microvilli system. As we have stated, similar kinds of cells are present in G.H. kidney tumors, being in close continuity to each other. Studies by Ito and Johnson [37] and Tannenbaum [41] indicate the presence of cilia in all the cases possessing an epithelial differentiation on human nephroblastoma. The cilium is very well developed, not only in the more epithelial tumorous cells but also in the undifferentiated variety. Other cellular characteristics such as desmosome junctions, compact arrangement of cells in close contact, a progressive evolution of the mesenchymal-blastemic cell-types into a more epithelial kind, and finally, a secretory activity of the cytoplasm with a very well developed RER and Golgi apparatus, are common features present in G.H. normal metanephrogenic tissue and in DES-induced kidney neoplasia. According to Tannenbaum [41], all these structures might be considered an expression of the embryonic character of nephroblastomas, either human or experimental ones.

The histological and electron-microscopic investigation of the tumors now reported by us, as compared with the metanephros of the developing kidney of the G.H., seems to determine an interstitial cell origin of the tumor and it might be considered an epithelial-blastemal type of nephroblastoma. To us it seems likely that the mesenchymal elements of metanephral origin remaining in the kidney stroma of the newborn G.H., as interstitial cells are responsible for the continuity of growth under DES stimulation.

Other factors might also contribute to the occurrence of renal tumors in the DES neoplasia of the G.H. No virus particles are present in primary tumors or in transplants of the hormone-dependent neoplasia, but as we have seen, [15, 42] the hormonal independence of tumorous cells is associated with the presence of virus-like particles (Oncornavirus C type) inside the RER of the transformed cells.

REFERENCES

1. A. Llombart Jr., Estudios experimentales sobre oncogenesis: la acción del tetracloruro de carbono y del estradiol en el desarrollo de los tumores renales malignos del hamster dorado. *Arch. Español Morf. Suppl.* **65,** 1 (1964).
2. A. Llombart Jr., Sur l'histogenese et la nature des neoplasies rénales produites par l'oestradiol chez le hamster doré. *Bull. Cancer (Paris)* **52,** 11 (1965).
3. H. Kirkman, Estrogen-induced tumors of the kidney in the Syrian hamster. *Nat. Cancer Inst. Monogr.* **1,** 1 (1959).
4. E. S. Homburger, The Syrian golden hamster in chemical carcinogenesis research. *Prog. exp. Tumor Res.* **10,** 203 (1968).
5. E. S. Horning, Endocrine factors involved in the induction, prevention and transplantation of kidney tumors in the male golden hamster. *Z. Krebsforch.* **61,** 1 (1956).
6. H. Kirkman and M. Robbins, Estrogen-induced tumors of the kidney. V. Histology and histogenesis in the Syrian hamster. *Nat. Cancer Inst. Monogr.* **1,** 93 (1959).
7. M. J. Guthrie, Tumorogenesis in the kidney of the estrogenized male golden hamster. *Anat. Rec.* **136,** 203 (1960).
8. R. I. Polkina, Kidney tumor induced by sinestrol and diethylstilbestrol in the hamster. *Prob. Oncol.* **7,** 26 (1961).
9. W. Dontenwill and M. Eder, Histogenese und biologische Verhaltensweise hormonell ausgelöster Geschwülste. *Beitr. path. Anat.* **120,** 270 (1959).
10. H. Kirkman and M. Robbins, Histogenesis of estrogen-induced renal tumors in golden hamster. *Anat. Rec.* **121,** 323 (1955).
11. A. S. K. Murthy and A. B. Sussfield, Dehydrogenases in diethylstilbestrol-induced kidney tumors of the Syrian hamster. *Experientia (Basel)* **24,** 60 (1968).
12. Kl. Mannweiler and W. Bernhard, Recherches ultrastructurales sur une tumeur rénale expérimentale du hamster. *J. Ultrastruc. Res.* **1,** 158 (1957).
13. A. Llombart Jr. and A. Peydro, Ultra-estructura ciliar en el riñón del hamster dorado intoxicado por dietilestilbestrol. Su relación con los llamados nefroblastomas diferenciados hormono dependientes. *Med. Española (Valencia)* **64,** 235 (1970).
14. T. Lacomba and M. Gabaldon, Biochemical studies of diethylstilbestrol-induced kidney tumors in the golden hamster. *Cancer Res.* **31,** 1251 (1971).
15. A. Llombart Jr. and A. Peydro, R. type virus particles (RTP) in hormone-independent transplants of a diethylstilbestrol (DES) induced kidney tumors in the Syrian golden hamster. *European Association for Cancer Research,* 2nd Meeting, 2–5 Oct. Heidelberg (1973).
16. A. Llombart Jr., A. Peydro and F. Llopis, Some histochemical alterations induced by the methylhydrazine derivative natulan on two experimental ascites tumors. *Oncology* **24,** 358 (1970).
17. W. Dontenwill, Experimentelle Erzeugung von Nieren und Lebertumoren durch Follikelhormon. *Verh. dtsch. Ges. Path.* **34,** 458 (1959).
18. E. S. Horning, The influence of unilateral nephrectomy on the development of stilbestrol-induced renal tumors in the male golden hamster. *Brit. J. Cancer* **8,** 627 (1954).
19. E. S. Horning, Observations on hormone dependent renal tumor in the golden hamster. *Brit. J. Cancer* **10,** 678 (1956).
20. E. S. Horning and J. W. Whittick, The histogenesis of stilbestrol-induced renal tumors in the male golden hamster. *Brit. J. Cancer* **8,** 451 (1954).
21. U. Ising, The effect of unilateral ureterotomy on the development of estrogen-induced renal tumors in male hamsters. *Acta path. microbiol. scand.* **39,** 168 (1956).
22. H. Kirkman, Relation of sex-hormones to the induction and control of renal tumors in the golden hamster. *Anat. Rec.* **109,** 311 (1951).
23. K. Apitz, Die Geschwülste und Gewebemissbildungen der Nierenrinde. *Virchows. Arch. path. Anat.* **311,** 285 (1943).
24. J. L. Bennington and R. M. Kradjian, *Renal Carcinoma.* W. B. Saunders, Philad. (1967).
25. J. S. King Jr., *Renal Neoplasia.* Little, Brown and Co., Boston (1967).
26. H. J. G. Bloom, Treatment of renal cell carcinoma with steroid-hormones. Observations with transplanted tumors in the hamster and incurable cancer in man. In *Renal Neoplasia.* Edited by J. Stanton King Jr., p. 605. Little, Brown and Co., Boston (1967).

27. H. J. G. Bloom, E. C. Dukes and B. C. Mitchley, Hormone-dependent tumors of the kidney. I. The oestrogen-induced renal tumor of the Syrian hamster. Hormone treatment and possible relationship to carcinoma of the kidney in man. *Brit. J. Cancer* **17,** 611 (1963).
28. J A. Arcadi, Glycogen-containing cells in the estrogen-induced renal tumor of the hamster. *Science* **142,** 592 (1963).
29. A. H. Dodge and H. Kirkman, Glucose-6-phosphatase dehydrogenase in primary and autonomous estrogen-induced renal tumors of the Syrian hamster. *J. Cell Biol.* (Suppl.) **47** (no. 2, part 2) p. 51a (abstr.) (1970).
30. A. H. Dodge, G-6-PD and LDH isoenzyme analysis of estrogen-dependent and independent renal tumors of the Syrian hamster. *Oncology* **28,** 253 (1973).
31. R. E. Bulger, C. C. Tischer, C. E. Myers and B. F. Trump, Human renal ultrastructure. II. The thin limb of Henle's Loop and interstitium in healthy individuals. *Lab. Invest.* **16,** 124 (1967).
32. C. E. Myers, R. E. Bulger, C. C. Tischer and B. F. Trump, Human renal ultrastructure. IV. Collecting duct of healthy individuals. *Lab. Invest.* **15,** 1921 (1966).
33. C. C. Tischer, R. E. Bulger and B. F. Trump, Human renal ultrastructure. I. Proximal tubule of healthy individuals. *Lab. Invest.* **15,** 1357 (1966).
34. J. Duffy and Y. Suzuki, Ciliated human renal proximal tubular cells. *Amer. J. Path.* **53,** 609 (1968).
35. H. Latta, In *Ultrastructure of the Kidney.* (Edited by A. J. Dalton and F. Haguenau) Vol. 2, p. 14. Academic Press, New York (1967).
36. M. Rossmen and P. Galle, Mise en évidence de cellules rénales ciliées chez l'homme. *Nephron* **5,** 426 (1968).
37. J. Ito and W. W. Johnson, Ultrastructure of Wilms' tumor. Epithelial cell. *J. nat. Cancer Inst.* **42,** 77 (1969).
38. H. Latta, A. B. Maunsbach and S. C. Madden, Cilia in different segments of the rat nephron. *J. biophys. biochem. Cytol.* **11,** 248 (1961).
39. L. Osvaldo and H. Latta, Interstitial cells of the renal medulla. *J. Ultrastruc. Res.* **15,** 589 (1966).
40. A. M. Balsaver, C. W. Gibley and C. F. Tessmer, Ultrastructural studies on Wilms's Tumor. *Cancer (Philad.)* **22,** 417 (1966).
41. M. Tannenbaum, Ultrastructural pathology of human renal cell tumors. In *Pathology Annual,* Vol. 6, p. 249. S. C. Sommers Butterworths, London (1971).
42. A. Llombart-Bosch, Particulas viricas tipo R (RTP virus) en transplantes del tumor renal hormono-independiente (RHI/GH) del hamster dorado sirio inducido por Dietilestilbestrol (DES). Primera comunicación. *Med. Española (Valencia)* **69,** 236 (1973).

Europ. J. Cancer Vol. 11, pp. 413–417. Pergamon Press 1975. Printed in Great Britain

Effect of Normal Immunosuppressive Protein (NIP) on Tumor Growth in Mice

H. OVADIA, N. HANNA and D. NELKEN

Lil and Ben Stein Transplantation Laboratory, Department of Immunology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Abstract—*The effect of normal immunosuppressive protein on the take and growth rate of a methylcholanthrene-induced tumor in C57B1 mice is described. NIP given in two injections (10 mg each), 24 hr before and 24 hr after tumor inoculation caused: (a) Enhanced tumor growth, most significantly observed at earlier stages after tumor inoculation, (b) Appearance of tumors in 25% of mice inoculated with only 10^3 cells, and in 100% of mice inoculated with 10^4 cells. Under similar conditions, in control groups no tumor takes could be observed in mice receiving 10^3 or 10^4 tumor cells.*

INTRODUCTION

AN ALPHA globulin-rich fraction isolated from human plasma has been found to inhibit both humoral and cellular immune responses [1, 2]. The active component which we called NIP markedly inhibited both PHA-induced and antibody-mediated lymphocyte cytotoxicity [3].

NIP levels were found to be elevated in a high percentage of patients suffering from various forms of malignant diseases [4]. It was suggested that elevated NIP levels may indicate a reduced immunological surveillance potential [5].

In the present study, experiments were performed to test the effect of NIP on the take and growth of a Methylcholanthrene-induced tumor in mice.

MATERIAL AND METHODS

Preparation of NIP

NIP was prepared from human plasma as described elsewhere [6]. Briefly, 1 l of plasma was diluted 1:2·5 with distilled water and acidified to pH 5·0. The euglobulin precipitate was discarded and the supernatant applied to a DEAE cellulose chromatography column. After thorough washing with 0·1M acetate buffer pH 5·0 NIP was eluted with 0·2M of same buffer.

The eluate was concentrated by ultrafiltration through Amicon diaflo membranes UM-2 (Amicon Corp. Lexington, Mass.) and dialised against large amounts of saline for 3 days. The concentrated NIP preparation was sterilized by filtration through a 0·45 μm millipore membrane and stored at −20°C.

Tumor and mice

Methylcholanthrene-induced tumor (MC-57T), kindly supplied by Prof. G. Klein, Karolinska Institute, was carried as a s.c. solid tumor in C57B1 mice.

Tumor cell-suspensions were prepared by trypsinization (0·25% trypsin solution) of solid tumors and washed 3 times in Hank's solution. Viability was tested by the trypan-blue dye exclusion method.

Experimental procedure

To test the effect of NIP on kinetics of tumor growth *in vivo*, two successive injections of NIP 10 mg protein each, were administered i.v., 24 hr before and 24 hr after tumor inoculation. Control mice received similar

Accepted 28 February 1975.

27. H. J. G. Bloom, E. C. Dukes and B. C. Mitchley, Hormone-dependent tumors of the kidney. I. The oestrogen-induced renal tumor of the Syrian hamster. Hormone treatment and possible relationship to carcinoma of the kidney in man. *Brit. J. Cancer* **17,** 611 (1963).
28. J A. Arcadi, Glycogen-containing cells in the estrogen-induced renal tumor of the hamster. *Science* **142,** 592 (1963).
29. A. H. Dodge and H. Kirkman, Glucose-6-phosphatase dehydrogenase in primary and autonomous estrogen-induced renal tumors of the Syrian hamster. *J. Cell Biol.* (Suppl.) **47** (no. 2, part 2) p. 51a (abstr.) (1970).
30. A. H. Dodge, G-6-PD and LDH isoenzyme analysis of estrogen-dependent and independent renal tumors of the Syrian hamster. *Oncology* **28,** 253 (1973).
31. R. E. Bulger, C. C. Tischer, C. E. Myers and B. F. Trump, Human renal ultrastructure. II. The thin limb of Henle's Loop and interstitium in healthy individuals. *Lab. Invest.* **16,** 124 (1967).
32. C. E. Myers, R. E. Bulger, C. C. Tischer and B. F. Trump, Human renal ultrastructure. IV. Collecting duct of healthy individuals. *Lab. Invest.* **15,** 1921 (1966).
33. C. C. Tischer, R. E. Bulger and B. F. Trump, Human renal ultrastructure. I. Proximal tubule of healthy individuals. *Lab. Invest.* **15,** 1357 (1966).
34. J. Duffy and Y. Suzuki, Ciliated human renal proximal tubular cells. *Amer. J. Path.* **53,** 609 (1968).
35. H. Latta, In *Ultrastructure of the Kidney.* (Edited by A. J. Dalton and F. Haguenau) Vol. 2, p. 14. Academic Press, New York (1967).
36. M. Rossmen and P. Galle, Mise en évidence de cellules rénales ciliées chez l'homme. *Nephron* **5,** 426 (1968).
37. J. Ito and W. W. Johnson, Ultrastructure of Wilms' tumor. Epithelial cell. *J. nat. Cancer Inst.* **42,** 77 (1969).
38. H. Latta, A. B. Maunsbach and S. C. Madden, Cilia in different segments of the rat nephron. *J. biophys. biochem. Cytol.* **11,** 248 (1961).
39. L. Osvaldo and H. Latta, Interstitial cells of the renal medulla. *J. Ultrastruc. Res.* **15,** 589 (1966).
40. A. M. Balsaver, C. W. Gibley and C. F. Tessmer, Ultrastructural studies on Wilms's Tumor. *Cancer (Philad.)* **22,** 417 (1966).
41. M. Tannenbaum, Ultrastructural pathology of human renal cell tumors. In *Pathology Annual,* Vol. 6, p. 249. S. C. Sommers Butterworths, London (1971).
42. A. Llombart-Bosch, Particulas viricas tipo R (RTP virus) en transplantes del tumor renal hormono-independiente (RHI/GH) del hamster dorado sirio inducido por Dietilestilbestrol (DES). Primera comunicación. *Med. Española (Valencia)* **69,** 236 (1973).

Table 1. *Volume of tumors in NIP-treated and in control mice*

Treatment	Number of cells inoculated	Days after inoculation 5	7	8	9	10	12	14	20
NIP	10^6	63·16 ± 13·2	167·08 ± 33·2	251·15 ± 41·2	300·7 ± 31·5	384·96 ± 33·5	507·2 ± 59	634·6 ± 57·7	1424·4 ± 188
	10^5	6·87 ± 0·4	41·56 ± 14·6	79·2 ± 22·8	122·7 ± 12	154·7 ± 11·5	223 ± 11·5	335·9 ± 27	1052·7 ± 93·4
	10^4	—	1·6 ± 0·4	16·5 ± 8·3	22·2 ± 9·3	37·8 ± 13.6	47·9 ± 9·7	85·53 ± 15·3	429·6 ± 78·8
	*10^3	—	—	—	0·4	3·2	10·8	28·8	84·7
Saline	10^6	25·3 ± 6·7	85·6 ± 19·7	139·3 ± 27	220·2 ± 23·6	293·2 ± 16·4	386·4 ± 46	519·2 ± 54·4	1120·7 ± 86·4
	10^5	—	6·93 ± 3·7	31·58 ± 4	56·8 ± 8·6	85·6 ± 11·5	119·1 ± 9·1	166·5 ± 25·5	616 ± 84·4
	10^4	—	—	—	—	—	—	—	—
	10^3	—	—	—	—	—	—	—	—
HSA	10^6	25 ± 8·6	85·8 ± 31·3	145·6 ± 43·1	207·3 ± 55·3	266·8 ± 65·8	419·7 ± 42	557·5 ± 68·9	1200·4 ± 94·5
	10^5	—	9·5 ± 3·3	30·8 ± 4·0	62·2 ± 4·0	87·2 ± 5·5	121·2 ± 12·3	165·6 ± 11·5	652 ± 86·5
	10^4	—	—	—	—	—	—	—	—
	10^3	—	—	—	—	—	—	—	—

Data are expressed as mean ± SEM (mm^3).
Each value represents the mean of 8 samples.
*Mean of 2 samples.

did not develop tumors at any stage during the follow-up period of the experiment. Control groups used in this experiment included animals injected with saline or with 20 mg HSA, which is the main contaminant of the NIP preparation. The administration of HSA is necessary in order to exclude non-specific effects or other factors, like antigenic competition, which might influence the immune response against the tumor cells. It was found that both treatments of control groups had no effect either on tumor growth rate at the higher doses (10^5 and 10^6), or on the take of tumors at the lower doses (10^3 and 10^4).

DISCUSSION

Previous studies from this and other laboratories have shown that alpha globulin-rich fractions isolated from mammalian plasma possess immunosuppressive properties [2, 8, 9]. Our material called normal immunosuppressive protein (NIP) has been found to depress both T and B lymphocyte functions [4]. Recent experiments in our laboratory using specific anti-NIP antibodies in passive hemagglutination inhibition tests have indicated that elevated NIP levels may be found frequently in the serum of patients suffering from various forms of malignant diseases. The anti-NIP serum was prepared by repeated immunizations of rabbits with human NIP which was further purified by acrylamide gel electrophoresis [4]. Indications of elevated α-globulin values in cases of malignancy were also reported by several other authors [10, 11].

In the present study, experiments were performed in order to examine the effect of NIP on tumor take and growth. To this aim, subthreshold doses of a methylcholanthrene-induced tumor in C57B1 mice were chosen. This tumor grows locally at the site of inoculation and may be measured easily. It also has a clear threshold dose, while 10^4 tumor cells are still rejected, doses of 10^5 and more cells grow progressively and rapidly.

In our experiments in NIP-treated mice, several animals inoculated with only 10^3 cells developed tumors, while all animals inoculated with 10^4 cells developed tumors. In the control groups however, not a single mouse receiving 10^3 or 10^4 cells developed a tumor. In the NIP-treated mice the tumor grew progressively and no regression could be observed during the experiment. A very marked increase of the tumor volume was observed in the NIP-treated mice receiving 10^5 tumor cells, compared to the control mice. The effect of NIP could be best observed during the first days after inoculation, when the tumor size was markedly greater than that in the control animals. However, the difference in volume between the two groups was diminished at a later stage. This is due most probably to the overwhelming dose of tumor cells against which the immune mechanism of the host has no effect. While in the control groups of mice receiving small doses of tumor cells, the host may cope completely or partially with the malignant cells. Similar small dose inocula given to NIP-treated mice have a pronounced effect on these animals. The early appearance of the tumor as well as its large volume seems to be an indication of the impairment of the immune response in these mice.

The observation that NIP was found to be elevated in sera of patients with various kinds of malignancy, and the finding that it suppresses both humoral and cellular immune response, may indicate that it can interfere with the immunological surveillance mechanism.

REFERENCES

1. M. Glaser, I. Cohen and D. Nelken, *In vitro* inhibition of plaque and rosette formation by alpha-globulin. *J. Immunol.* **108**, 286 (1972).
2. M. Glaser, I. Ofek and D. Nelken, Inhibition of plaque formation, rosette formation and phagocytosis by alpha-globulin. *Immunology* **23,** 205 (1972).
3. N. Hanna, R. Kalderon and D. Nelken, Inhibition of T and B lymphocyte functions by Normal Immunosuppressive Protein. Immunology. In press (1975).
4. D. Nelken, N. Hanna and R. Kalderon, Normal Immunosuppressive Protein: Quantitative estimation and effect on T and B lymphocytes. *Transpl. Proc.* In press (1975).
5. D. Nelken, Normal Immunosuppressive Protein and immunological surveillance. *Lancet* **1,** 1443 (1973).
6. D. Nelken, Normal Immunosuppressive Protein (NIP). *J. Immunol.* **110,** 1161 (1973).

7. M. A. Atia and D. W. Weiss, Immunology of spontaneous mammary carcinomas in mice. V. Acquired tumor resistance and enhancement in strain A mice infected with mammary tumor virus. *Cancer Res.* **26,** 1787 (1966).
8. B. B. Kamrin, Successful skin homografts in mature non-littermate rats treated with fractions containing alpha-globulins. *Proc. Soc. exp. Biol. (N.Y.)* **100,** 58 (1959).
9. S. R. Cooperband, H. Bondevik, K. Schmid and J. A. Mannick, Transformation of human lymphocytes: inhibition by homologous alpha-globulin. *Science* **159,** 1243 (1968).
10. R. W. Sunderman and F. W. Sunderman Jr., *Serum Proteins and Dysproteinemias.* J. P. Lippincot, Philadelphia (1964).
11. R. J. Ablin, Interference of immunologic surveillance by immunoregulatory alpha-globulin—A hypothesis. *Neoplasma (Bratis P.)* **20,** 159 (1973).

Europ. J. Cancer Vol. 11, pp. 419–423. Pergamon Press 1975. Printed in Great Britain

Radiation-induced Synchronization of the Walker Carcinoma *In Vivo**

WALFRIED A. LINDEN, FRIEDRICH ZYWIETZ, HEINZ BAISCH and JÖRN SKIBA

Institut für Biophysik und Strahlenbiologie der Universität Hamburg, Martinistrasse 52, *D*-2000 *Hamburg* 20, *Germany*

Abstract—*Exponentially growing Walker tumours of a weight of* $1{\cdot}0 \pm 0{\cdot}3$ *g were irradiated at a* 200 kV_p *X-ray unit with single and fractionated doses ranging from* 50 *to* 900 *rads. The influence of the radiation on the cell cycle of the carcinoma was studied using a pulse-cytophotometer. By this method DNA distribution patterns of the tumour cells are obtained. The mathematical analysis of these DNA distributions using a computer program gives the percentage of cells belonging to the various phases of the cell cycle. The results show a reversible accumulation of cells in the* G_2*-phase and a partly synchronous passage of cells from* G_2*-phase into mitosis and* G_1*-phase. With the highest dose applied, the fraction of cells in* G_2 *could be increased from* 14 *to* 48%. *The degree of synchrony obtained is compared with the values determined after chemical treatment* in vivo.

INTRODUCTION

FOR SEVERAL years human tumour therapy has tried to use the differential sensitivity of the phases of the cell cycle for better regimens of therapy [1–3]. For these new methods of therapy a synchronous passage of tumour cells through the cell cycle is a prerequisite. Partial synchronization of cell division *in vitro* has been obtained with considerable success by the application of several techniques. A review was given by Nias and Fox [4]. However, little is known about methods giving a satisfactory degree of synchrony under *in vivo* conditions. Promising results for practical application have been obtained with block and release methods using either chemicals or ionizing radiation. Rajewsky [5] observed a synchronized fraction of S-cells of about 0·5 in a solid rat carcinoma after temporary inhibition of DNA synthesis by hydroxyurea. Partial synchronization after X-ray exposure is also a well known phenomenon [6–9]. In a more recent investigation, Göhde [10] achieved a reversible accumulation of about 70% of Ehrlich ascites tumour cells in the G_2-phase by irradiation with 300 rads of neutrons. Our paper is concerned with studies on the synchronization of the Walker carcinoma *in vivo* by treatment with single and fractionated doses of X-rays. The cell kinetics are determined using the technique of pulse-cytophotometry.

Accepted 29 January 1975.

*Supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg.

MATERIAL AND METHODS

The experiments were carried out with male rats of the inbred strain BD1 weighing 200 ± 15 g. The Walker carcinosarcoma 256 was implanted s.c. in the tails of the animals by the trocar technique. On the 7th day after transplantation, tumours of a weight of $1{\cdot}0 \pm 0{\cdot}3$ g were selected and irradiated at a 200 kV_p X-ray unit (filter 0·5 mm Cu), the dose rate being 100 rads/min. The radiation doses ranged from 50 to 900 rads. The fractionated X-ray treatment was carried out with a total dose of 900 rads given in 3 fractions of 300 rads at a time interval of 8 hr. For each determination 2 rats were sacrificed at 6-hr intervals p. irr. up to 48 hr and from this time up to 96 hr at

12 hr intervals respectively. From the tumour tissue excised one part was taken for histological studies, the other for pulse-cytophotometric measurements. Histological slides were Feulgen-stained and at least 2000 cells were scored for mitotic figures.

The determination of the synchronization effect was carried out using the pulse-cytophotometer (Impulscytophotometer ICP 11, PHYWE, Göttingen) developed by Göhde and Dittrich [10, 11]. This instrument basically represents an automatic fluorescence microscope. It allows a quick determination of the DNA content of single cells in suspension after staining with a fluorescent dye, thus providing DNA distribution patterns (DNA histograms) of cell populations. The preparation of tumour material for investigation with the pulse-cytophotometer was carried out as described previously [12, 13]. A sample of tumour tissue was cut to small pieces. The cells were isolated by a treatment with pronase (20 min). The methanol fixed cells were washed, treated with RNase (1 hr) and Pepsin (15 min) and stained with the fluorescent dye, ethidium bromide. A cytologic study of the cell suspension after staining with hematoxylin–eosin showed that more than 90% of the cells isolated were neoplastic cells.

Each DNA histogram represents measurements from 100,000 cells taking about 2 min to complete. From the DNA distribution patterns the fractions of cells in G_1-, S- and (G_2+M)-phase were calculated by a computer program [14].

RESULTS AND DISCUSSION

The influence of an irradiation with a single or fractionated dose of 900 rads on cell cycle progression in the Walker carcinoma is shown in Fig. 1. The DNA distribution patterns were obtained at different times after irradiation. The histograms marked 0 hr refer to the unirradiated controls. The left peak represents cells in G_1 (DNA content 2C), the right peak cells in G_2+M having double the DNA content (4C). The area between the 2 peaks corresponds to S-cells with variable DNA content between 2C and 4C. Twenty-four hr after irradiating cells with a single dose of 900 rads, an accumulation of cells in (G_2+M)-phase can be observed (Fig. 1A), corresponding to smaller fractions of cells in G_1- and S-phase. At a time of 60 hr the fraction of (G_2+M)-cells has decreased, while the fraction of G_1-cells has increased again. Thus, the accumulation of

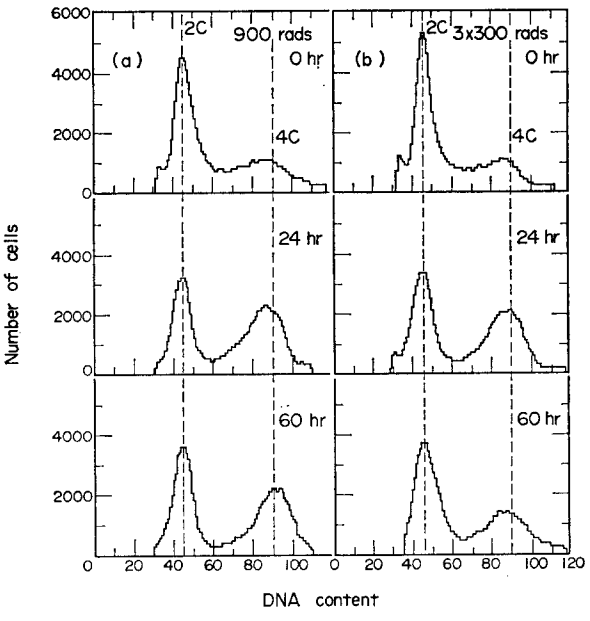


Fig. 1. Pulse-cytophotometric DNA histograms of Walker carcinoma cells 24- and 60-hr after beginning of irradiation. A, irradiation dose 900 rads. B, irradiation dose 3 × 300 rads at 8-hr intervals.

cells in the (G_2+M)-phase seems to be reversible. Analogous results were obtained by fractionating the radiation dose, as is demonstrated in Fig. 1B.

A quantitative evaluation of a series of histograms is set out in Fig. 2, giving the percentage of cells that are contained in the various phases of the cell cycle at different times after irradiation with 300 rads, 900 rads and 3 × 300 rads, respectively. As G_2- and mitotic cells are represented in a single peak in the DNA histograms the mathematical analysis yields the percentages of G_2- and M-cells as a single figure. By substracting the mitotic index we obtained the fraction of G_2-cells. For a dose of 300 rads (Fig. 2A) the G_2-curve shows a maximum of 32% at 10 hr after irradiation. Eight point five hr after irradiation there is a minimum in the G_1-curve. In the following hours the fraction of G_1-cells increases again and becomes greater than in the controls, showing that the accumulation of cells in G_2 is reversible and that the cells are leaving the G_2-phase partly synchronized. This is also confirmed by a maximum of the M-curve 12 hr after irradiation. The reversible accumulation of cells in the G_2-phase is more pronounced after an irradiation dose of 900 rads (Fig. 2B). The G_2-curve shows two maxima, one maximum of 44% 12 hr after irradiation and a second maximum of 48% 40 hr after irradiation. The maximal accumulation of cells in G_2 is reached at later time than after irradiation with the

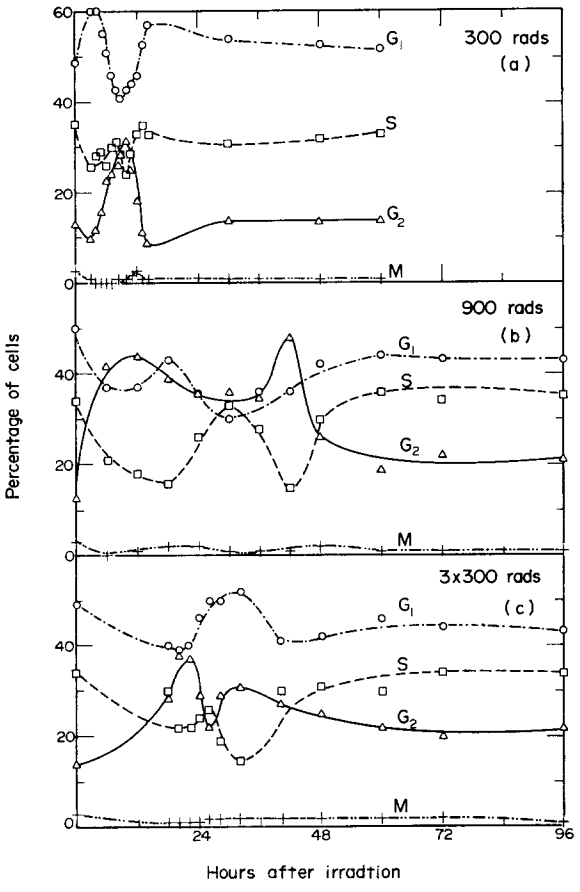


Fig. 2. Percentage of cells in the various phases of the cell cycle at different times after irradiation. A, irradiation dose 300 rads. B, irradiation dose 900 rads. C, irradiation dose 3 x 300 rads at 8-hr intervals. G_1(○)–·–, S (□)– – –, G_2 (△)——, M (+) –··–.

lower dose. At 18 hr after irradiation there is a maximum in the G_1-curve showing that the cells are leaving the G_2-phase partly synchronized. At 96 hr after irradiation the fraction of cells with a DNA content of 4C is 21% and still higher than the control value of 13%. Whether this greater percentage of cells with increased DNA content is caused by diploid G_2-cells or tetraploid G_1-cells from endomitosis can not be decided from pulse-cytophotometric measurements.

Fractionated treatment with 3 x 300 rads has a smaller effect on cell cycle progression (Fig. 2C) than one single dose. Twenty hr after the first irradiation, 38% of cells are accumulated in the G_2-phase. After a second maximum of 31% 32 hr after irradiation, the fraction of G_2-cells decreases. A higher percentage of cells with a 4C DNA content, however, can be seen up to 96 hr after irradiation.

The effect of different radiation doses is demonstrated in Fig. 3, where the percentage of cells in the various phases of the cell cycle

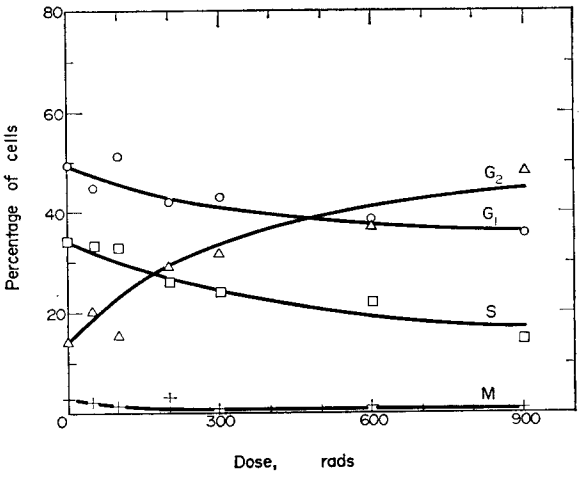


Fig. 3. Percentage of cells in the various phases of the cell cycle at the time of maximum G_2 arrest as function of irradiation dose. G_1 (○), S (□), G_2 (△), M (+).

at the time of maximum G_2 arrest is plotted versus radiation dose. An increasing accumulation of cells in the G_2-phase is observed with increasing radiation dose and simultaneously a decrease of the fraction of G_1-cells as well as of S-cells.

In previous investigations [9, 12] we found that the radiation-induced accumulation of L-cells in the G_2-phase could be considerably increased by repetitive irradiation. In the Walker carcinoma repetitive irradiation had only a slightly higher effect than a single dose. For example, after irradiation with a dose of 300 rads 32% of cells are accumulated in the G_2-phase while after irradiation with 3 x 300 rads at 8 hr intervals 38% of cells are arrested in this phase. The effect of the split dose technique is however smaller than the effect of a single irradiation with the total dose of 900 rads which leads to an accumulation of 48% of cells in the G_2-phase.

The highest degree of *in vivo* synchrony reported thus far had been reached using hydroxyurea as a synchronizing agent. The results of Rajewsky [5], obtained with this method, have been cited above. Evidence for partial cell synchrony in C_3H mouse mammary tumours upon recovery from hydroxyurea inhibition was shown by Dethlefsen and Riley [15]. Gillette *et al.* [16] studied the synchronization of epithelial cells of mouse small intestine after intraperitoneal application of hydroxyurea. The authors observed two waves of S-cells at 8 hr intervals each corresponding to about 50% of the total cell number. A partial synchronization of human malignant melanoma cells *in vivo* was reached by Barranco *et al.* [17] using low doses of Bleomycin.

We have shown that irradiation of the Walker carcinoma with a dose of 900 rads caused a reversible accumulation of about 50% of cells in the G_2-phase. These results are consistent with those of Raju *et al.* [18], who irradiated KHT tumour cells in mice. These authors used two X-ray doses of 300 rads and 3500 rads. After irradiation with 300 rads, the accumulation of cells in G_2-phase was reversible while after treatment with 3500 rads most of the cells were blocked indefinitely. An accumulation of ascites tumour cells in G_2-phase was shown in a cytophotometric study of Frindel *et al.* [19]. After a continuous irradiation of 40 hr, the total dose being 2000 rads of ^{60}Co-γ-rays, 61% of the cells were in G_2-phase versus 5% in the controls.

There is no generally accepted criterion to specify the degree of synchony in *in vivo* studies. Rajewsky [5] used the difference between the peak value and the subsequent minimum value of the labelling index curve and obtained for the BICR/M1R rat carcinoma a value of 0·5. Applying this criterion to the second maximum of the G_2-curve obtained after irradiation with a single dose of 900 rads (Fig. 2B) we calculated a degree of synchrony of 0·3. A comparison of these values is somewhat doubtful, as different techniques (autoradiography and pulse-cytophotometry respectively) had been used to characterize the changes in cell kinetics induced by the synchronizing agents. However, a comparison of cell cycle parameters of L-cells determined by autoradiography and pulse-cytophotometry demonstrated that the results of the two methods were within the frame of statistical error at $P = 0{\cdot}05$ [20]. Our unpublished results show strong agreement of the two methods also for the Walker carcinoma cells *in vivo*. These findings confirm the validity of the pulse-cytophotometric method.

It should be pointed out, that the cells accumulated in G_2 after X-ray irradiation will contain a fraction of "doomed cells" having a limited proliferation capacity. Hermens and Barendsen [21] showed that after irradiation of a rat rhabdomyosarcoma with 2000 rads of X-rays the fraction of cells having retained the capacity for unlimited proliferation was only 0·008. "Doomed Cells" which are incapable of forming viable clones may well continue cell division for two or three cycles. These cells are included in our analysis. This might be taken as a slight disadvantage of the pulse-cytophotometric method compared with the method of time-lapse photography which yields pedigrees of clones after irradiation [22]. The clonogenic tumour cells are the only ones that are of significance for therapeutic studies. Thus, for therapeutic applications pulse-cytophotometric studies should be supported by the determination of the clonogenecity of the tumour cells.

From our data we may conclude that it is possible to obtain a partial synchronization of the solid Walker carcinoma *in vivo* by treatment with single or fractionated doses of X rays. As the effect on cell progression and also the damage to the cells is restricted to the radiation field synchronization of tumours by X rays has some advantages compared with the chemical treatments. Thus, synchronization by radiation might well become a useful tool in human tumour "synchronization therapy".

Acknowledgements—We thank Mrs. D. Lensch, Mrs. H. Winter and Miss H. Kölling for careful technical assistance.

REFERENCES

1. H. R. Nitze, U. Ganzer and K.-H. Vosteen, Die Strahlenbehandlung maligner Tumoren nach Synchronisation des Zellteilungsrhythmus. Erfahrungen nach zweijähriger klinischer Anwendung. *Strahlentherapie* **143,** 329 (1972).
2. H. O. Klein, K. J. Lennartz, R. Gross, M. Eder and R. Fischer, *In-vivo*- und *In vitro*-Untersuchungen zur Zellkinetik und Synchronisation menschlicher Tumorzellen. Ihre Bedeutung für die zytostatische Tumortherapie. *Dtsch. med. Wschr.* **97,** 1273 (1972).
3. M. Wannenmacher, E. Esser, J. Glupe and J. Schumann, Klinische und experimentelle Untersuchungen zur Strahlenbehandlung inoperabler Tumoren nach Teilsynchronisation. *Strahlentherapie* **147,** 1 (1974).
4. A. H. W. Nias and M. Fox, Synchronization of mammalian cells with respect to the mitotic cycle. *Cell Tiss. Kinet.* **4,** 375 (1971).
5. M. F. Rajewsky, Synchronisation *in vivo*: Kinetics of a malignant cell system following temporary inhibition of DNA synthesis with hydroxyurea. *Exp. Cell Res.* **60,** 269 (1970).
6. M. M. Elkind, H. Sutton and W. E. Moses, Postirradiation survival kinetics of mammalian cells grown in culture. *J. cell. comp. Physiol.* **58,** suppl. 1, 113 (1961).

7. R. F. KALLMAN, G. SILINI and H. M. TAYLOR III, Recuperation from lethal injury by whole-body irradiation. II. Kinetic aspects in radiosensitive BALB/c mice, and cyclic fine structure during the four days after conditioning irradiation. *Radiat. Res.* **29,** 362 (1966).
8. D. BOOTSMA, Mitotic delay of human cells in tissue culture irradiated at different phases of the generation cycle. *Progr. Radiol.* **2,** 1404 (1967).
9. W. A. LINDEN, H. PRÉVÔT JR., C. SCHNEIDER, H. BERG and A. LÜGGER, Synchronisation des Teilungszyklus von Säugetierzellen *in vitro* durch fraktionierte Röntgenbestrahlung. *Strahlentherapie* **140,** 706 (1970).
10. W. GÖHDE, Automation of cytofluorometry by use of the impulsemicrophotometer. In *Fluorescence Techniques in Cell Biology* (Edited by A. A. THAER and M. SERNETZ) p. 79. Springer-Verlag, Berlin, Heidelberg, New York (1973).
11. W. GÖHDE and W. DITTRICH, Impulsfluorometrie—ein neuartiges Durchflußverfahren zur ultraschnellen Mengenbestimmung von Zellinhaltsstoffen. *Acta histochem. (Jena)* **10,** 429 (1971).
12. W. A. LINDEN, F. ZYWIETZ, H. LANDEN and C. WENDT, Synchronisation des Teilungszyklus von L-Zellen in der G_2-Phase durch fraktionierte Röntgenbestrahlung und Daunomycin. Quantitative Bestimmung des Synchronisationsgrades mit dem Impulscytophotometer. *Strahlentherapie* **146,** 216 (1973).
13. F. ZYWIETZ, S. WEHBE, W. A. LINDEN, H. BAISCH and J. STRAATMANN, Impulscytophotometrische Untersuchungen zur Synchronisation des Walker-Karzinoms der Ratte mit Daunomycin. *Strahlentherapie* **147,** 514 (1974).
14. H. BAISCH, W. GÖHDE and W. A. LINDEN, Analysis of PCP-data to determine the fraction of cells in the various phases of cell cycle. *Rad. environ. Biophys.* In press (1975).
15. L. A. DETHLEFSEN and R. M. RILEY, Hydroxyurea effects in the C_3H mouse. II. Mammary Tumor Cell Kinetics. *Cell Tiss. Kinet.* **6,** 173 (1973).
16. E. L. GILLETTE, H. R. WITHERS and J. F. TANNOCK, The age sensitivity of epithelial cells of mouse small intestine. *Radiology* **96,** 639 (1970).
17. S. C. BARRANCO, J. K. LUCE, M. M. ROMSDAHL and R. M. HUMPHREY, Bleomycin as a Possible Synchronizing Agent for Human Tumor Cells *in vivo*. *Cancer Res.* **33,** 882 (1973).
18. M. R. RAJU, T. T. TRUJILLO, P. F. MULLANEY, A. ROMERO, J. A. STEINKAMP and R. A. WALTERS, The distribution in the cell cycle of normal cells and of irradiated tumour cells in mice. *Brit. J. Radiol.* **47,** 405 (1974).
19. E. FRINDEL, G. M. HAHN, D. ROBAGLIA and M. TUBIANA, Responses of Bone Marrow and Tumor Cells to Acute and Protracted Irradiation. *Cancer Res.* **32,** 2096 (1972).
20. S. B. REDDY, W. ERBE, W. A. LINDEN, H. LANDEN and C. BAIGENT, Die Dauer der Phasen im Zellzyklus von L-929-Zellen. Vergleich von impulscytophotometrischen und autoradiographischen Messungen. *Biophysik* **10,** 45 (1973).
21. A. F. HERMENS and G. W. BARENDSEN, Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after X-irradiation. *Europ. J. Cancer* **5,** 173 (1969).
22. L. H. THOMPSON and H.D. SUIT, Proliferation kinetics of X-irradiated mouse L cells studied with time-lapse photography. I. Experimental methods and data analysis. *Int. J. Radiat. Biol.* **13,** 391 (1967).

Europ. J. Cancer Vol. 11, pp. 425–431. Pergamon Press 1975. Printed in Great Britain

Resting Stages of the JB-1 Ascites Tumour in the Irradiated Mouse*

P. BICHEL and P. DOMBERNOWSKY

The Institute of Cancer Research, Radiumstationen, Aarhus, Denmark, and the Finsen Institute, Department of Internal Medicine, Copenhagen, Denmark

Abstract—*In previous investigations of the JB-1 ascites tumour we demonstrated increasing fractions of resting cells with G_1 DNA content (Q_1 cells) as well as of resting cells with G_2 DNA content (Q_2 cells) with increasing age and mass of the tumour.*

The present study of the JB-1 ascites tumour in immuno-suppressed mice (subjected to 400-rad whole-body irradiation 24 hr before inoculation) was undertaken in order to investigate the role of a possible immune reaction against the tumour cells for the accumulation of these Q_1 and Q_2 cells.

The experimental data, viz. growth curve, and continuous labelling experiments combined with cytophotometry, showed, first, identical growth of the tumours in the irradiated and non-irradiated mice and, secondly, preserved accumulation of Q_1 and Q_2 cells in the irradiated mice. These non-cycling cells re-entered the cell cycle after removal of most of the tumour in the plateau phase, or after transplantation of cells from plateau tumours to new hosts.

The present results, which are identical with those previously obtained with the tumour carried by normal mice, lend further support to the assumption that the accumulation of non-cycling cells in the ageing JB-1 ascites tumour is of a non-immunological nature.

INTRODUCTION

THE GROWTH rate of most ascites tumours decreases with increasing tumour mass and time after inoculation [1–10]. During a study of this phenomenon in the JB-1 ascites tumour we observed a considerable fraction of resting cells with G_1 DNA content as well as of resting cells with G_2 DNA content (Q_1 and Q_2 cells) [10, 11].

In the Ehrlich ascites tumour, DeCosse and Gelfant [12] likewise observed non-cycling G_1 and G_2 cells. After treatment with anti-lymphocyte serum [13] or hydrocortisone [14] non-cycling G_2 cells were immediately released into mitosis, and the authors suggested that some non-cycling cells may be restrained by immune inhibition.

Most, if not all, experimental tumours carry tumour-specific antigens [15, 16] capable of eliciting specific immune responses in the primary host or when transplanted to isogenic hosts; as a result of this reaction tumour growth may be retarded [17].

Although, since its spontaneous appearance in 1950 [18], the JB-1 ascites tumour has been maintained in the syngeneic host, the tumour-host relationship may have changed, giving rise to minor antigenic differences. In order to study the role of the possible antigenicity of the JB-1 tumour in the syngeneic AKR/Aa strain for the accumulation of resting cells we decided to examine the growth curve and the

Accepted 28 January 1975

*This investigation was supported by grants from the Daell Foundation, the Danish Cancer Society, The Danish Hospital Foundation for Medical Research, Region of Copenhagen, the Danish Medical Research Council, the Hasselbalch Foundation and the Michaelsen Foundation.

resting stages in the plateau phase of JB-1 ascites tumours carried by mice whose immune mechanisms were suppressed by sublethal whole-body irradiation.

MATERIAL AND METHODS

The JB-1 ascites tumour used in the present study is a hypotetraploid transplantable plasmacytoma maintained syngeneically [19] in inbred AKR/Aa mice by i.p. injection of 5×10^6 tumour cells every ninth or tenth day. The mice used in the experiments were 6–10 weeks old.

Except for the retransplantation experiments in which the mice received 100×10^6 JB-1 cells, all experimental animals used were inoculated with $2{\cdot}5 \times 10^6$ tumour cells. Twenty-four hr before inoculation the irradiated mice received a single whole-body dose of 400 rads from a Cobolt source.

To establish the tumour growth curve, mice were killed by cervical dislocation on days 4, 7 and 10 after inoculation, and the total number of ascites tumour cells per mouse was determined according to the method of Klein and Révész [2].

Labelling of the tumour cells was performed by i.p. injection of tritiated thymidine, ^{3}H-TdR (specific activity 2·0 Ci/mM, the Radiochemical Centre, Amersham, England). The amount of ^{3}H-TdR and the time of injection are reported in detail in the appropriate sections on the results.

Serial aspirations of less than 0·05 ml ascitic fluid were performed at short intervals and prepared for autoradiography. The tumour cells were smeared on cleaned glass slides coated with gelatine and fixed for 1 hr in a mixture of 85% methanol, 10% formalin and 5% acetic acid. The autoradiographs were covered with Ilford K2 liquid emulsion diluted 1:1, exposed in the dark for 10 days at 4°C, and developed with D 170 amidol developer.

An error in the experimental work is the grain limit of 3 or more grains used in the evaluation of the autoradiographs. This grain limit is low and only slightly above the mean background (average 1–2 grains). Consequently, the experimental error is an overestimation of the percentage of labelled cells and an underestimation of the percentage of non-cycling cells.

The fraction of labelled cells was determined in 1000 tumour cells per mouse, and the number of labelled mitoses was determined from 100 mitotic cells per mouse. The mitotic index (MI) (late prophase to early telophase) was obtained by counting 1000 tumour cells on smears stained with May–Grünwald–Giemsa.

Single-cell DNA content was determined by means of a scanning microscope-photometer 05 (Cytoscan, C. Zeiss, Oberkochen, West Germany) on Feulgen-stained tumour cells as previously described [10]. In all tumours, about 100 consecutive unlabelled ($\leqq 3$ grains) cells were measured.

The number of lymphocytes per μl was determined in blood from a tail vein by counting nucleated cells in a haemocytometer followed by differential counting on smears stained with May–Grünwald–Giemsa.

The Wilcoxon two-tailed test [20] for two samples was used in the statistical evaluation.

RESULTS

Radiation effect on circulating lymphocytes

Figure 1 shows the variation in the number of lymphocytes per μl tail blood in normal and tumour-bearing mice, with time after irradiation compared with controls. A marked depression in the number of circulating lymphocytes was evident in the irradiated mice on both days as compared with the non-irradiated mice.

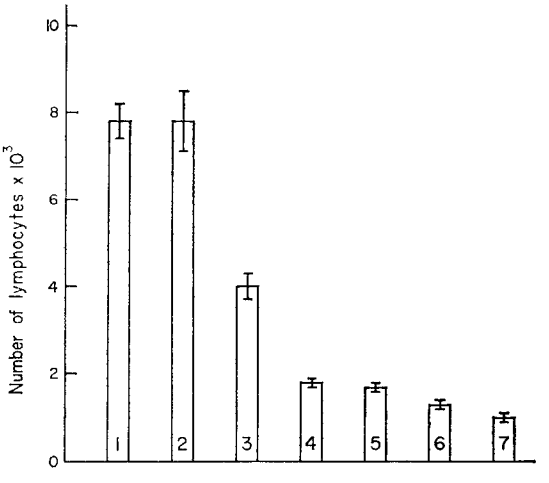


Fig. 1. *Number of lymphocytes per* μl *tail blood. Mean of* 5 *mice.*

No. 1. *Non-irradiated mice.*

No. 2. *Non-irradiated mice bearing* 6*-day JB-*1 *ascites tumours.*

No. 3. *Non-irradiated mice bearing* 10*-day JB-*1 *ascites tumours.*

No. 4. *Non-tumour bearing mice* 7 *days after* 400 *rad.*

No. 5. *Non-tumour bearing mice* 11 *days after* 400 *rad.*

No. 6. *Six-day JB-*1 *ascites tumours,* 7 *days after* 400 *rad.*

No. 7. *Ten-day JB-*1 *ascites tumours,* 11 *days after* 400 *rad.*

Vectors represent standard error of the mean.

The growth curve

Four, 7 and 10 days after i.p. inoculation of $2{\cdot}5 \times 10^6$ JB-1 cells the total number of ascites tumour cells was determined in 10 irradiated and 10 non-irradiated mice.

It appears from Table 1 that the total i.p. number of JB-1 cells increased nearly identically in the two groups ($P > 0{\cdot}1$) and day 10 after inoculation reached values of 510×10^6 and 542×10^6, respectively.

Table 1. *Number of i.p. JB-1 cells* $\times 10^6 \pm$ *S.D. in irradiated* (400 *rad whole-body dose given* 24 *hr before transplantation) and non-irradiated mice after inoculation of* $2{\cdot}5 \times 10^6$ *tumour cells. Mean of* 10 *mice*

	Days after inoculation		
	4	7	10
Irradiated mice	103 ± 8	298 ± 41	510 ± 67
Non-irradiated mice	95 ± 6	325 ± 59	542 ± 63

Table 2. *Results of microspectrophotometry of unlabelled tumour cells in the plateau stage after labelling with* ^{3}*H-TdR every fourth hr for* 24 *hr. Percentage of labelled cells and MI are from the same tumours.*

Hr after start of the experiment	Mouse No.	No. of unlabelled cells measured	Percentage distribution			
			$G_1 + Q_1$	Per cent labelled cells	$G_2 + Q_2$	MI%
0	1	98	51.1	31.5	16.6	0.8
	2	102	57.0	31.7	9.8	1.5
	3	97	45.5	30.4	23.4	0.7
Total		297	$51.2^{+}_{-}3.3$	$31.2^{+}_{-}0.4$	$16.6^{+}_{-}3.9$	$1.0^{+}_{-}0.3$
8	1	100	45.7	39.1	15.2	1.0
	2	100	52.2	40.0	7.8	1.0
	3	105	34.9	48.4	16.7	1.5
Total		305	$44.3^{+}_{-}5.0$	$42.5^{+}_{-}3.0$	$13.2^{+}_{-}2.8$	$1.2^{+}_{-}0.2$
24	1	104	48.0	39.8	12.2	1.0
	2	102	37.7	51.3	11.0	1.3
	3	102	40.9	44.4	14.7	0.8
Total		308	$42.2^{+}_{-}3.0$	$45.2^{+}_{-}3.3$	$12.6^{+}_{-}1.1$	$1.0^{+}_{-}0.1$

Non-cycling cells in the plateau stage

Ten days after inoculation a continuous labelling experiment was initiated. The mice received i.p. injections of ^{3}H-TdR (0·3 μCi/g body weight) every 4 hr for 24 hr. Fifteen min after the injections, at 0, 8 and 24 hr, tumour cells were prepared for autoradiography, and the percentage of labelled cells was scored ($30{\cdot}9 \pm 2{\cdot}9$, $39{\cdot}2 \pm 2{\cdot}8$ and $42{\cdot}2 \pm 1{\cdot}8$ per cent, respectively). The relative amounts of nuclear DNA in unlabelled tumour cells were determined by cytophotometry at 0, 8 and 24 hr after the initiation of continuous labelling in three of these mice. Table 2 shows the number of unlabelled tumour cells measured in the individual tumours, the percentage distribution of labelled cells, unlabelled cells in $G_1 + Q_1$ and $G_2 + Q_2$, respectively, and the mitotic index. It appears from the table that even after 24 hr continuous labelling, 42·2 and 12·6 per cent of all cells remained unlabelled with G_1 and G_2 DNA content, respectively.

Non-cycling cells in recurrent growth

As much as possible of the tumour was aspirated from 6 irradiated mice carrying plateau tumours. Twenty-four hr later continuous labelling and cyctophotometric determination of single cell DNA were performed in the same way as in the experiments described under "the plateau stage".

In the continuous labelling experiments, the percentages of labelled cells at 0, 8 and 24 hr were $59{\cdot}8 \pm 2{\cdot}5$, $73{\cdot}2 \pm 2{\cdot}0$ and $88{\cdot}3 \pm 1{\cdot}6$, respectively. Cytophotometry of 3 tumours showed that after 24 hr continuous labelling the amount of unlabelled tumour cells with G_1 and G_2 DNA content comprised only 9·0 and 3·1 per cent, respectively, of all cells (Table 3).

A comparison of Tables 2 and 3 shows that the fraction of unlabelled cells with G_1 and G_2 DNA content after 24 hr of continuous labelling is considerably reduced in the aspirated tumours. This means that the growth fraction

Table 3. Results of microspectrophotometry of unlabelled tumour cells in recurrent growth after labelling with ^{3}H-TdR every fourth hr for 24 hr. Percentage of labelled cells and MI are from the same tumours

Hr after start of the experiment	Mouse No.	No. of unlabelled cells measured	Percentage distribution			
			$G_1 + Q_1$	Per cent labelled cells	$G_2 + Q_2$	MI%
0	1	96	25.5	56.4	16.9	0.8
	2	89	17.4	62.7	18.7	1.2
	3	101	25.8	55.5	17.7	1.0
Total		286	22.9±2.8	58.2±2.3	17.8±0.5	1.0±0.1
8	1	99	21.1	74.2	4.7	1.1
	2	93	20.0	77.9	2.1	1.3
	3	98	25.5	71.3	3.2	0.9
Total		290	22.2±1.7	74.5±1.9	3.3±0.8	1.1±0.1
24	1	95	9.7	87.0	3.3	1.3
	2	78	7.9	89.4	2.7	1.0
	3	95	9.3	87.5	3.2	1.8
Total		268	9.0±0.5	88.0±0.7	3.1±0.2	1.4±0.2

has increased owing to a release of resting cells into the cell cycle [10].

$Q_1 - G_1$ and $Q_2 - G_2$ transition in recurrent growth

After 24 hr of continuous labelling of plateau JB-1 ascites tumours in irradiated mice, as much as possible of the tumours was removed by percutaneous aspiration. At 0, 4, 8 and 24 hr after aspiration tumour cells from these mice were prepared for autoradiography and cytophotometry.

Table 4 shows the cytophotometric results. It appears from the table that immediately after aspiration, 77% of the unlabelled cells had G_1 DNA content, 22% had G_2 DNA content and 1% intermediary DNA content. At 8 and 24 hr after aspiration, the number of unlabelled cells with G_1 DNA content had decreased to 72% and 61%, respectively. At the same times, the number of unlabelled cells in S increased to 9% and 14%, respectively. These results indicate that resting Q_1 cells were released to the cell cycle after aspiration.

Figure 2 shows the MI and the percentage of labelled mitoses as a function of time after

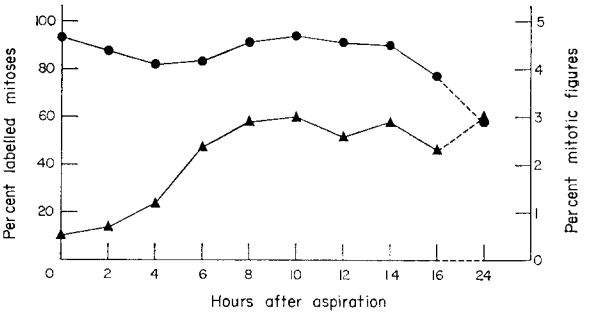


Fig. 2. Percentage of labelled mitoses ●—● and MI ▲—▲ after aspiration from irradiated mice of plateau JB-1 ascites tumours prelabelled with ^{3}H-TdR for 24 hr at 4 hr intervals.

aspiration. It appears from the curve that the MI, after a short latency period, increased to a maximum value of about 3% at 8–10 hr after aspiration. The percentage of labelled mitoses decreased significantly ($P < 0{\cdot}01$) from the initial value of 93% to 82 and 83% at 4 and 6 hr after aspiration. At 10 hr, the percentage of labelled mitoses had reached the initial value, and from 16 to 24 hr after aspiration,

Table 4. Percentage distribution of DNA content in unlabelled cells after aspiration of plateau JB-1 ascites tumours in irradiated mice, prelabelled with ^{3}H-TdR for 24 hr at 4 hr intervals

Hr after aspiration	No. of unlabelled cells measured	Percentage distribution of unlabelled tumour cells			
		$G_1 + Q_1$	S	$G_2 + Q_2$	Per cent labelled cells
0	102	77	1	22	51
4	99	78	2	20	50
8	103	72	9	19	47
24	102	61	14	25	50

Table 5. Percentage distribution of DNA content in unlabelled cells after transplantation to new hosts of plateau JB-1 ascites tumour cells, prelabelled with ^{3}H-TdR in the irradiated donors every fourth hr for 24 hr

Hr after retransplantation	No. of unlabelled cells measured	Percentage distribution of unlabelled tumour cells			Per cent labelled cells
		$G_1 + Q_1$	S	$G_2 + Q_2$	
0	99	80	3	17	63.0
2	100	81	0	19	64.1
4	100	74	7	19	68.5
6	100	78	10	12	59.2
8	95	65	17	18	60.7

a new decrease occurred, viz. to 77 and 58% labelled mitoses, respectively. The shape of the curve indicates a short minor wave of cells released from Q_2 and a major one released from Q_1.

$Q_1 - G_1$ and $Q_2 - G_2$ transition after transplantation

Three irradiated mice carrying plateau JB-1 ascites tumours were given i.p. injections of ^{3}H-TdR (0·3 μCi/g body weight) every 4 hr for 24 hr. Fifteen min after the last injection the tumours were pooled and 100×10^6 cells were transplanted to fresh hosts. Every hr for the following 8 hr, tumour cells were aspirated from the new hosts, prepared for autoradiography and cytophotometry.

Table 5 shows the percentage distribution of unlabelled tumour cells with G_1, S and G_2 DNA content, determined by cytophotometry, as a function of time after transplantation. The marked increase in the number of unlabelled cells with S DNA content from 4 to 8 hr after transplantation at the extent of unlabelled cells with G_1 DNA content indicates a release of resting cells into the cell cycle from a resting Q_1 compartment.

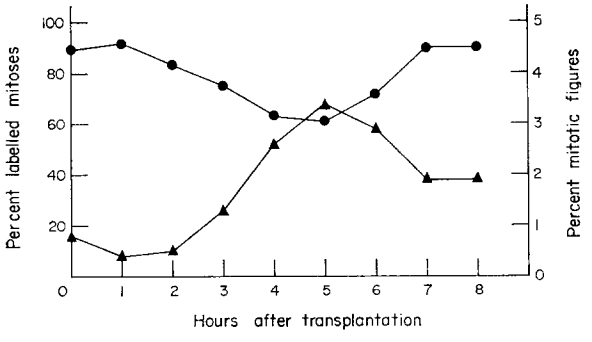


Fig. 3. Percentage of labelled mitoses ●—● and MI ▲—▲ after transplantation to new hosts of plateau JB-1 ascites tumours prelabelled with ^{3}H-TdR in the irradiated donors for 24 hr at 4 hr intervals.

Figure 3 shows the MI and the percentage of labelled mitoses as a function of time after transplantation. A considerable increase is apparent in the mitotic index from 3 to 6 hr while at the same time the percentage of labelled mitoses shows a significant ($P < 0{\cdot}01$) decrease, indicating a release of Q_2 cells into the cell cycle.

DISCUSSION

In recent years it has been demonstrated conclusively that most experimental tumours possess specific antigens which may evoke immune responses in the primary host as well as in isogenic animals upon transplantation [15, 16]; it has also been reported that when mice were subjected to whole-body irradiation prior to transplantation a marked acceleration of tumour growth occurred [17].

In the ELD Ehrlich ascites tumour, DeCosse and Gelfant [13, 14] demonstrated that after continuous labelling for a period exceeding the cell-generation time some tumour cells remained unlabelled. Addition of antilymphocyte serum [14] or hydrocortisone [15] to these tumour-bearing mice was rapidly followed by the occurrence of unlabelled mitotic cells, and the authors concluded that some non-cycling tumour cells were probably restrained by immune inhibition.

In our previous studies on the JB-1 ascites tumour we observed that with increasing age and decreasing growth rate of the tumour an increasing proportion of the tumour-cell population was transferred to resting compartments (Q_1 and Q_2) corresponding to the G_1 and the G_2 phase [10]. A release of both Q_1 and Q_2 cells to the cell cycle was observed after (a) transplantation of cells from plateau tumours to new hosts [11], (b) aspiration of most of the tumours from mice bearing JB-1 ascites tumours

in the plateau phase of growth [21], and (c) cytostatic treatment of plateau tumours [22].

In order to find out whether an immune reaction against the tumour cells plays any significant role in the decelerating growth and the accumulation of resting cells in the JB-1/AKR tumour–host system, we studied the growth of the tumour carried by mice whose immunological defence mechanisms had been impaired by sublethal irradiation.

A number of studies in mice have shown that a whole-body dose of 400 rads given 24 hr before sensitization results in a severe depression of the immune competence which lasts from 3 to 5 weeks after irradiation [23–27]. It has, furthermore, been demonstrated that maximum suppression of the immune response is obtained when the antigen is injected about 24 hr after irradiation [27]. Immune reactions to tumour-specific antigens have recently been investigated by comparing the growth of antigenic tumours transplanted to intact animals and to animals made immunologically deficient by whole-body exposure to X-rays [17]. It was found that when irradiation preceded transplantation of the mice with tumour cells known to be antigenic by 24 hr, tumour growth increased with the amount of radiation up to 300 rads. Higher doses of radiation did not produce further increases in tumour growth. Even 4 months after irradiation some acceleration was noted [17].

In the present study, we observed a parallel increase in the number of i.p. tumour cells in the irradiated and non-irradiated mice after inoculation of $2{\cdot}5 \times 10^6$ JB-1 cells. Furthermore, we observed that in the plateau tumours a considerable number of tumour cells with both G_1 and G_2 DNA content remained unlabelled after 24 hr continuous labelling, which is the mean of $T_{G2} + 3$ S.D. calculated from the percentage labelled mitoses curve [28].

Twenty-four hr after aspiration, i.e. in the regenerative or recurrent phase of growth, a large number of non-cycling cells had returned to the cell cycle and participated in the cell division. Investigations during the initial 24 hr after aspiration and immediately after transplanting cells from plateau tumours to new hosts revealed that cells from both Q_1 and Q_2 were released into the cell cycle under these circumstances. The re-entry of these cells into the cell cycle indicates that they are true resting cells.

The strong agreement between the growth patterns from irradiated and non-irradiated mice [10, 11, 21], together with the observation that the G_1 and G_2 blocking substances found in the old cell-free JB-1 ascitic fluid have molecular weights below 50,000 Daltons [29] suggests that the decelerating growth of the ageing tumour and the restraint of cells in the cell cycle in our system are at least partially of a non-immunological nature.

The results from our previous investigations of the JB-1 ascites tumour in normal mice [10, 11, 19, 21, 22] suggest that the growth of the tumour is regulated by a negative feedback mechanism, and the present study indirectly supports this conception.

Acknowledgements—The excellent technical assistance of Miss J. Andersen, Mrs. N. Arnkjaer and Mrs. J. Olsen is gratefully acknowledged.

REFERENCES

1. H. M. Patt, M. E. Blackford and J. L. Drallmeier, Growth characteristics of the Krebs ascites tumor. *Proc. Soc. exp. Biol. (N.Y.)* **83,** 520 (1953).
2. G. Klein and L. Révész, Quantitative studies on the multiplication of neoplastic cells *in vivo*. I. Growth curves of the Ehrlich and MC1M ascites tumors. *J. nat. Cancer Inst.* **14,** 229 (1953).
3. P. K. Lala and H. M. Patt, Cytokinetic analysis of tumor growth. *Proc. nat. Acad. Sci. (Wash.)* **56,** 1735 (1966).
4. E. R. Burns, Initiation of DNA synthesis in Ehrlich ascites tumor cells in their plateau phase of growth. *Cancer Res.* **28,** 1191 (1968).
5. I. F. Tannock, A comparison of cell proliferation parameters in solid and ascites Ehrlich tumors. *Cancer Res.* **29,** 1527 (1969).
6. E. Frindel, A. J. Valleron, F. Vassort and M. Tubiana, Proliferation kinetics of an experimental ascites tumour of the mouse. *Cell. Tiss. Kinet.* **2,** 51 (1969).
7. L. Simpson-Herren and H. H. Lloyd, Kinetic parameters and growth curves for experimental tumor systems. *Cancer Chemother. Rep.* **54,** 143 (1970).
8. K. G. Hofer and M. Hofer, Kinetics of proliferation, migration, and death of L1210 ascites cells. *Cancer Res.* **31,** 402 (1971).

9. P. Dombernowsky and N. R. Hartmann, Analysis of variations in the cell population kinetics with tumor age in the L1210 ascites tumor. *Cancer Res.* **32,** 2452 (1972).
10. P. Dombernowsky, P. Bichel and N. R. Hartmann, Cytokinetic analysis of the JB-1 ascites tumour at different stages of growth. *Cell Tiss. Kinet.* **6,** 347 (1973).
11. P. Bichel and P. Dombernowsky, On the resting stages of the JB-1 ascites tumour. *Cell Tiss. Kinet.* **6,** 359 (1973).
12. J. DeCosse and S. Gelfant, Demonstration of subpopulations in ascites tumor cells. *Proc. Amer. Ass. Cancer Res.* **7,** 17 (1966).
13. J. DeCosse and S. Gelfant, Noncycling tumor cells: mitogenic response to antilymphocytic serum. *Science* **162,** 698 (1968).
14. J. DeCosse and S. Gelfant, Effect of hydrocortisone on cell division of tumor cells. *Surg. Forum* **18,** 80 (1967).
15. G. Klein, Tumor antigens. *Ann. Rev. Microbiol.* **20,** 223 (1966).
16. L. J. Old and E. A. Boyse, Immunology of experimental tumors. *Ann. Rev. Med.* **15,** 167 (1964).
17. W. Rosenau and H. D. Moon, Suppression of the immune response to antigenic tumors in isogenic mice by whole-body irradiation. *Cancer Res.* **27,** 1973 (1967).
18. J. Bichel, A transplantable plasma cell leukemia in mice. *Acta path. microbiol. scand.* **29,** 464 (1951).
19. P. Bichel, Autoregulation of ascites tumour growth by inhibition of the G-1 and the G-2 phase. *Europ. J Cancer* **7,** 349 (1971)
20. Documenta Geigy—Scientific Tables. Edited by Geighy, Geighy, Basel (1963).
21. P. Dombernowsky, P. Bichel and N. R. Hartmann, Cytokinetic studies of the regenerative phase in the JB-1 ascites tumour. *Cell Tiss. Kinet.* **7,** 47 (1974).
22. P. Dombernowsky and P. Bichel, Recycling of resting cells after cytostatic treatment. *Abstracts XIth Int. Cancer Congr.*, Florence 1974, p. 421.
23. P. P. H. De Bruyn, Lymph node and intestinal lymphatic tissue. In *Histopathology of Irradiation* (Edited by W. Bloom), p. 348. McGraw-Hill, New York (1948).
24. M. S. Silverman and P. H. Chin, The effect of whole body X-irradiation of mice on immunity to tetanus toxoid. II. The delayed immune response to injections of tetanus toxoid. *J. Immunol.* **77,** 266 (1956).
25. T. Makinodan and N. Gengozian, Primary antibody response to a distantly related heterologous antigen during maximum depression period after varying doses of X-radiation. *J. Immunol.* **81,** 150 (1958).
26. H. S. Micklem and J. A. H. Brown, Rejection of skin grafts and production of specific iso-haemagglutinins by normal and X-irradiated mice. *Immunology* **4,** 318 (1961).
27. W. H. Taliaferro, L. G. Taliaferro and B. N. Jaroslow, *Radiation and Immune Mechanisms.* Academic Press, N.Y. and London (1964).
28. M. Takahashi, Theoretical basis for cell cycle analysis. I. Labelled mitosis wave method. *J. theoret. Biol.* **13,** 202 (1966).
29. P. Bichel, Further studies on the self-limitation of growth of JB-1 ascites tumours. *Europ. J. Cancer* **9,** 133 (1973).

Europ. J. Cancer Vol. 11, pp. 433–441. Pergamon Press 1975. Printed in Great Britain

Differential Effects of Two Chemotherapeutic Agents, Streptozotocin and Chlorozotocin, on the Mammalian Cell Cycle*

ROBERT A. TOBEY†, MELVIN S. OKA‡ and HARRY A. CRISSMAN‡

†*Cellular and Molecular Radiobiology Group*
‡*Biophysics and Instrumentation Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico* 87544 *U.S.A.*

Abstract—*Streptozotocin and chlorozotocin were examined for effects on survival and cell-cycle progression in exponentially growing (cycling) populations of CHO (Chinese hamster) cells and in non-cycling G1-arrested cells. On a weight basis, chlorozotocin was approximately* 40 *times more toxic for non-cycling cells and nearly* 20 *times more toxic for cycling cells in comparison to streptozotocin. While low concentrations of streptozotocin were more cytotoxic to cycling cells as compared to non-cycling cells, non-cycling cells were more sensitive than cycling cells at all concentrations of chlorozotocin examined. Streptozotocin did not affect the rate of traverse through G1 (pre-DNA synthetic phase), produced only a slight prolongation in duration of S phase (DNA-synthetic phase), and caused a delay in progression through G2 (pre-mitotic phase) of approximately* 4 *hr, although most cells ultimately divided. Therefore, populations treated with streptozotocin exhibited a modest increase in the fraction of cells in G2, primarily at the expense of the G1 population. Chlorozotocin, administered at an isosurvival concentration, did not affect rate of traverse of G1, approximately doubled the time required for completion of DNA synthesis, and caused cells to accumulate preferentially within the first* 10 *min of G2, with only a small fraction of cells capable of dividing. These population redistributions were observed whether cells were treated with either agent in G1, G1-arrest, S, or G2. Both compounds induced mitotic non-disjunction and polyploidy. Therefore, even though chlorozotocin is a derivative of streptozotocin, the two compounds elicit very different patterns of kinetic response in susceptible cell populations.*

INTRODUCTION

THE DESIGN of meaningful chemotherapy regimens involves consideration of a number of factors, one of which is the kinetic response of surviving cells following treatment of a susceptible population with one or more agents. Based upon the behavior of cells following primary treatment, additional drugs may be administered which are maximally active against the types of cells encountered in the surviving population. In this manner, knowledge of the kinetic response can be utilized to increase the cell kill capacity.

Accepted 22 January 1975.

*This study was supported by Contract NIH-CR-(71)-56 from the division of Cancer Treatment, National Cancer Institute, N.I.H., under interagency agreement with the U.S. Atomic Energy Commission.

To provide general guideline information on the kinetic response of susceptible cells to treatment with toxic agents, we have designed a model system employing cells in culture which allows us to monitor *in vitro* the cell-cycle kinetics of both cycling and non-cycling treated populations [1, 2]. The advantages of our system include ease and speed of operation and low cost, coupled with *complete cell accountability*. Our intent is to provide information to be used in conjunction with data obtained from other experimental model systems. Data obtained in our system to date with a limited number of compounds for which corresponding clinical data are available suggest that our results generally mimic the response of susceptible tumor cells *in vivo* [1, 2].

In this report, we have investigated the effects of streptozotocin (a 2-deoxy-D-glucose derivative of *N*-methyl-*N*-nitrosourea) and chlorozotocin (chloroethyl-streptozotocin) on the cell cycle. Streptozotocin is reportedly effective in the treatment of pancreatic islet cell carcinomas [3–6] and metastatic carcinoid tumors [4, 7]. Chlorozotocin appeared promising in early trials against experimental tumors; therefore, its properties were investigated and compared to those of the parent compound, streptozotocin. Our results indicate that, at drug concentrations yielding comparable levels of survival, chlorozotocin affects cycle progression to a greater extent than does streptozotocin.

MATERIAL AND METHODS

Cells and survival measurements

Chinese hamster cells (line CHO, originally obtained from Dr. T. T. Puck) were maintained free of contamination with *Mycoplasma* in F-10 medium supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. Assay of survival of both cycling and non-cycling cells was carried out as described previously [2]. Basically, known numbers of cells from exponentially growing suspension cultures (*i.e.*, cycling cells) were placed in plastic tissue culture plates and, after 1·5 hr to permit cell attachment, drug solutions were added. Two hr later, the medium was removed and replaced with drug-free medium. Following incubation for 7 days, the surviving colonies (50 or more cells) were washed, fixed, and stained with crystal violet solution prior to counting.

Non-cycling G1-arrested cells were prepared by transferring cells from an exponentially growing culture to F-10 medium containing suboptimal quantities of isoleucine [8–10]. After 36 hr, the cells accumulated in G1 while retaining viability and remained capable of synthesizing a number of macromolecules without entering a state of gross biochemical imbalance [8–10]; following restoration of isoleucine, these cells resumed proliferation [8–10]. For analysis of survival, the G1-arrested cells in suspension culture were placed in tissue culture plates, allowed to attach, and then exposed to drug for a 2-hr period. The drug-containing medium was then removed and replaced with drug-free medium containing isoleucine, allowing surviving cells to resume proliferation. Colonies were scored as above after a 7-day incubation period. The results presented represent the average of three experiments.

Cell-cycle-traverse measurements

Suspension cultures of either asynchronous (cycling) cells or G1-arrested (non-cycling) cells were treated with appropriate concentrations of drugs for 2 hr, after which the cells were placed in drug-free complete medium. The cell number was determined at this time ($t = 0$) along with an analysis of population DNA content by means of flow microfluorometric (FMF) analysis. Two duplicate cultures were prepared from each dilution, and one culture received ^{3}H-thymidine to a final concentration of 0·05 μCi/ml. The label was maintained in the culture throughout the entire 30 hr of the experiment, after which the fraction of labeled cells (*i.e.*, fraction of cells capable of at least initiating DNA synthesis) was determined via autoradiography. The cell number was determined at $t = 30$ hr to provide an estimate of the fraction of cells which divided during the test period. An aliquot of cells was fixed and stained at 30 hr for determination of population DNA content distribution (*i.e.*, cell-cycle distribution) via FMF analysis. As a supplement to FMF data, at 30 hr an aliquot of cells was pulse-labeled for 15 min with 2 μCi/ml ^{3}H-thymidine, and the labeled fraction was scored autoradiographically to reveal the fraction of cells in S phase at the time of sampling for FMF analysis.

DNA content distribution measurements

Cells for FMF analysis were either dispersed, fixed, and stained with acriflavine [11, 12] or resuspended directly in 25% ethanol containing 15 mM $MgCl_2$ and 100 μg/ml of the fluorescent antibiotic mithramycin [13]. Cell DNA content was determined with the Los Alamos flow microfluorometer [14, 15] utilizing laser wavelength settings of 488 and 457 nm, respectively,

for cells treated with acriflavine and mithramycin.

Chemicals

Both streptozotocin (NSC #85998) and chlorozotocin (NSC #178248) were obtained through Drug Research and Development, Division of Cancer Treatment, National Cancer Institute. Drug solutions were prepared immediately prior to use by dissolving them in serum-free F-10 medium, followed by dilution in the same medium. Methyl-^{3}H-thymidine (6 Ci/mmole) was purchased from Schwarz/Mann, Orangeburg, New York, U.S.A. Supplies of mithramycin were obtained both from the Division of Cancer Treatment, National Cancer Institute, and as a gift, generously provided by Pfizer Laboratories Division, New York, New York, U.S.A.

RESULTS

An essential parameter in any consideration of experimental chemotherapeutic scheduling is the survival capacity of both cycling and non-cycling populations following treatment with test agents. Results of the survival experiments in which cycling (exponentially growing) and non-cycling cells (G1-arrested) were exposed to drugs for 2 hr are illustrated in Fig. 1.

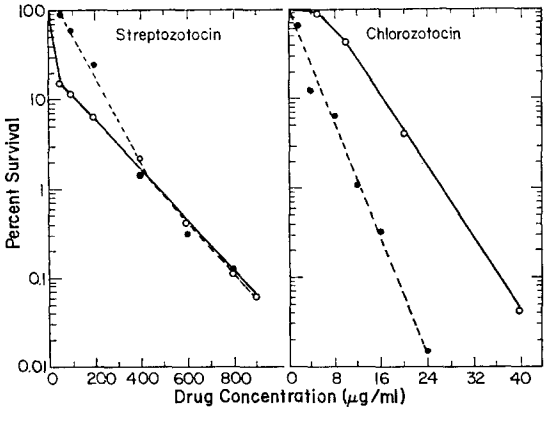


Fig. 1. Effects of streptozotocin and chlorozotocin on survival of cycling CHO cells (open circles) and on non-cycling (G1-arrested) cells returned to cycle following drug removal (solid circles). Cells were exposed to drug for 2 hr, after which fresh drug-free medium was added back, and the plates were incubated for 7 days prior to scoring the surviving colonies. Details are provided in the Material and Methods section.

Low concentrations of streptozotocin were more cytotoxic to cycling cells than to non-cycling cells although, at 400 μg/ml or higher, the survival capabilities of both cycling and non-cycling populations were essentially identical. In contrast, chlorozotocin was much more toxic to CHO cells than streptozotocin, with non-cycling cells more sensitive than cycling cells over the range of drug concentrations investigated. At survival levels of 1% or less, chlorozotocin was approximately 40 times as lethal to non-cycling cells and nearly 20 times as toxic for cycling cells in comparison to streptozotocin.

The effect of streptozotocin and chlorozotocin on cell-cycle progression is presented in Tables 1 and 2. The results include the distribution of cells in different phases derived from FMF measurements, along with the fraction of cells capable of initiating DNA synthesis and the fraction of cells dividing during the 30-hr period after removal of the drug. Note first the dramatic change in cell-cycle distributions in the drug-free control culture at the start and at the conclusion of the experiment utilizing G1-arrested cells returned to a proliferation mode by addition of isoleucine. Because of the naturally occurring loss of synchrony, the cell-cycle distribution after 30 hr is essentially that of an exponentially growing culture, as expected from previous studies [10]. Since cells in isoleucine-limiting medium accumulate in early G1, maximally distant from mitosis, upon return to cycle these cultures do not commence dividing for 12 or 13 hr, and there is only sufficient time for slightly more than one parasynchronous division wave during the 30-hr test period [8, 10].

Examination of the data in Table 1 reveals that the primary effect of streptozotocin on cycling cells is a modest accumulation of cells in G2, primarily at the expense of the G1 cell fraction. Cells from non-cycling populations treated with streptozotocin, then returned to a cycling state, showed a more dramatic preferential accumulation of cells in G2, particularly evident in the culture receiving 800 μg/ml of drug; once again the predominant phase of the cell cycle from which cells were lost during redistribution was the G1 phase. At all concentrations of streptozotocin studied, essentially the entire population was able to initiate DNA synthesis in both cycling and non-cycling populations when the drug was removed. There was a concentration-dependent reduction in the number of dividing cells, although even 800 μg/ml failed to totally inhibit cell division.

The effect of chlorozotocin on both cycling and non-cycling cells (Table 2) was much more dramatic than the effects of streptozotocin. For example, at the highest drug concentrations, nearly all cells accumulated in G2, with

Table 1. *Effect of streptozotocin on CHO cell-cycle progression capacity*

Drug concentration (μg/ml)	% of cells in G1	S	G2	M	Cells initiating DNA synthesis (%)	Cells dividing (%)
Cycling cells						
Control (t = 0)	57	34	6	3	—	—
Control (30 hr)	56	32	10	2	98	150
50	37	37	25	1	98	121
100	29	38	31	2	97	97
200	27	45	27	1	98	89
400	27	42	30	1	98	89
600	25	32	42	1	97	71
800	33	32	34	1	96	33
Non-cycling cells						
Control (t = 0)	97	1	2	0	—	—
Control (30 hr)	59	32	7	2	98	115
50	24	41	34	1	99	88
100	20	41	39	0	98	75
200	27	51	22	0	99	58
400	32	43	25	0	98	55
600	27	30	42	1	99	36
800	16	22	61	1	98	15

Cultures of cycling or non-cycling cells were exposed for 2-hr periods to different concentrations of streptozotocin, then resuspended in complete drug-free medium containing low levels of ^{3}H-thymidine for 30 hr. The cell number increase and percentage of cells incorporating ^{3}H-thymidine into DNA and scored autoradiographically were determined along with cell-cycle distributions by standard FMF analysis by the acriflavine–Feulgen procedure. Further details are given in the Material and Methods section.

Table 2. *Effect of chlorozotocin on CHO cell-cycle progression capacity*

Drug concentration (μg/ml)	% of cells in G1	S	G2	M	Cells initiating DNA synthesis (%)	Cells dividing (%)
Cycling cells						
Control (t = 0)	46	39	13	2	—	—
Control (30 hr)	55	26	17	2	98	198
4	48	29	21	2	99	130
8	33	23	41	3	99	69
12	20	30	48	2	97	46
16	14	19	66	1	98	30
24	12	16	71	1	97	20
32	7	23	69	1	98	14
Non-cycling cells						
Control (t = 0)	95	<1	5	0	—	—
Control (30 hr)	47	35	15	3	99	125
4	40	30	28	2	99	67
8	21	20	55	4	99	16
12	4	13	81	2	99	<1
16	<1	14	86	0	99	<1
24	<1	7	93	0	99	<1
32	<1	7	93	0	99	<1

Details are as in Table 1.

both the G1 and S fractions of cells grossly depleted. Traverse from G1 to S (*i.e.*, ability to initiate DNA synthesis) once again was not affected by chlorozotocin. In initially non-cycling populations, none of the cells in the cultures receiving 12 μg/ml or more of chlorozotocin were able to traverse to mitosis during the test period. In cycling populations treated with high concentrations of chlorozotocin, only cells in G2 and M (mitotic phase) at the time of drug addition were able to divide (*i.e.*, 14–20% divided in cultures receiving 24–32 μg/ml of chlorozotocin, compared to 15–19% of cells in G2 and M in the control cultures).

Additional information can be derived from direct examination of the DNA distribution patterns obtained by FMF analysis. For example in Fig. 2 are the DNA distribution patterns of an exponentially growing culture and the same culture after treatment with 8 μg/ml of chlorozotocin for 2 hr, followed by incubation for 30 hr in drug-free medium. The peak of the lower DNA content (scale value of approximately 21) represents cells with a 2C (G1) DNA content, while the peak at scale value 43 represents cells in G2 and M with a 4C DNA content. In between the 2C and 4C peaks are cells in S phase which have replicated different amounts of DNA, reflecting different degrees of progression through the DNA-synthetic phase of the cell cycle. It is possible not only to calculate the cell-cycle distribution of the drug-treated culture (shown in Table 2) but also to detect peculiarities in cell division. Drug treatment caused a pronounced broadening of the base of the G1 peak (Fig. 2(b)) which was never observed in untreated cultures (Fig. 2(a)). This broadening reflects non-disjunctive errors occurring during mitosis and cell division, resulting in an unequal partitioning of DNA between daughter cells. Determination of the induced non-disjunctive errors utilizing FMF analysis is documented elsewhere [15]. A similar phenomenon was observed in cultures treated with chlorozotocin. These findings suggest that many of the cells which divided in the presence of streptozotocin or chlorozotocin had abnormal complements of chromosomes. Furthermore, both drugs produced small quantities of polyploid cells.

To determine the effect of the drugs on the *rate* of progression through the cell cycle, G1-arrested (synchronized) cells were exposed to either 300 μg/ml streptozotocin or 8 μg/ml chlorozotocin. These levels of the drugs killed 95% of the cells. A third culture served as drug-free control. After exposure to drug for

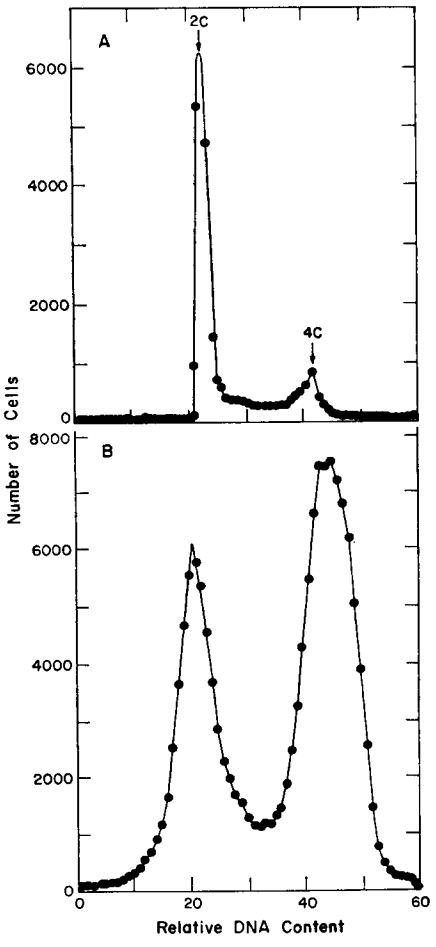


Fig. 2. FMF-derived DNA distribution for a culture of exponentially growing CHO cells (A) and the same culture following treatment for 2 hr with 8 μg/ml of chlorozotocin, then incubated for 30 hr in drug-free medium (B). Cells were dispersed, fixed, and stained by our standard acriflavine-Feulgen procedure [11, 12].

2 hr, the cells from all three cultures were resuspended in fresh drug-free medium containing isoleucine, in which cell-cycle traverse was again possible. The results, shown in Fig. 3, demonstrate that neither streptozotocin nor chlorozotocin affected the rate of traverse through G1. The rate of progression through S phase was only slightly reduced in the streptozotocin-treated cells. In contrast, cells treated with chlorozotocin required approximately twice as long a time period to traverse the DNA-synthetic phase as did the drug-free control culture. Cells treated with streptozotocin were delayed in progression through G2 for nealry 4 hr but thereafter entered mitosis at approximately the rate observed in the control culture. The number of cells which divided in the streptozotocin-treated culture was only about 70% of the number dividing in the control. The chlorozotocin-treated cells were delayed in entering G2 and then began

accumulating in G2 during the latter stages of the experiment, with less than 10% of the cells dividing; the cells that did divide underwent mitotic non-disjunction similar to the results shown in Fig. 2(b). Thus, at isosurvival levels, chlorozotocin was far more effective in reducing the rate of cell-cycle progression through S and G2 than was streptozotocin.

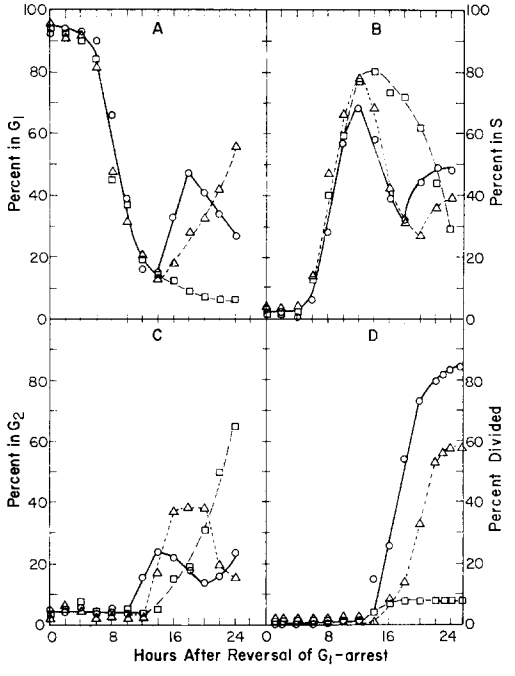


Fig. 3. Progression of synchronized CHO cells through the cell cycle following treatment with streptozotocin or chlorozotocin. Cells arrested in G1 by cultivation in isoleucine-deficient medium for 36 hr were treated for a 2 hr period with either 300 μg/ml of streptozotocin (triangles) or 8 μg/ml of chlorozotocin (squares), then returned to cycle by resuspension in fresh complete medium. An untreated culture served as control (circles). The drug concentrations employed yielded a survival value of approximately 5%. Following return to complete F-10, samples were removed for determination of the fraction of cells in each phase of the cell cycle by means of combined FMF analysis employing mithramycin and ^3H-thymidine autoradiography. Cells were pulse-labeled with 2 μCi/ml of ^3H-thymidine for 15 min periods and collected at the time of collection of samples for FMF analysis. Cell number determinations were made with an electronic cell counter. Divided fraction represents $N/N_0 - 1$ so that a true population doubling would appear as an increase from 0–1 on the scale provided.

The data presented thus far suggest that treatment with chlorozotocin leads to the accumulation in G2 of essentially all cells in the population. However, the point of accumulation within G2 can be localized in the following manner. Briefly, if chlorozotocin is added to an exponentially growing culture of cells, the time between drug addition and change in growth rate determines the time back in the cell cycle preceding M/G1 (*i.e.*, cell division) at which the drug exerted its terminal effect. That is, cells beyond this temporal marker will continue through the cell cycle and will divide, even in the presence of chlorozotocin, whereas cells located at earlier stages in the cell cycle will be prevented from progressing at the normal rate. Figure 4 presents data obtained by treating an exponentially growing culture of CHO cells with 32 μg/ml of chlorozotocin. The cells continued dividing at a constant rate for approximately 210 min following drug addition, after which cell division stopped. Similar results were obtained when 15–40 μg/ml of chlorozotocin was used (not shown). These results suggest that chlorozotocin-treated cells accumulate at 210 min

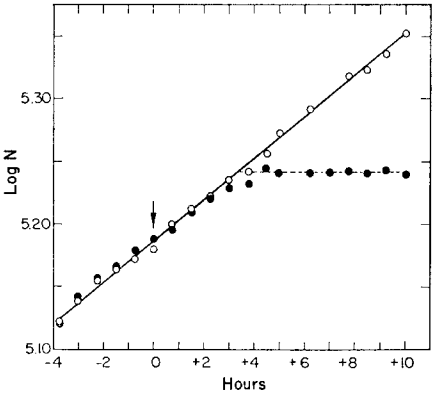


Fig. 4. Effect of chlorozotocin on cell division. An aliquot of exponentially growing CHO cells was split into two cultures, and at t = 0 one of the cultures received chlorozotocin to a final concentration of 32 μg/ml. The other culture was maintained as a drug-free control. Cell number determinations were made with an electronic cell counter.

ahead of the M/G1 boundary. This concentration of chlorozotocin caused a nearly complete accumulation of cells in G2 (Table 2). The duration of the G2 and M phases in the CHO cell is approximately 220 min under these culture conditions (*i.e.*, the beginning of G2 is located 220 min ahead of the M/G1 boundary) [16]. Therefore, we conclude that chlorozotocin causes accumulation of cells *within the first* 10 *min of the G2 period.* Similar experiments with streptozotocin (not shown) indicated that the terminal point of action for that drug was located at approximately 60 min into G2, or 126 min before prophase.

DISCUSSION

Although chlorozotocin is a derivative of streptozotocin, there are a number of differences in kinetic responses of CHO cells following treatment with the two compounds. For

example, non-cycling cells were much more sensitive than cycling cells to the cytotoxic effects of chlorozotocin, whereas the cycling cell population was more sensitive than the non-cycling cells to treatment with low doses of streptozotocin (Fig. 1). Furthermore, on a weight basis, chlorozotocin was 20–40 times as effective in killing CHO cells than was an equivalent concentration of streptozotocin. While streptozotocin caused a modest increase in the fraction of cells in G2 at the expense of the G1 population, with little effect upon the fraction in S phase (Table 1), chlorozotocin induced a near-depletion of both G1 and S cells, with the displaced cells accumulating in G2 (Table 2). At isosurvival levels, chlorozotocin was far more effective in reducing the rate of progression through S and G2 than was streptozotocin (Fig. 3). The terminal points of action in G2 for streptozotocin and chlorozotocin were located at 60 min and at 10 min, respectively, from the S/G2 boundary. For cells treated with either drug and progressing through mitosis, the likelihood of successful completion of mitosis was reduced due to non-disjunctive errors or the formation of polyploid cells (Fig. 2(b)). Finally, both drugs had a greater effect upon cell division than on DNA replication capacity (Tables 1 and 2). Note that cells exposed briefly to streptozotocin or chlorozotocin while in either the G1-arrest, G1, or S phases traversed to G2 and then slowed or ceased cycle progression (Table 2), while cells initially in G2 and M during exposure to the drug were able to divide but were ultimately affected during the next division cycle. These results suggest that (a) drug effects were irreversible and were not circumvented by returning the cells to drug-free medium and (b) drugs were effective against cells in all stages of the cell cycle.

How do our data compare with results from previous studies? We were unable to find any previous information with which to compare our results on the biological effects of chlorozotocin. However, earlier studies of streptozotocin effects in cultured cells revealed the following: (a) survival curves for cycling cells were generally similar to our results in Fig. 1, although there was no indication of the biphasic response observed in our studies [17, 18]; (b) division and reproductive capacities were more sensitive to drug effects than was DNA synthesis capability [17, 19]; (c) progression of cells was blocked in mid-G2, resulting in an accumulation of cells in that phase of the cell cycle [17]; (d) chromosome aberrations were observed along with alterations in chromosome surface structure, yielding chromosomes that tended to stick together [17, 19] (and which could have been involved in the non-disjunctive errors observed in our studies); and (e) cells in all phases of the cell cycle were sensitive to drug effects [17, 19]. Thus, we feel that our findings concerning streptozotocin are in generally good agreement with the results obtained in other laboratories utilizing different analytical systems.

Comparison of the results obtained with chlorozotocin to our earlier studies of the effects of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) [2] and 1-*trans*(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (Me-CCNU) [2] reveals that all three of these compounds produced similar kinetic responses in susceptible cells. For example, all three agents (a) caused a gross reduction in rate of traverse of S and G2, resulting ultimately in nearly pure G2 populations; (b) were much more cytotoxic for non-cycling cells than for cycling cells; and (c) induced mitotic non-disjunction and polyploidization. Because of these similarities, we would expect chlorozotocin to behave more nearly like CCNU and Me-CCNU than streptozotocin in terms of expected kinetic response of susceptible cells.

We are attempting to provide a simple model system which may be utilized as a predictive guide for the expected kinetic response of *susceptible* (and only susceptible) tumor cells *in vivo*. Non-responding cells *in vivo* obviously will not behave in the kinetic fashion described in our system. In our previous studies with antimetabolites and several nitrosourea derivatives, the results obtained in our *in vitro* system were generally in good agreement with the kinetic responses of susceptible cells observed *in vivo* [12, 16]. If our model system may also be utilised as a valid predictive guide for *in vivo* responses to treatment with chlorozotocin, then we wish to make the following suggestions concerning clinical use of this drug. It is our intention that the recommendations should be interpreted with caution and constitute *only general guideline suggestions*, providing one input for the clinician to consider in design of therapeutic treatment schedule.

In view of the grossly extended duration of the G2 phase and subsequent enrichment of the G2 fraction, we would expect maximum cell kill to be achieved by administration of G2-specific cytotoxic agents after a prolonged interval (in excess of 24–36 hr) following primary treatment with chlorozotocin. That is, partially synchronize *in vivo* the tumor cell population with chlorozotocin, then treat

with a G2-specific agent to reduce the accumulated G2 cell fraction. Note that *in vivo* synchronization of cells has been demonstrated by appropriate treatment with drugs [20, 21]. The decisive factor in flexibility of chemotherapy regimen design obviously is the cumulative toxicity arising from the use of multiple agents.

In this and in our preceding study [2] involving effects of CCNU, Me-CCNU, and 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU), our cells were exposed to drug in serum-containing medium. Use of serum-containing medium during exposure of cells to alkylating agents has been questioned in a recent report [22]. That study provided evidence that the survival of cells treated with alkylating agents was more highly variable when exposure of cells took place in serum-containing medium rather than in balanced salt solution. As a result, the order of magnitude of difference in survival between cycling and plateau phase cells was *less* when cells were exposed to BCNU in serum-free medium than it was in serum-containing medium [22]. The primary difference between these results [22] and those reported by Barranco *et al.* [23] and also by us [2] is the degree of greater sensitivity of non-cycling cells in comparison with cycling cell populations; however, the same general conclusion was obtained in all three studies: non-cycling cells were somewhat more sensitive than cycling cells to the effects of BCNU.

A final decision regarding the advisability of serum-free or serum-containing medium during the period of exposure of cultured cells to alkylating agents must await the accumulation of extensive clinical data documenting the response of human tumor cell populations in patients to treatment with alkylating agents. That is, *in vitro* conditions will be selected that yield results most comparable to responses observed *in vivo*.

Until sufficient *in vivo* data are available, we shall continue to utilize serum-containing medium in our studies, since we believe that there are two major objections to the use of balanced salt solution: (1) cells maintained in balanced salt solution for significant periods may well be operating under biochemical stress, with the result that drug cytotoxic effects may be potentiated; and (2) drug administration in balanced salt solution under non-physiological conditions runs counter to the serum-containing environment encountered *in vivo*.

Acknowledgements—The excellent technical assistance of Mrs. Phyllis Sanders, Mrs. Priscilla Jose, and Ms. Elvira Bain is gratefully acknowledged.

REFERENCES

1. R. A. Tobey and H. A. Crissman, Use of flow microfluorometry in detailed analysis of effects of chemical agents on cell-cycle progression. *Cancer Res.* **32,** 2726 (1972).
2. R. A. Tobey and H. A. Crissman, Comparative effects of three nitrosourea derivatives on mammalian cell-cycle progression. *Cancer Res.* **35,** 460 (1975).
3. L. E. Broder and S. K. Carter, Pancreatic islet cell carcinoma—II. Results of therapy with streptozotocin in 52 patients. *Ann. intern. Med.* **72,** 108 (1973).
4. D. C. Stolinsky, L. Sadoff, J. Braunwald and J. R. Bateman, Streptozotocin in the treatment of cancer. Phase II study. *Cancer (Philad.)* **30,** 61 (1972).
5. L. Sadoff, Effects of streptozotocin in a patient with islet carcinoma. *Diabetes* **18,** 675 (1969).
6. W. G. Blackard, A. R. Garcia and C. L. Brown, Jr., Effect of streptozotocin in qualitative aspects of plasma insulin in a patient with a malignant islet cell tumor. *J. clin. Endocrin.* **31,** 215 (1970).
7. J. M. Feldman, K. E. Quickel, Jr., R. L. Marecek and H. E. Lebovitz, Streptozotocin treatment of metastatic carcinoid tumors. *Sth. med. J. (Bgham, Ala.)* **65,** 1325 (1972).
8. R. A. Tobey and K. D. Ley, Isoleucine-mediated regulation of genome replication in various mammalian cell lines. *Cancer Res.* **31,** 46 (1971).
9. M. D. Enger and R. A. Tobey, Effects of isoleucine deficiency on nucleic acid and protein metabolism in cultured Chinese hamster cells. Continued ribonucleic acid and protein synthesis in the absence of deoxyribonucleic acid synthesis. *Biochemistry* **11,** 269 (1972).
10. R. A. Tobey, Production and characterization of mammalian cells reversibly arrested in G1 by growth in isoleucine-deficient medium. In *Methods in Cell Biology*, Edited by D. M. Prescott, Vol. 6, p. 67. Academic Press, New York (1973).

11. R. A. Tobey, H. A. Crissman and P. M. Kraemer, A method for comparing effects of different synchronizing protocols on mammalian cell-cycle traverse. The traverse perturbation index. *J. Cell Biol.* **54,** 638 (1972).
12. H. A. Crissman, P. F. Mullaney and J. A. Steinkamp, Methods and applications of flow systems for analysis and sorting of mammalian cells. In *Methods in Cell Biology*, Edited by D. M. Prescott, Vol. 9, Academic Press, New York (1974). In press.
13. H. A. Crissman and R. A. Tobey, Cell-cycle analysis in twenty minutes. *Science* **184,** 1297 (1974).
14. D. M. Holm and L. S. Cram, An improved flow microfluorometer for rapid measurement of cell fluorescence. *Exp. Cell Res.* **80,** 105 (1973).
15. P. M. Kraemer, L. L. Deaven, H. A. Crissman and M. A. van Dilla, DNA constancy despite variability in chromosome number. In *Advances in Cell and Molecular Biology*, Edited by E. J. Du Praw, Vol. 2, p. 47. Academic Press, New York (1972).
16. R. A. Tobey, Arrest of Chinese hamster cells in G2 following treatment with the anti-tumor drug Bleomycin. *J. Cell. Physiol.* **79,** 259 (1972).
17. B. K. Bhuyan, The action of streptozotocin on mammalian cells. *Cancer Res.* **30,** 2017 (1970).
18. B. K. Bhuyan, L. G. Scheidt and T. J. Fraser, Cell cycle phase specificity of antitumor agents. *Cancer Res.* **32,** 398 (1972).
19. F. Kelley and M. Legator, The effects of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and streptozotocin on mammalian cell cultures. *Mutation Res.* **12,** 183 (1971).
20. R. S. Verbin, G. Diluiso, H. Liang and E. Farber, Synchronization of cell division *in vivo* through the combined use of cytosine arabinoside and Colcemid. *Cancer Res.* **32,** 1489 (1972).
21. S. C. Barranco, J. K. Luce, M. M. Romsdahl and R. M. Humphrey, Bleomycin as a possible synchronizing agent for human tumor cells *in vivo*. *Cancer Res.* **33,** 882 (1973).
22. G. M. Hahn, L. F. Gordon and S. D. Kurkjian, Responses of cycling and non-cycling cells to 1,3-*Bis*(2-chloroethyl)-1-nitrosourea and to Bleomycin. *Cancer Res.* **34,** 2373 (1974).
23. S. C. Barranco, J. K. Novak and R. M. Humphrey, Response of mammalian cells following treatment with Bleomycin and 1,3-*Bis*(2-chloroethyl)-1-nitrosourea during plateau phase. *Cancer Res.* **33,** 691 (1973).

Europ. J. Cancer Vol. 11, pp. 443–449. Pergamon Press 1975. Printed in Great Britain

Effect of Endogenous and Exogenous Murine Leukemia Virus Infection on Immunologic Response*

DINO COLLAVO, GIOVANNI BIASI, ALFONSO COLOMBATTI and LUIGI CHIECO-BIANCHI

Laboratory of Experimental Oncology, Institute of Pathological Anatomy, University of Padova, Padova, Italy

Abstract—*Thymus derived (T) and bursa equivalent derived (B) lymphocyte functions of AKR and C58 mice, natural carriers of endogenous Gross–MuLV, were compared to those of normal, passage A Gross– and Graffi–MuLV neonatally injected CBA mice. The same percentage of θ positive T lymphocytes was found in blood, spleen and lymph node from normal donors of the three strains. Results of PHA stimulation of spleen cells* in vitro *disclosed depressed values for the leukemia-prone strains. Moreover, only C58 spleen cell cultures stimulated by LPS were depressed. However, no significant reduction in PHA and LPS* in vitro *response was observed in spleen cultures from passage A Gross and Graffi–MuLV neonatally injected, leukemia-free CBA mice.*

Following in vivo *immunization with SRBC, AKR mice produced significantly less PFC than both C58 and CBA mice. After immunization with LPS, both AKR and C58 mice produced significantly less PFC than CBA mice. In leukemia-free, passage A Gross– and Graffi–MuLV neonatally injected CBA mice similarly immunized with SRBC and LPS, a slight but significant reduction in the PFC number against SRBC was found only in Graffi–MuLV injected mice. The remaining groups gave values similar to their controls.*

INTRODUCTION

IT IS well established that certain animal viruses can interfere with the host immune response [1]. This observation, together with the hypothesis that tumor cells may elude immune surveillance through a non-specific immune depression caused by the oncogenic agent itself, suggests that the oncogenic viruses may provide a model system for the study of the relationship between virus-induced immune depression and tumor induction.

In this regard, the murine leukemia viruses (MuLVs) have received close attention since they may affect cells committed to antigen recognition and elimination. There is general agreement that Friend (F–) and Rauscher (R–) MuLVs exert a suppressive effect mostly on humoral immunity [2–6]. These viruses induce neoplastic proliferation of the erythroid cells within a few days following infection. Contradictory results have been reported for other MuLVs such as passage A Gross (G)–MuLV which selectively transform lymphatic cells and induce leukemia after a long latent period. In fact, while a depressed immune reactivity is easily determined in overtly leukemic passage A G–MuLV infected mice, before the clinical onset of leukemia immunologic deficiency seems to be rather subtle, if any [7].

Recently, we compared the immunologic response pattern of leukemia-prone AKR mice, natural carriers of endogenous G–MuLV, with

Accepted 16 January 1975.

*This work has been supported in part by grants from Consiglio Nazionale delle Ricerche, Roma, and the Associazione per la Promozione delle Ricerche sul Cancro, Milano.

The skillful technical assistance of Gabriella Miotti and Silvio Mezzalira is gratefully acknowledged.

that of CBA mice, which present a low incidence of spontaneous leukemia. We observed that AKR mice exhibit a reduced *in vitro* thymus derived (T) lymphocyte reactivity to mitogen stimulation together with a low *in vivo* response following immunization with thymus dependent or independent antigens [8,9]. The question then arose whether the immunologic deficit observed in AKR mice might be due to the high titer of endogenous G–MuLV normally present in these mice. Consequently, the *in vitro* and *in vivo* T and bursa equivalent derived (B) lymphocyte functions of C58 mice (natural carriers of endogenous G–MuLV) were compared to those of normal, passage A G–, and Graffi (Gi–)MuLV neonatally infected CBA mice. This paper reports the results of this study.

MATERIAL AND METHODS

Mice

Inbred AKR and CBA mice were from our colony. C58/J mice were purchased from the Jackson Laboratory, Bar Harbor, Maine, U.S.A.

Viruses

Passage A Gross and Graffi leukemogenic viruses were maintained by inoculation of cell-free extracts in newborn CBA and C57BL mice, respectively. Cell-free extracts, prepared from leukemic lymph node, spleen and thymus by homogenization and centrifugation as described [10], were diluted 1:5 weight/volume (w/v) with Hank's balanced solution and 0·05 ml of this extract was injected s.c. into 1–3 day old mice.

Cell suspensions

Spleens and lymph nodes were minced and pressed through a multilayer nylon sieve using RPMI 1640 medium (Eurobio, Paris, France) containing 15% heat inactivated fetal calf serum (FCS) (Grand Island Biol. Co., Grand Island, N.Y., U.S.A.) and antibiotics. The cells in suspension were then washed at 4°C. The number of viable cells was determined using the Eosin Y exclusion method. No FCS was added to the medium when cells were used for cytotoxic and hemolytic plaque forming cell assays (see below).

Determination of θ antigenicity

Anti-θ C3H serum was a gift from Dr. M. C. Raff. Anti-θ AKR serum was prepared in CBA mice by repeated i.p. injection of AKR thymocytes according to the method of Reif and Allen [11]. Specificity of this last serum was demonstrated by the fact that AKR but not CBA brain completely absorbed out the cytotoxic activity. Both anti-θ sera were used in 1:4 dilution (the 50% endpoint lysis on lymph node cells was 1:16 for anti-θ C3H, and 1:256 for anti-θ AKR). The cytotoxic assay, based on direct cell observation under the phase contrast microscope, was performed as previously described [12]. Fresh guinea pig serum diluted 1:3 and absorbed with agarose according to Cohen and Schlesinger [13], was employed as source of complement.

Spleen cell cultures

Spleen cells were resuspended in complete medium to reach a final concentration of 2×10^6 cells/ml. Triplicate tubes, containing 1 ml cell suspension, received PHA (Wellcome, Beckenham, England) at a final concentration of 1:100, or 25 μg Lipopolysaccharide BE. Coli (LPS, 055:B5, Difco, Detroit, Michigan, U.S.A.) All doses were calculated to give a maximum mitogenic response. Triplicate tubes without mitogens served as controls. Culture vials consisted of 2 ml disposable plastic tubes (Stayne Continental S.A., Jumet Diarbois, Belgium). Cultures were incubated for 72 hr at 37°C in 5% CO_2 moist atmosphere. Previous experiments had demonstrated that a maximum response in all mouse strains is seen after 72 hr incubation. For DNA synthesis analysis, 2 μCi Thymidine-Methyl-^{3}H(^{3}H-TdR, specific activity 2·0 Ci/mM, NEN, Frankfurt, Germany) were added to each culture 12 hr before harvesting. Cultures were then processed and the ^{3}H-TdR uptake was calculated by the method of liquid scintillation counting [14].

Antigens

SRBC (Sclavo, Siena, Italy) were washed twice with saline and 4×10^8 cells in 0·1 ml were injected i.p. 0·02 mg LPS in 0·1 ml saline was injected i.p.

Detection of antibody-producing cells

The number of direct hemolytic plaque forming cells (PFC) against SRBC was determined according to Jerne *et al.* [15]. The number of direct PFC against LPS-coated SRBC was determined using the method described by Möller [16]. Coating was achieved by incubating 1 mg of boiled LPS in 1 ml of saline containing 0·5 ml packed SRBC for 30 min at 37°C. Thereafter, the coated SRBC were washed three times and plated at a concentration of 4×10^8 cells/plate. Agar plate containing the cell suspensions adjusted to give

about 50–100 PFC/plate, were incubated at 37°C for 1 hr, after which 3 ml of 1:10 guinea pig serum (Sclavo, Siena, Italy) were added. The plates were incubated again at 37°C for 1 hr to develop hemolytic plaques. PFC were counted at × 10 magnification.

Statistical analysis

Student's *t* test was used to compare the experimental results.

RESULTS

Evaluation of θ positive lymphocytes in blood, lymph node and spleen

A preliminary study was carried out to determine the number of θ positive T-lymphocytes in CBA, AKR and C58 mice. Blood, spleen and lymph node cells were tested by the cytotoxic assay using anti θ sera. As shown in Table 1, the same percentage of θ positive cells was found in blood and peripheral lymphoid organs of the 3 mouse strains.

Response of spleen cultures to PHA and LPS stimulation

We had already observed that 2–4 month old AKR mice respond similarly to CBA mice when stimulated with a B-lymphocyte selective mitogen (LPS); whereas following stimulation with T-lymphocyte selective mitogens (PHA and Con A), the AKR response is reduced [8. 9]. Therefore, we compared the ^{3}H-TdR uptake following PHA or LPS stimulation of spleen cell cultures from individual 2–4 month old C58 mice to that of AKR and CBA cultures. In Table 2 the results of 3 different experiments with a total of 6 mice for each strain are reported. After PHA stimulation, the ^{3}H-TdR absolute uptake of spleen cells from AKR and C58 mice was similar but both values were lower than that for CBA mice ($P < 0{\cdot}01$). However, when the stimulation index (S.I.) was calculated, S.I. values for C58 cultures were lower than CBA and higher than AKR; this last finding depends on the low spontaneous ^{3}H-TdR uptake observed in C58 mice.

Following LPS stimulation, C58 cultures showed both lower ^{3}H-TdR absolute uptake ($P < 0{\cdot}001$) and S.I. than CBA and AKR cultures. These results indicate that while AKR mice present an impairment only in T-lymphocyte reactivity, C58 mice exhibit an impairment mainly in B-lymphocyte reactivity.

Effect of exogenous MuLV infection on PHA and LPS lymphocyte stimulation

Leukemia free, 2–3 month old CBA mice, which had received passage A G–MuLV or Gi–MuLV infection at birth, were used as spleen cell donors. We have observed repeatedly that neonatal infection of CBA mice with both these viruses induces a high incidence of leukemia which generally arises in the thymus.

Table 1. *θ positive cells in blood, lymph node and spleen*

Strain	No. mice*	% θ positive cells† Blood	Lymph node	Spleen
CBA	6	65.2 ± 7·5	79·6 ± 3·0	37·3 ± 6·3
AKR	7	72·8 ± 8·3	79·0 ± 11·7	35·3 ± 12·4
C58	6	67·0 ± 5·3	73·8 ± 2·7	34·6 ± 8·7

*Two month old mice were used as donors.
†Each value represents the mean ± S.D.

Table 2. *Response of spleen cultures to PHA and LPS stimulation**

Strain	Unstimulated	PHA-stimulated	S.I.†	LPS-stimulated	S.I.†
CBA	1606 ± 278	11180 ± 4012	7·2	9566 ± 2855	5·9
AKR	1897 ± 175	5736 ± 1713‡	3·1	9868 ± 1316	5·3
C58	1061 ± 131	5466 ± 2238‡	5·1	2947 ± 1070§	2·7

*DNA synthesis was determined by ^{3}H-TdR uptake in counts/min. Each value represents the mean ± S.D. of triplicate cultures from 6 different donors.
†S.I. = Stimulation index: mean ratio of cpm in stimulated/unstimulated cultures.
‡$P < 0{\cdot}01$ and §$P < 0{\cdot}001$ in comparison to CBA mice.

*Table 3. Response of normal and MuLV-neonatally injected CBA mice to PHA and LPS stimulation**

MuLV	Unstimulated	PHA-stimulated	S.I.†	LPS-stimulated	S.I.†
—	1652 ± 182	10113 ± 3150	6·1	8986 ± 2543	5·5
Passage A Gross	1635 ± 225	9836 ± 2095	6·0	9129 ± 1984	5·5
Graffi	1535 ± 281	8970 ± 2684	5·8	7676 ± 2123	5·0

*DNA synthesis was determined by ^{3}H-TdR uptake in counts/min. Each value represents the mean ± S.D. of triplicate cultures from 6 different donors.
†S.I. = Stimulation index: mean ratio of cpm in stimulated/unstimulated cultures.

Spleen cell cultures were prepared from MuLV injected CBA mice. As reported in Table 3, no significant reduction in the response to PHA and LPS stimulation was found in comparison to uninjected controls. These data suggest that infection with both MuLV strains used does not modify CBA T or B lymphocyte reactivity to mitogen stimulation.

In vivo *response to SRBC and LPS immunization*

Previous results showed that AKR mice produced a much lower number of PFC than CBA mice following immunization with thymus dependent (SRBC) or independent (LPS) antigens [9]. It was considered of interest to evaluate also the response pattern of C58 mice. As reported in Table 4, following SRBC immunization, C58 mice produced, both per million nucleated cells and per spleen, a PFC number similar to that of CBA donors and thus significantly higher than that of AKR mice ($P < 0{\cdot}001$). Following LPS immunization (Table 5), C58 like AKR mice produced a much lower number of PFC than CBA donors ($P < 0{\cdot}001$). These data show that while the immunologic deficit in AKR mice involves both the thymus dependent and independent antigens tested, only the response to thymus independent antigens seems to be reduced in C58 mice.

In vivo *response of MuLV injected mice to SRBC and LPS immunization*

To determine whether exogenous passage A G– or Gi–MuLV impairs the immune response to SRBC or LPS, direct PFC production was studied in MuLV neonatally injected CBA mice. As shown in Table 6, after SRBC immunization no differences were observed between controls and passage A G–MuLV injected mice. However, Gi–MuLV injected mice produced a lower number of PFC when calculated both per million nucleated cells and per spleen ($P < 0{\cdot}01$ and $P < 0{\cdot}02$ respectively). Following LPS immunization, passage

*Table 4. PFC production following SRBC immunization**

Strain	No. mice	PFC/10^6 Cells†	PFC/Spleen†
CBA	5	2·335 ± 0·069	4·388 ± 0·086
AKR	6	1·710 ± 0·063‡	3·881 ± 0·112‡
C58	6	2·431 ± 0·050	4·481 ± 0·041

*Direct PFC evaluated on day 5 after immunization.
†Log_{10} ± S.E. of the means of the number of PFC.
‡$P < 0{\cdot}001$ in comparison to CBA mice.

*Table 5. PFC production following LPS immunization**

Strain	No. mice	PFC/10^6 Cells†	PFC/Spleen†
CBA	20	2·356 ± 0·062	4·514 ± 0·069
AKR	17	1·543 ± 0·091‡	3·553 ± 0·103‡
C58	7	1·451 ± 0·132‡	3·503 ± 0·189‡

*Direct PFC evaluated on day 4 after immunization.
†Log_{10} ± S.E. of the means of the number of PFC.
‡$P < 0{\cdot}001$ in comparison to CBA mice.

*Table 6. PFC production in normal and MuLV-neonatally injected CBA mice following SRBC immunization**

MuLV injected	No. mice	PFC/10^6 Cells†	PFC/Spleen†
—	11	2·114 ± 0·027	4·283 ± 0·037
Passage A Gross	9	2·095 ± 0·043	4.312 ± 0·073
Graffi	8	1·937 ± 0·059‡	4·111 ± 0·056§

*Direct PFC evaluated on day 5 after immunization.
†Log_{10} ± S.E. of the means of the number of PFC.
‡$P < 0{\cdot}01$, and § $P < 0{\cdot}02$ in comparison to control mice.

A G–MuLV injected mice produced less PFC per 10^6 cells than controls ($P < 0{\cdot}01$). On the other hand, no significant differences between MuLV-injected and control mice were found when the PFC were evaluated per spleen (Table 7). Following SRBC or LPS immunization, however, the majority of MuLV-injected mice exhibited a more prominent splenomegaly than their controls. Accordingly, if the PFC number is calculated per 10^6 cells, its reduction is more likely due to a PFC dilution caused by the proliferation of non-antibody producing cells rather than to an actual decrease in PFC production. Thus, a slight but significant reduction of PFC following SRBC immunization was evident only in Gi–MuLV neonatally injected mice. Passage A G–MuLV infection did not modify the *in vivo* response to both SRBC and LPS antigens.

*Table 7. PFC production in normal and MuLV-neonatally injected CBA mice following LPS immunization**

MuLV injected	No. mice	PFC/10^6 Cells†	PFC/Spleen†
—	8	2·258 ± 0·052	4·491 ± 0·091
Passage A			
Gross	11	2·093 ± 0·121‡	4·547 ± 0·103
Graffi	10	2·221 ± 0·107	4·520 ± 0·076

*Direct PFC evaluated on day 4 after immunization.
†Log_{10} ± S.E. of the means of the number of PFC.
‡$P < 0{\cdot}01$ in comparison to control mice.

DISCUSSION

Our previous [8, 9] and present results suggest that, in comparison to CBA mice, an impairment of T and/or B lymphocyte functions exists in leukemia-prone AKR and C58 mice. Since mice of these three strains share the same histocompatibility-2 happotype (H-2^k) the expectancy of similar behavior, at least for some immune reactions such as those governed by the immune response (Ir) genes linked with the H-2 region [17] seemed reasonable. This was further strengthened by the observation that the mouse strains studied have approximately the same number of T cells in their blood and peripheral lymphoid organs.

However, in comparison to CBA, the AKR T cell response to PHA stimulation *in vitro* was lower than that of C58 mice, whereas the B lymphocyte response to LPS appeared normal in AKR and reduced in C58 mice. The results following *in vivo* immunization with thymus dependent (SRBC) or independent (LPS) antigens are somewhat in line with these *in vitro* findings. In fact, despite their normal *in vitro* LPS response, AKR mice showed a reduced direct PFC production both to SRBC and to LPS. It is possible that dissociation between the mitogenic and immunogenic properties of LPS [18] is responsible for this discrepancy. On the other hand, in C58 mice, whose B cell response appeared to be mainly affected *in vitro*, only the PFC production to LPS-coated erythrocytes was decreased. Thus, although leukemic transformation in both strains involves primarily the T lymphocytes (unpublished results), AKR mice show a more severe impairment in T cell reactivity, while mainly the B lymphocytes seem defective in C58 mice. Therefore, the possibility that the endogenous MuLV present in both mouse strains might induce the observed immune deficiency had to be considered.

The effect of exogenous MuLV infection on host immune reactivity has been studied extensively. Definite evidence exists for immunodepression exerted by F- and R-MuLV as judged by several parameters, both *in vitro* and *in vivo* [2–6, 19, 20], even if the associated Rowson–Parr MuLV seems to contribute in some way to this effect [21, 22]. On the other hand, contrasting results have been reported for other viruses, such as passage A G–MuLV and Moloney (M–)MuLV. For instance, in neonatally injected passage A G–MuLV mice, skin graft rejection across weak non H-2 loci has been found to be either delayed [23] or normal [24]; and following M–MuLV infection, an impairment in antibody production has been observed during the preleukemic period [25] or exclusively in leukemic animals [2].

In the present study, spleen cells from leukemia-free CBA mice, neonatally injected with passage A G–MuLV or Gi–MuLV, were fully competent to stimulation by both PHA and LPS. Moreover, *in vivo* immunization revealed that the overall response of passage A G–MuLV-injected mice is not changed, while Gi–MuLV-injected mice showed a diminished response only towards SRBC. This last finding may indicate that Gi–MuLV affects preferentially the T cell population possessing helper functions, as recently shown for the radiation (Rad-)MuLV [26].

It is well-known that among the laboratory strains of MuLVs, passage A G–MuLV possesses biologic and pathogenic properties very similar to the endogenous G–MuLV from which it was derived. Consequently, it seems unlikely that the depression in immune responses observed in AKR and C58 mice is

due to the endogenous G–MuLV infection. In this connection, Celada and Åsjö [27] have shown that antibody forming cells (and their precursors) may be infected by M–MuLV and still maintain their capacity for specific antibody synthesis to SRBC. Thus, while different exogenous MuLV strains may or may not induce immunodepression involving T and/or B lymphocyte subpopulations, our previous and present results favor the hypothesis that the immunodeficiencies detected in AKR and C58 mice represent an intrinsic defect which, together with other genetic factors, are merely the cause and not the consequence of early full expression of endogenous MuLV. While it would appear that there is no general correlation between the immunodepressive and oncogenic effects exerted by MuLVs, an observation reported also by Haran Ghera *et al.* for both endogenous [28] and passaged MuLVs [29], the biological role of the newly-reported xenotropic endogenous MuLVs [30] should be clarified before any final conclusions can be drawn.

REFERENCES

1. A. L. Notkins, S. E. Mergenhagen and R. J. Howard, Effect of virus infections on the function of the immune system. *Ann. Rev. Microbiol.* **24,** 525 (1970).
2. W. Ceglowski and H. Friedman, Suppression of the primary antibody plaque response of mice following injection with Friend disease virus. *Proc. Soc. exp. Biol. (N.Y.)* **126,** 662 (1967).
3. M. H. Salaman and N. Wedderburn, The immunodepressive effect of Friend virus. *Immunology* **10,** 445 (1966).
4. N. Wedderburn and M. H. Salaman, The immunodepressive effect of Friend virus. II. Reduction of splenic haemolysin-producing cells in primary and secondary responses. *Immunology* **15,** 439 (1968).
5. B. V. Siegel and J. I. Morton, Depressed antibody response in the mouse infected with Rauscher leukemia virus. *Immunology* **10,** 559 (1966).
6. W. S. Ceglowski and H. Friedman, Immunosuppressive effects of Friend and Rauscher leukemic disease viruses on cellular and humoral antibody formation. *J. nat. Cancer Inst.* **40,** 983 (1968).
7. P. B. Dent, Immunodepression by oncogenic viruses. *Progr. med. Virol.* **14,** 1 (1972).
8. G. Biasi, D. Collavo, A. Biasi and L. Chieco-Bianchi. Evaluation of T-lymphocyte reactivity in leukemia prone AKR mice. In *Characterization of Human Tumors.* Edited by W. Davis and C. Maltoni, p. 261. Excerpta Medica, Amsterdam (1974).
9. D. Collavo, G. Biasi, A. Colombatti and L. Chieco-Bianchi, *In vitro* and *in vivo* evaluation of T and B lymphocyte functions in AKR mice. In preparation (1975).
10. L. Fiore-Donati and L. Chieco-Bianchi, Influence of host factors on development and type of leukemia induced in mice by Graffi virus. *J. nat. Cancer Inst.* **32,** 1083 (1964).
11. A. E. Reif and J. M. Vallen, The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. exp. Med.* **120,** 413 (1964).
12. G. Tridente, D. Collavo, G. M. Cappuzzo and L. Chieco-Bianchi, *In vitro* cytotoxic activity of normal and leukemic anti-leukocyte murine sera. *Tumori* **54,** 321 (1968).
13. A. Cohen and M. Schlesinger, Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. *Transplantation* **10,** 130 (1970).
14. M. J. Doenhoff, A. J. S. Davies, E. Leuchars and V. Wallis, The thymus and circulating lymphocytes of mice. *Proc. R. Soc. B.* **176,** 69 (1970).
15. N. H. Jerne, A. A. Nordin and C. Henry, The agar plaque technique for recognizing antibody-producing cells. In *Cell Bound Antibodies.* Edited by H. Koprowski and B. Amos, p. 107. Wistar Institute Press, Philadelphia (1963).
16. G. Möller, 19s antibody production against soluble LPS antigens by individual lymphoid cells *in vitro*. *Nature (Lond.)* **207,** 1177 (1965).
17. H. O. McDevitt, K. B. Bechtol and G. J. Hämmerling, Histocompatibility-linked genetic control of specific immune responses. In *Cellular Selection and Regulation in the Immune Response.* Edited by G. M. Edelman, p. 101. Raven Press, New York (1974).

18. J. ANDERSSON, O. SJÖBERG and G. MÖLLER, Mitogens as probes for immunocyte activation and cellular cooperation. *Transplant. Rev.* **11,** 131 (1972).
19. P. HÄYRY, D. RAGO and V. DEFENDI, Inhibition of phytohemagglutinin and allo-antigen induced lymphocyte stimulation by Rauscher leukemia virus. *J. nat. Cancer Inst.* **44,** 1311 (1970).
20. R. F. MORTENSEN, W. S. CEGLOWSKI and H. FRIEDMAN, *In vitro* assessment of cellular immunity to Rauscher leukemia virus. *J. Immunol.* **111,** 657 (1973).
21. R. L. CARTER, F. C. CHESTERMAN, K. E. E. ROWSON, M. H. SALAMAN and N. WEDDERBURN, A new virus of minimal pathogenicity associated with Friend virus. II. Histological changes and immunodepressive effect. *Int. J. Cancer* **5,** 103 (1970).
22. M. BENDINELLI, Immunodepression by Rowson–Parr virus. In *Virus Tumorigenesis and Immunogenesis.* Edited by W. S. CEGLOWSKI and H. FRIEDMAN, p. 181, Academic Press, New York, London (1973).
23. P. B. DENT, R. D. PETERSON and R. A. GOOD, A defect in cellular immunity during the incubation period of passage A leukemia in C3H mice. *Proc. Soc. exp. Biol. (N.Y.)* **119,** 869 (1965).
24. L. CHIECO-BIANCHI, Fenomeni immunologici nei tumori linforeticolari sperimentali. *Atti Soc. It. Canc.* **6,** 159 (1967).
25. N. E. CREMER, D. O. N. TAYLOR and S. J. HAGENS, Antibody formation, latency and leukemia: Infection with Moloney virus. *J. Immunol.* **96,** 495 (1966).
26. A. PELED and N. HARAN-GHERA, The cellular basis of immunodepression caused by the Radiation Leukemia Virus. *Immunology* **26,** 323 (1974).
27. F. CELADA and B. ÅSJÖ, Studies on Moloney leukemia virus infection of antibody-forming cells. In *Virus Tumorigenesis and immunogenesis.* Edited by W. S. CEGLOWSKI and H. FRIEDMAN, p. 263, Academic Press, New York, London (1973).
28. N. HARAN-GHERA, M. BENYAAKOV, A. PELED and Z. BENTWICH, The immune status of SJL/J mice in relation to age and spontaneous tumor development. *J. nat. Cancer Inst.* **50,** 1227 (1973).
29. A. PELED and N. HARAN-GHERA, Immunosuppression by the radiation leukemia virus and its relation to lymphatic leukemia development. *Int. J. Cancer* **8,** 97 (1971).
30. J. A. LEVY, Xenotropic viruses: Murine leukemia viruses associated with NIH Swiss, NZB and other mouse strains. *Science* **182,** 1151 (1973).

Europ. J. Cancer Vol. 11 pp. 451–455. Pergamon Press 1975. Printed in Great Britain

On The Nature of Blocking Factors and Their Lymphoid Target Cells in an Allogeneic Tumor System

ALBERTO MANTOVANI and FEDERICO SPREAFICO

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62–20157 Milano, Italy.

Abstract—*Employing the allogeneic tumor system of C3H mice transplanted with the L_{1210} leukemia, evidence was obtained that serum blocking activity can be due to antigen-antibody complexes. In this system, the cytotoxic activity induced in normal splenocytes by arming antibody is not modified by exposure to blocking sera. It is suggested that in allogeneic tumor systems, blockade can be due to antigen-antibody complexes and that blocking factors and arming antibodies act on different lymphoid populations.*

INTRODUCTION

IT HAS been shown repeatedly in the last few years that serum factors can effectively modulate the activity of lymphoid cells, either inducing or impairing their cytotoxicity towards target cells. Blocking by serum of cell-mediated immunity (CMI) has in fact been shown to occur in syngeneic as well as allogeneic conditions [1–6] and more recently, evidence was presented supporting the participation of the phenomenon of antibody-dependent cellular cytotoxicity in the mechanisms of target cell destruction [7–9].

The nature of blocking factors is still a matter of discussion especially as regards allogeneic tumor systems, a condition for which little information is presently available. There is also insufficient knowledge concerning the type of effector population susceptible to serum inhibition. The findings reported here may be relevant to both these questions; evidence in fact is presented supporting the concepts that serum blocking and arming factors act on different lymphoid cell populations and that antigen-antibody complexes can be responsible for blocking in allogeneic tumor conditions.

Accepted 2 January 1975.

MATERIAL AND METHODS

Female C3H (H-2^K) mice were used for all experiments. The animals were injected i.p. with 10^7 cells of the allogeneic L_{1210} leukemia (H-2^d); in these conditions an initial phase of tumor growth occurs reaching its peak on days 6–7, as ascertained by counting the leukemia cell numbers in the peritoneal cavity [10]; thereafter tumor rejection ensues which is generally complete by day 12 [11]. Sera were obtained from pools of 4–6 tumor allografted mice either during active tumor growth (days 4–7) or after rejection, e.g. over 30 days after leukemia inoculation, and heat inactivated at 56°C for 30 min.

These sera are hereafter referred to as S4, S5, etc., depending on the day of collection. Serum (S4Rx and S5Rx) was also obtained from C3H mice submitted to 800® whole body X-irradiation 24 hr before receiving the same tumor inoculum. In this latter condition mean survival time was $4{\cdot}8 \pm 0{\cdot}5$ days and the animals

showed no evidence of humoral or cellular immune reactivity against the L_{1210} leukemia when their splenocytes or serum were assayed *in vitro* employing the same testing conditions described below.

Cellular cytotoxicity of C3H splenocytes against L_{1210} leukemia elements was evaluated by a short term ^{51}Cr release assay, essentially as described by Brunner *et al.* [2]; to investigate the interactions between early and late sera on lymphocyte cytotoxicity, 0·2 ml of undiluted early serum was mixed with 0·1 ml of a 1:10 dilution of late serum, ^{51}Cr-labelled leukemia cells (5×10^4 in 1 ml) were then added to the tubes (Cat. No. 1090, Nunc. Denmark) immediately followed by 5×10^6/0·2 ml normal or immune C3H splenocytes (e.g. collected 8 days after the i.p. injection of 10^7 cells unless otherwise stated). In control tubes which contained only early or late sera, final volume was achieved by adding appropriately diluted normal C3H serum and further control tubes containing only normal serum were run in every test. For serum dilution and preparation of cell suspensions, RPMI 1640 medium containing 10% fetal calf serum was used. The samples, 4 replicates per experimental group, were incubated for 4 hr at 37°C in a humidified atmosphere of 5% CO_2 in air.

Specific cytotoxicity was calculated on individual tubes as described by Plata *et al.* [12]; the isotope release in the presence of normal spleen cells was substracted from the values measured on the tubes with immune cells and normal and test sera. Spontaneous isotope release from the target cells was 13 ± 1·5%. In the 3 experiments performed, no significant modifications from this value were seen when normal or test sera were added to the tubes in the absence of spleen cells, thus indicating that the observed modifications by test sera of cellular toxicity were not attributable to "feeder" or toxic effects on the target cells.

Serum arming and potentiating activities, *i.e.*, the ability of sera to render specifically cytotoxic normal lymphocytes and to increase the cytocydal efficiency of immune elements respectively, as well as serum blocking activity were calculated as proposed by Hellström *et al.* [13]. Results presented are representative data of 3 experiments performed.

RESULTS

In sera obtained from irradiated or untreated mice 4 and 5 days after tumor allografting, no blocking or potentiating activities could be detected (Table 1). On the other hand, when S6 and S7 were tested, a definite blocking activity was observed, the reductions in specific cytotoxicity being 20 and 26% respectively. Sera taken from animals which had rejected the tumor allograft were found not only to possess a direct C-dependent cytotoxic capacity

Table 1. *Effect of early and late sera singly or in combination on the cytotoxic activity of immune splenocytes*

Serum	% Specific Cytotoxicity (± S.E.)	% Change*
Normal	21·3 ± 0·6	—
S4	21·8 ± 0·5	—
S4 Rx	21·2 ± 0·4	—
S5	20·3 ± 0·8	—
S5 Rx	21·1 ± 0·9	—
S6	17·0 ± 0·7	−20†
S7	15·8 ± 0·9	−26†
Normal	17·8 ± 1·3	—
S4 Rx	18·6 ± 1·3	—
S53	21·3 ± 0·6	+20‡
S53 + S4 Rx	13·4 ± 1·6	−25‡
Normal	16·7 ± 1·1	—
S5 Rx	19·3 ± 3·9	—
S41	25·6 ± 1·1	+53‡
S41 + S5 Rx	10·1 ± 1·0	−39‡

* − and + signs indicate blocking and potentiation respectively.
† = $P < 0.01$ by Dunnet test.
‡ = $P < 0.01$ by analysis of variance.

but also to exhibit a clear potentiating activity, the increase in specific cytotoxicity displayed by day 8 immune splenocytes after exposure, for instance, to S41 and S53, being of 53% and 20% respectively. When these late sera were mixed *in vitro* with S4Rx or S5Rx in the conditions described, it was found that not only a disappearance of the potentiating activity of the former occurred but that actually a definite blocking of cytotoxicity resulted (Table 1).

In the presence of normal spleen cells, immune late sera such as S33, S41 and S53 showed a clear arming capacity with 93, 55 and 59% increases in specific cell killing respectively. This arming activity completely disappeared when these regressor sera were mixed *in vitro* with S4Rx or S5Rx (Table 2); the same suppression was seen also when late sera were combined with early sera obtained from non-irradiated mice, as shown in Table 2 by the representative S4-S53 combination; results obtained with other mixtures of arming and early sera such as S4-S33 and S5-S41 were qualitatively analogous. However, when an arming serum such as S33, was combined with a directly blocking serum, *i.e.*, S6 or S7, no reduction in the arming capacity of the former could be seen; in fact the cytotoxicity values observed in the presence of the serum mixture were not significantly different from those found when only arming serum had been added to the tubes (Table 2).

In another series of experiments we investigated whether the cytotoxic capacity of splenocytes obtained at different times after the tumor allograft showed a different susceptibility to the synergistic or antagonistic activity of serum factors. Therefore spleen cells were collected 7, 9 and 11 days after tumor allografting, *i.e.*, before, concomitantly with and after the peak of CMI which, in our conditions occurred on day 9 (Fig. 1). Sera were obtained either during (S9) or immediately after rejection (S13) and their arming activities determined. They were then employed at the highest dilution still showing significant activity. Specifically, S9 induced a 6% increase in specific cytotoxicity (corresponding to 60% arming) at the final dilution of 1:12 employed; for S13 the value was 10% (100% arming) at a 1:120 dilution. It can be seen from Fig. 1 that both S9 and S13 significantly potentiated the cytotoxicity of day 7 splenocytes with an average 11% increase in specific cytotoxicity, however the cell killing capacity of days 9 and 11 immune splenocytes was clearly decreased after exposure to these same sera; in fact the

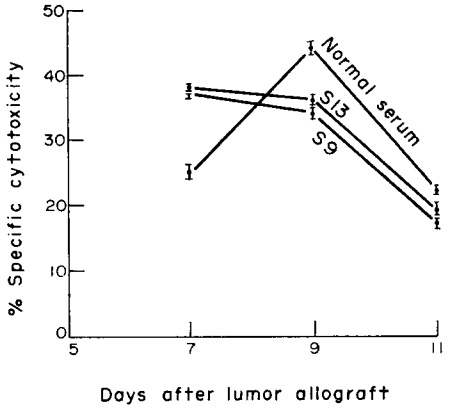


Fig. 1. Effect of S9 and S13 on the cytotoxic activity of immune splenocytes collected at different times after tumor allograft.

Table 2. Cytotoxic activity of normal splenocytes in the presence of single or combinations of early and late sera from allografted mice

Serum	% ^{51}Cr release (± s.e.)	% Arming
Normal	11·9 ± 1·0	—
S4 Rx	12·5 ± 0·5	—
S4	13·2 ± 0·5	—
S53	18·9 ± 1·3	59*
S53 + S4 Rx	12·6 ± 0·9	—
S53 + S4	14·0 ± 1·9	—
Normal	15·0 ± 0·8	—
S6	16·1 ± 0·7	—
S7	17·1 ± 1	—
S33	29·1 ± 0·6	93*
S33 + S6	30·1 ± 1·2	103*
S33 + S7	28·3 ± 1·0	89*

*$P < 0·01$.

specific cytotoxicity values were from 4 to 10% lower ($P < 0{\cdot}05$) in the presence of these test sera than when observed with normal C3H serum.

DISCUSSION

In that no evidence of immune reactivity against the allogeneic leukemia nor towards other stimuli such as foreign erythrocytes, could be detected in the irradiated, tumor-allografted animals, it seems reasonable to conclude that the factor present in early sera from tumor-bearing mice responsible for the abolition of the potentiating capacity of late sera is represented by circulating alloantigen(s). Since the existence of *in vivo* preformed complexes or of possibly blocking antibodies in these irradiated hosts can, on the same grounds, also presumably be excluded, then the appearance of a blocking activity after the *in vitro* combination of early with late sera can be ascribed to complexing of the circulating antigenic material in the former serum with the antibody present in the latter. These data would thus seem to indicate that antigen–antibody complexes can be responsible for blockade in allogeneic tumor systems as observed in syngeneic conditions [14, 15]. This conclusion thus confirms, by a different experimental approach, the results recently obtained by Cohen *et al.* [6] employing fractionation techniques.

In our system, *in vitro* treatment with rabbit antitheta serum [16] and complement can abolish the cytocydal capacity of immune spleen cells; however, such a treatment was without influence on the cytotoxic capacity of armed splenocytes, a finding which points towards the conclusion that non-T cells are the target population for arming antibody as proposed by various authors [9, 17].

In view of our observation that S6 and S7 possessing per se a blocking activity on immune splenocytes, did not however interfere with the capacity of late sera to arm non-immune lymphocytes, it seems reasonable to conclude that blocking factors and arming antibody act on different cell populations. It remains to be established whether this, (to our knowledge, a novel finding) also holds true for syngeneic tumor systems.

The proposition that different lymphocyte populations are the targets for blocking and arming serum activities, more specifically, that armed non-T cells are not susceptible to the same blocking factors which can act on T lymphocytes, may offer a basis for explaining the finding that the same sera which increased the cytotoxic activity of immune splenocytes harvested 7 days after tumor allograft, impaired the cell-killing capacity of days 9 and 11 splenocytes. It has been demonstrated [18] that in allogeneic tumor systems, early CMI depends mainly on the cytocydal activity of non-T, presumably B cells, whereas at later times, T elements contribute in a relatively larger extent to cellular cytotoxicity. Since, as concluded here, armed non-T cells are not susceptible to serum blockade, a synergistic effect of serum on cellular cytotoxicity would thus more likely be expected to occur at earlier times, whereas later on in the course of the response when T cells predominate, inhibition of CMI by blocking factors would then be the more easily observable finding.

REFERENCES

1. K. E. Hellström and I. Hellström, Immunological enhancement as studied by cell cultures techniques. *Ann. Rev. Microbiol.* **24,** 373 (1970).
2. K. T. Brunner, J. Mauel, J. C. Cerottini and B Chapuis, Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells *in vitro*; inhibition by iso-antibody and by drugs. *Immunology* **14,** 181 (1968).
3. T. G. Canty and J. R. Wunderlich, Quantitative *in vitro* assay of cytotoxic cellular immunity. *J. nat. Cancer Inst.* **45,** 761 (1970).
4. R. F. Todd III, R. D. Stulting and G. Berke, Mechanism of blocking by hyperimmune serum of lymphocyte-mediated cytolysis of allogeneic tumor cells. *Cancer Res.* **33,** 3203 (1973).
5. S. C. Bansal, I. Hellström, K. E. Hellström and P. W. Wright, Cell-mediated immunity and blocking serum activity before and after breakage of allograft tolerance in rats. *Transplantation* **16,** 610 (1973).
6. J. M. Cohen, S. S. Yang and L. W. Law, Abrogation of cell-mediated immunity by hyperimmune alloantiserum: mechanisms and correlation with allograft enhancement. *Int. J. Cancer* **13,** 463 (1974).
7. R. Kiessling and E. Klein, Cytotoxic potential of mouse spleen cells on H-2 antibody-treated target cells. *J. exp. Med.* **137,** 527 (1973).

8. E. W. LAMON, B. ANDERSSON, H. WIGZELL, E. M. FENYÖ and E. KLEIN, The immune response to primary MSV tumors in BALB/c mice: cellular and humoral activity of long-term regressors. *Int J. Cancer* **13,** 91 (1974).
9. P. PERLMANN, H. PERLMANN and H. WIGZELL, Lymphocyte-mediated cytotoxicity *in vitro*. Induction and inhibition by humoral antibody and nature of effector cells. *Transplant. Rev.* **13,** 91 (1972).
10. A. MANTOVANI, A. VECCHI, A. ANAGLERIO and F. SPREAFICO, A new technique for the measurement of ascites tumor cell numbers. *Cancer Chemother. Rep.* **58,** Pt. 1, 741 (1974).
11. A. MANTOVANI, A. VECCHI and F. SPREAFICO, Evidence for an *in vivo* role of antibody-dependent cellular cytotoxicity in tumor allograft resistance. Submitted for publication.
12. F. PLATA, E. GOMARD, J.-C. LECLERC and J. P. LEVY, Further evidence for the involvement of thymus-processed lymphocytes in syngeneic tumor cell cytolysis. *J. Immunol.* **111,** 667 (1973).
13. I. HELLSTRÖM, K. E. HELLSTRÖM and G. A. WARNER, Increase of lymphocyte-mediated tumor-cell destruction by certain patient sera. *Int. J. Cancer* **12,** 348 (1973).
14. H. O. SJÖGREN, I. HELLSTRÖM, S. C. BANSAL and K. E. HELLSTRÖM, Suggestive evidence that the "blocking antibodies" of tumor-bearing individuals may be antigen–antibody complexes. *Proc. nat. Acad. Sci. (Wash.)* **68,** 1372 (1971).
15. R. W. BALDWIN, M. R. PRICE and R. A. ROBINS, Blocking of lymphocyte-mediated cytotoxicity for rat hepatoma cells by tumor-specific antigen–antibody complexes. *Nature New Biol.* **238,** 185 (1972).
16. E. S. GOLUB, Brain-associated theta antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol.* **2,** 353 (1971).
17. J. A. VAN BOXEL, J. D. STOBO, W. E. PAUL and I. GREEN, Antibody-dependent lymphoid cell-mediated cytotoxicity: no requirement for thymus-derived lymphocytes. *Science* **175,** 194 (1971).
18. J. FORMAN and S. BRITTON, Heterogeneity of the effector cells in the cytotoxic reaction against allogeneic lymphoma cells. *J. exp. Med.* **137,** 369 (1973).

Europ. J. Cancer Vol. 11, pp. 457–458. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer — *June, 1975*

H. F. Jeejeebhoy: Immunological studies of women with primary breast carcinoma.

G. Klein and E. Klein: Are methylcholanthrene induced sarcoma associated, rejection inducing (TSTA) antigens, modified forms of H-2 or linked determinants?

J. S. Greenberger, J. R. Stephenson and S. A. Aaronson: Temperature sensitive mutants of murine leukemia virus V: Impaired leukemogenic activity *in vivo*.

B. Glimelius, K. Nilsson and J. Pontén: Lectin agglutinability of non-neoplastic and neoplastic human lymphoid cells *in vitro*.

M. Moore, N. Lawrence and N. W. Nisbet: Tumour inhibition mediated by BCG in immunosuppressed rats.

P. Brookes, P. Jones and J. Amos: The nature of the deoxyribonucleosides involved in the binding of carcinogenic hydrocarbons to the DNA of mouse embryo cells.

R. S. Kerbel, H. F. Pross and E. V. Elliott: Origin and partial characterization of F_c receptor-bearing cells found within experimental carcinoma and sarcomas.

R. Kiessling, G. Petranyi, G. Klein and H. Wigzell: Genetic variation of *in vitro* cytolytic activity and *in vivo* rejection potential of non immunized semisyngeneic mice against a mouse lymphoma line.

J. W. Pearson, K. Perk, M. A. Chirigos, J. W. Pryor and F. S. Fuhrman: Histological and combined chemo-immunostimulation therapy studies against a murine leukemia.

A. Billiau, V. G. Edy, E. De Clercq, H. Heremans and P. De Somer: Influence of interferon on virus-particle synthesis in oncornavirus carrier lines. III. Survey of effects on A-, B-, and C-type oncornaviruses.

F. Lešnik and L. J. N. Ross: Immunization against Marek's disease using Marek's disease virus-specific antigens free from infectious virus.

M. Small and N. Trainin: Inhibition of syngeneic fibrosarcoma growth by lymphocytes sensitized on tumor cell monolayers in the presence of the thymic humoral factor.

L. D. Berman: Lack of correlation between growth characteristics, agglutinability by plant lectins and the malignant phenotype.

T. Fleischmann and F. Bodor: Chromosome banding anomalies in acute myeloid leukaemia.

M. A. Wainberg, Y. Markson, F. Doljanski and D. W. Weiss: Reactivity of serum from Rous sarcoma-bearing chickens with autochthonous and with allogeneic tumor cells: Preferential autochthonous recognition.

I. C. M. MacLennan, D. G. L. Gale and J. Wood: Resistance of certain leukaemic myeloblasts to immunological attack.

J. Werner, H. Wolf, J. Apodaca and H. zur Hausen: Lymphoproliferative disease in a cotton-topped marmoset after inoculation with infectious mononucleosis-derived Epstein–Barr virus.

D. M. P. Thomson: Soluble tumour-specific antigen and its relationship to tumour growth.

British Journal of Cancer *June, 1975*

I. P. Griffith, N. E. Crook and D. O. White: Protection of mice against cancer by immunization with membranes, but not purified virions, from virus -infected cancer cells.

G. R. Flannery, H. K. Muller and R. C. Nairn: Lymphoreticular response to a syngeneic rat tumour: Gravimetric and histological studies.

A. P. P. Nind, N. Matthews, E. A. V. Pihl, J. M. Rolland and R. C. Nairn: Analysis of inhibition of lymphocyte cytotoxicity in human colon carcinoma.

J. M. Birch, M. Moore and A. W. Craig: Cell surface antigen expression on chemically-induced murine leukaemias.

T. C. M. Morris, T. A. McNeill and J. M. Bridges: Inhibition of normal human *in vitro* colony-forming cells from leukaemic patients.

P. Ebbesen and L. Olsson: Chalone-like effect abrogated by dextran sulphate and heparin polyanion pretreatment of target cells.

M. S. Al-Adnani, J. A. Kirrane and J. O'D. McGee: Inappropriate production of collagen and prolyl hydroxylase by human breast cancer cells *in vivo*.

E. Langvad and B. Jemec: Prediction of local recurrence in colo-rectal carcinoma an LDH isoenzymatic assay.

J. S. Harington, N. D. McGlashan, E. Bradshawe, E. W. Geddes and L. R. Purves: A spatial and temporal analysis of four cancers in African gold miners from Southern Africa.

J. L. Foster, I. R. Flockhart, S. Dische, A. Gray, I. Lenox-Smith and C. E. Smithen: *Short Communication.* Serum concentration measurements in man of the radio-sensitizer Ro-07-0582: Some preliminary results.

A. Wolf and K. A. Steele: *Short Communication.* Separation of a tumour-specific transplantation-type antigen from the ascitic fluid of mice bearing a syngeneic lymphoma.

D. Blakeslee, M. Chen and J. C. Kennedy: *Short Communication.* Aggregation of Chlorambucil *in vitro* may cause misinterpretation of protein binding data.

E. A. Rogers, J. S. Wishnok and M. C. Archer: *Short Communication.* Effect of diet on den clearance and carcinogenesis in rats.

John J. Gart: *Letter to the Editor.*
R. Peto: *Letter to the Editor (Reply).*

I. Todd: *Book Review.* Fundamentals of radiation therapy and cancer chemotherapy.
O. G. Dodge: *Book Review.* Tumours of the major salivary glands.

Europ. J. Cancer Vol. 11, pp. 459–460. Pergamon Press 1975. Printed in Great Britain

Errata

J. NORELLE LICKISS, KAREN A. CANE and ALBERT G. BAIKIE: *In vitro* drug selection in antineoplastic chemotherapy. *Europ. J. Cancer* **10,** 809 (1974).

Due to a publisher's error the caption to Figure 4 was omitted. The complete figure and caption are reproduced below.

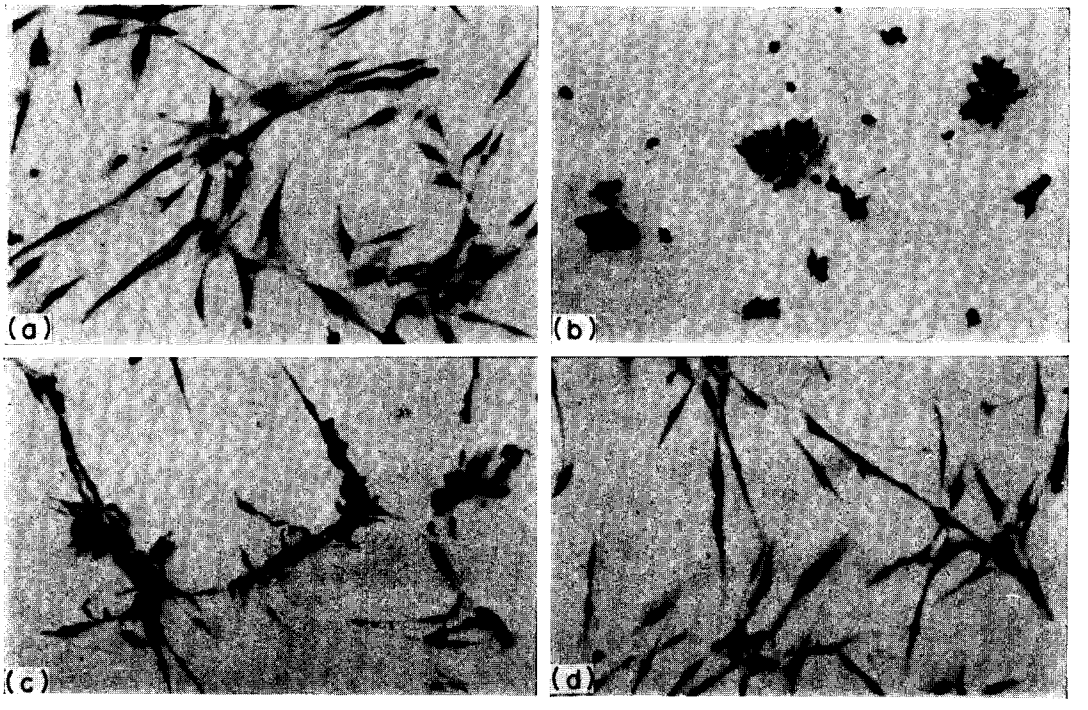


Fig. 4. Coverslip cultures from osteolytic lesions of rib, probably reticulum cell sarcoma, 48 hr after 48 hr of exposure to drugs. (a) No drugs; (b) Vincristine; (c) Cyclophosphamide; (d) fluorouracil.

HANNELORE BRAUNSBERG: The determination of high-affinity oestrogen receptors in breast tumours. *Europ. J. Cancer* **11,** 101 (1975).

Page 102, column two, line nine should read:

$$A = \frac{P+Q}{F} - \frac{Q_O}{K_Q}$$

Europ. J. Cancer Vol. 11, p. 461. Pergamon Press 1975. Printed in Great Britain

Announcement

A course in Malignant Disease in Childhood

London — Manchester — Oxford

1-3 February 1976

This London-based course, directed by Professor R. M. Hardisty of the Institute of Child Health, will be chiefly devoted to recent advances in the subject of paediatric oncology, including theoretical, experimental and epidemiological aspects, as well as up-to-date information on diagnosis and treatment and the organisation of clinical services. It includes visits to the Institute of Cancer Research, Surrey, the Radcliffe Infirmary, Oxford, and the Christie Hospital and Holt Radium Institute, Manchester.

Topics to be covered include: Cell kinetics and cytogenetics in malignant disease; Principles of cancer chemotherapy and immunotherapy; Statistical design of therapeutic trials; Epidemiology of malignant disease in childhood; Immunodeficiency and the incidence of malignant disease; Effects of radiotherapy and chemotherapy on lymphocyte and granulocyte function; Acute leukaemias; Burkitt's lymphoma; Intracranial neoplasms; Recent advances in treatment of neuroblastoma, Wilms' tumour etc; Protective environments, granulocyte transfusion and other supportive measures; Catecholamine excretion and its determination; Investigation of cardiac function in patients receiving cardiotoxic drugs; Psychological effects of malignant disease; Regional organisation of paediatric oncology services.

The course is open to: experienced paediatric oncologists and interested general paediatricians, who are completely proficient in the English language.

Fee: £305 (demi-pension—London, full pension elsewhere)

Apply to: Director, Courses Department
The British Council
65 Davies Street
London W1Y 2AA, U.K.

Application Deadline: 1 October, 1975

Europ. J. Cancer Vol. 11, pp. 463–472. Pergamon Press 1975. Printed in Great Britain

Hypoxanthine Phosphoribosyltransferase Activity in Normal, Developing, and Neoplastic Tissues of the Rat*

ROBERT M. WOHLHUETER†
Laboratory for Experimental Oncology, Indiana University, School of Medicine, Indianapolis, IN, U.S.A.

Abstract—*The activity of hypoxanthine phosphoribosyltransferase, an enzyme of purine salvage, has been measured in ten normal tissues of the rat, in regenerating liver, in developing liver, in starved and glucocorticoid-treated rats, and in several Morris hepatomas. A new method was employed, making a multiple-time-point assay practicable. Liver contained the highest activity of those tissues assayed; activity was maximum in this tissue at 32 days after birth. No correlation of enzyme activity with tissue growth rate was apparent.*

Kinetic studies on the enzymes from normal liver and Morris hepatoma 3924A indicated half-maximal velocities were attained at concentrations of about 4 μM hypoxanthine, and about 5 μM phosphoribosylpyrophosphate.

INTRODUCTION

HYPOXANTHINE phosphoribosyltransferase (HPRT, E. C. 2.4.2.8) catalyzes the transfer of the 5-phosphoribose moeity from phosphoribosylpyrophosphate (PRPP, α-D-ribofuranose-1-pyrophosphate-5-phosphate) to hypoxanthine or guanine, thereby salvaging these free purines and returning them to the purine nucleotide monophosphate pools. In mammals, activity with both hypoxanthine and guanine resides in a single enzyme [1], existent perhaps as isozymes [2]. Another enzyme catalyzes the analogous reaction with adenine [3].

Atkinson, Murray and their coworkers have contributed much to our knowledge of the purine phosphoribosyltransferases in normal tissues and ascites tumors of laboratory animals [4–7]. Yet the significance of purine salvage to normal cell physiology remains an open question [8, 9]. Congenital absence of HPRT in humans, though, is associated with a profound upset of purine metabolism [10], and elevated excretion of oxypurines. This disease has been the focal point of a good deal of research on purine phosphoribosyltransferases [11].

Of particular importance to cancer chemotherapy is the fact that HPRT is responsible for the conversion of purine analogs to their pharmacologically active nucleotide forms [12].

The present study was undertaken as part of a larger project to delineate the pattern of activities of purine synthetic and degradative enzymes in the Morris series of experimental hepatomas. Specifically, we consider here the matters of tissue distribution of HPRT, of a kinetic comparison of normal and neoplastic versions of the enzyme, and of modulation of HPRT activity as a necessary accessory to rapid cell proliferation.

Accepted 8 April 1975.

*This investigation was supported by the United States Public Health Service grant no. CA-13526 (National Cancer Institute).

†Correspondence to author's present address: Department of Microbiology, University of Minnesota School of Medicine, Minneapolis, MN 55455, U.S.A.

MATERIAL AND METHODS

Material

Sodium 5-phosphorylribose-1-pyrophosphate was obtained from Sigma Chemical Co. (St. Louis, Mo.). Where reaction dependence on the concentration of PRPP ([PRPP]) was studied, the actual PRPP content of the stock solution was determined by the spectrophotometric method of Kornberg *et al.* [13] to be 53 % of nominal content. Routinely, stocks were considered 50 % pure.

[8-^{14}C]hypoxanthine (59 Ci/mole) was purchased from Amersham/Searle (Arlington Heights, Ill.) and diluted with non-radioactive hypoxanthine (Sigma Chemical Co.) to about 7 dis/min per pmole. Concentration and specific radioactivity of stock solutions were verified by spectrometry (ε = 10800, [14]) and internally calibrated scintillation counting.

Pre-washed, thin-layer chromatography sheets of polyethyleneimine-impregnated cellulose (Macherey and Nagel "Polygram Cel 300 PEI") were obtained from Brinkmann Instruments (Westbury, N.Y.). When used for chromatography, these were soaked in 10 % (w/v) NaCl and rinsed in distilled water; for routine sequestration of reaction products such pretreatment was unnecessary.

Triamcinolone diacetate ("Aristocort") was the product of Lederle Laboratories (Pearl River, N.Y.). Other chemicals were analytical grade from standard suppliers, and were used as delivered.

Experimental animals

Rats were kept in individual cages in air-conditioned quarters illuminated 6 a.m.–7 p.m. Purina laboratory chow and water were available *ad libitum*, unless otherwise noted. Rats were killed between 9 and 10 a.m. Males were used in all experiments except those with newborns.

Hepatomas of different growth rates [15] were transplanted s.c. bilaterally in Buffalo or ACI/N rats at the laboratory of Harold P. Morris at Howard University and transported by air to Indianapolis. The tumors were harvested when they reached a diameter of about 1·5 cm and dissected free of hemorrhagic, necrotic, and connective tissue. Normal rats of the same strain, sex, and age were killed concurrently, and their livers assayed for enzyme activity as a control tissue.

Partial hepatectomy was performed under ether anesthesia by the procedure of Higgins and Anderson [16], which removes about two-thirds of the liver. Rats subjected to a sham operation served as controls. Livers were removed for assay 24 hr after surgery.

Tissue preparations

Tissues were excised from stunned, decapitated animals and placed in beakers on ice. They were minced finely with a scissors and homogenized (5 % fresh w/v) with a Teflon-pestle tissue grinder (Thomas Scientific, Philadelphia, Pa.) in 0·15 M KCl, whose pH was adjusted to 7·4 with $KHCO_3$. The homogenates were then centrifuged at $10^5 \times g$ for 30 min at 4°, and the supernates taken as source of enzyme.

Assay of HPRT activity

The method of assay was based in principle on that of Atkinson and Murray [4], namely the separation of product [^{14}C]nucleotide from substrate [^{14}C]base by binding the former on an anion-exchanger. Use of PEI-cellulose plates as ion exchanger, however, simplifies the separation, and makes possible batch processing, thus rendering practicable an assay with consecutive aliquots of the reaction mixture.

We adopted the following standard assay: A reaction mixture of 100 µl contained 200 mM triethanolamine/HCl buffer, pH 7·8, 5 mM $MgCl_2$, 0·2 mM [^{14}C]hypoxanthine (typically 7·3 dis/min per pmole), *ca.* 0·2 mM PRPP, and 10 µl of tissue supernate (corresponding to 0·5 mg fresh tissue). The mixture, less PRPP, was brought to 30° and the reaction started by addition of 10 µl of approximately 2 mM PRPP. Twenty µl aliquots were removed with an Oxford Sampler (Oxford Laboratories, Foster City, Cal.) at various times, normally 4, 8, 12 and 16 min, spotted on PEI-cellulose plates, and dried with a hot air blower which developed about 70° at the surface of the plate. The appearance of PEI-bound radioactivity is a measure of enzyme activity.

In practice, a 10 × 20 cm sheet of PEI-cellulose was penciled into 32 numbered fields, 2·5 × 2·5 cm, each row of 4 accommodating the 4 aliquots from a single reaction. Finished sheets were accumulated until all reactions were completed, then cut into 5 × 10 cm pieces and placed in alternate slots of multi-plate chromatography tanks (Brinkmann Instruments). Unreacted hypoxanthine was washed off the plates by filling the tank (*ca.* 600 ml) with distilled water, letting it stand 5 min without agitation, then pouring it off. Plates were thus washed 3 times with water and once with absolute methanol. After drying in air, the individual

fields were cut out, placed upright in scintillation vials, and 10 ml of toluene scintillator (3·0 g PPO (2,5-Diphenyloxazole) + 0·2 g POPOP (1,4-Bis-[2-(5-phenyloxazolyl)]-benzene) per liter) added. Scintillation counting was done in a Model 3380 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.).

Except in studies of reaction kinetics, data were not plotted normally, but analyzed (Programma 101 computer, Olivetti-Underwood Corp., New York, N.Y.) by linear regression to give a slope and its s.e. [17]. When the s.e. was less than 5 % of the slope, the reaction was considered linear, and the slope calculated as international enzyme units, taking the specific radioactivity of the hypoxanthine and the ^{14}C-counting efficiency on PEI-plates into consideration.

Cell counts

The cell counts were made as described by Weber and Cantero [18]; cellularity was expressed as millions of nuclei per gram of fresh tissue.

Protein determination

Protein content of supernatant fractions was determined by the Lowry method as described by Dawson *et al.* [14] with bovine serum albumin as standard.

RESULTS

Assay of HPRT activity

The method of assay developed here is technically undemanding and capable of measuring HPRT activity of 100 μg of rat liver. Centrifugation of crude homogenates is essential. Whole homogenate of liver yields as little as 1/6 the activity apparent in high-speed supernates. Preliminary experiments indicate much of the disparity is accounted for by 5'-nucleotidase, a membrane-bound enzyme [19]. The efficiency of extraction in 0·15 M KCl of activity from liver could not be surpassed by homogenization in H_2O or in 0·25 M sucrose, by sonication, nor by addition of 1 mM dithioerythritol to the medium. Supernatant activity was stable for 8 hr on ice, and for at least 18 days at −25°.

Sampling at four times ensures that the activity measurement is based on an initial period of linear reaction velocity; proportionality of activity with amount of supernate added is demonstrated for liver and hepatoma 3924A in Fig. 1. It appears that the reaction is adequately stopped by binding of substrate PRPP upon contact with PEI-cellulose and the subsequent rapid drying. [^{14}C]Hypoxanthine was quantitatively removed by the washing procedure, leaving, typically, backgrounds of 200 counts/min of a total of 22000 counts/min applied. Radioactive inosine monophosphate acid (IMP) bound to PEI-cellulose plates was not diminished by repeated washing with water or methanol. Neither hypoxanthine nor IMP was eluted from plates by toluene scintillator, and counting efficiency on the plates was found to be 77 %.

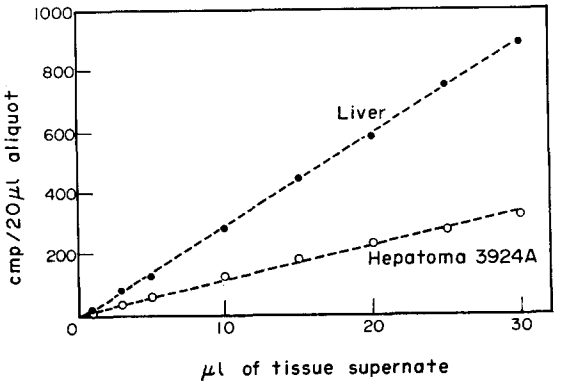


Fig. 1. Velocity of the hypoxanthine phosphoribosyltransferase reaction as a function of amount of tissue supernate. Reaction mixtures contained, in a total volume of 100 *μl,* 200 *mM triethanolamine/HCl, pH* 7·8, 5 *mM* $MgCl_2$, ca. 0·2 *mM PRPP,* 0·2 *mM* [8-^{14}C]*hypoxanthine* (7·3 *dis/min per pmole*), *and* 0–30 *μl of supernate prepared from* 5% (*w/v*) *homogenates of liver or hepatoma* 3924*A in* 0·15 *M KCl. Reactions* (30°) *were started with PRPP and four* 20 *μl samples taken periodically up to* 16 *min for assay of nucleotide product. Reaction velocities are calculated from the slopes of the product appearance curves. Each point represents a single reaction; the lines were drawn according to the least-squares principle and their slopes have s.e. of* 1 *and* 2% *for liver and hepatoma, respectively.*

Reaction velocities in the absence of liver or hepatoma supernates, or of PRPP were generally negligible, and not regularly carried out. Caution, though, is in order, since with larger quantities of liver (corresponding to ≧ 1 mg tissue) or minimal amounts of intestinal mucosa, an accelerating reaction became apparent which was not dependent on Mg^{2+} or PRPP. The nature of this reaction has not been established. Such aberrations, though, are detected by the criterion of linearity outlined above.

The pH profiles of activity in supernates from liver and hepatoma 3924A were qualitatively similar. In Tris–maleate–glycine buffer (37·5 mM each) with 0·2 mM PRPP and

5 mM $MgCl_2$ there was a near-plateau at pH 7·8 and a sharp peak of activity at pH 9·5. The shallow slope at pH 7·8 was deemed a more suitable region for assay than the peak, since the positions of the peak and the sharp drop on its alkaline side were contingent on the concentration of Mg^{2+}([Mg^{2+}]), presumably reflecting changes in complexation of PRPP. At pH 7·8, activity at 37° was 1·3 times that at the standard assay temperature of 30°.

As a consequence of the washing procedure, all PEI-bound radioactivity is interpreted as product of the HPRT reaction. In fact, the product is largely IMP. Thus, when a reaction mixture with liver supernate, in which 44 % of the initial hypoxanthine had been converted to PEI-bound material, was chromatographed with 1 M LiCl as mobile phase [20], 92 % of this material cochromatographed with IMP, and 4 % each with AMP and ADP.

Kinetic properties of HPRT from liver and hepatoma 3924A

To optimize assay conditions in the crude enzyme preparations, and possibly to identify kinetic differences between normal and neoplastic forms of the enzyme, the dependence of initial reaction velocity on the concentration of substrates and $MgCl_2$ was studied in high-speed tissue supernates from liver and hepatoma 3924A.

Figure 2 shows the results when [PRPP] was varied at [$MgCl_2$] = 5 mM and [hypoxanthine] = 0·2 mM. The abscissa gives actual initial concentrations of PRPP as determined in the stock solution. No detrimental effect on enzyme activity can be ascribed to the substantial impurities in this PRPP preparation. The first parts of the curves correspond to rectangular hyperbolae, and double reciprocal plots of these extrapolate to Michaelis constants of 5 μM for both enzymes.

As with PRPP, there is a trend for apparent maximum velocity to increase slightly as $MgCl_2$ concentration was increased to 50 mM (not shown). Half maximal velocities were in the range of 0·2 to 0·4 mM for enzymes of liver and tumor origin, nearly 10^2 higher than that for PRPP. In up to 50 mM $MgCl_2$ we observed no inhibition attributable to formation of Mg_2PRPP complexes [21].

With hypoxanthine as variable substrate, curves typified by Fig. 3 have been obtained consistently. For the enzyme from hepatoma 3924A, the curve is a rectangular hyperbola with K_m = 4 μM. 3 μM hypoxanthine gave half-maximal velocity with the enzyme from liver.

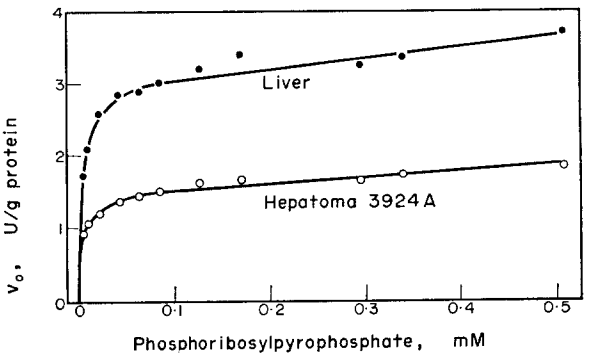


Fig. 2. Dependence of initial reaction velocity of hypoxanthine phosphoribosyltransferase on the concentration of PRPP. Reaction mixtures contained 200 mM triethanolamine/HCl, pH 7·8, 5 mM $MgCl_2$, 0·2 mM [8-^{14}C]hypoxanthine, 7·3 dis/min per pmole, up to 509 μM PRPP, and 10 μl of the high-speed supernate from a 5% tissue homogenate (45 μg liver protein or 28 μg hepatoma protein) in a final volume of 100 μl. The reactions (30°) were started with PRPP and 20 μl samples removed at 0·5, 1·5, 4·5 and 13·5 min for adsorption of product nucleotide on PEI-cellulose plates. Concentrations given are based upon the actual content of PRPP (53% of nominal) as determined spectrophotometrically by the method of Kornberg et al. [13].

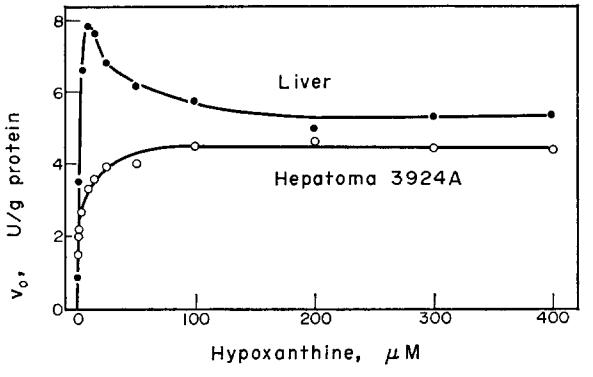


Fig. 3. Dependence of initial reaction velocity of hypoxanthine phosphoribosyltransferase on concentration of hypoxanthine. Reaction mixtures contained 200 mM triethanolamine/HCl, pH 7·8, 5 mM $MgCl_2$, 0·2 mM PRPP, 1 to 400 μM [8-^{14}C]hypoxanthine (7·3 dis/min per pmole), and high-speed supernate equivalent to 0·15 mg fresh liver (7·9 μg protein) or 0·5 mg hepatoma (33·8 μg protein) in a total volume of 100 μl. The reactions (at 30°) were started by addition of PRPP, and 20 μl samples were removed at 0·5, 1·5, 4·5 and 13·5 min for adsorption of product nucleotide on PEI-cellulose plates.

We have no information allowing us to decide whether this peculiar substrate dependence of the liver enzyme is some artefact of using crude enzyme preparations, or whether it indeed reflects a mechanistic feature, such as hypoxanthine's binding to 2 forms of the enzyme. In neither case could it be of physiological significance. For purposes of the standard assay a concentration of 200 μM was selected.

Tissue distribution

HPRT activity was found in all tissues measured (Table 1), generally at levels less than in liver. Results with intestinal mucosa were inconclusive: apparent initial reaction velocity was about half that of liver, but it was accelerating and independent of exogenous PRPP. The reaction(s) responsible for conversion of hypoxanthine to a PEI-bound anion in this case have not been established.

HPRT activity in developing liver

The changes in liver activity from the neonatal to the adult rat are tabulated in Table 2. On a tissue weight basis (and more pronouncedly, on a supernate protein basis) the developmental curve passes through a maximum at around 30 days. This maximum was not reflected in total liver activity, which rose monotonically to the adult level, nor in activity per average cell, which remained essentially constant after 30 days. The average size of a liver cell increases throughout the post-natal period.

Starvation and glucocorticosteroids

When rats were deprived of food, HPRT activity fell (Table 3). After six days, total liver activity was only 15% of that in fed controls. Decreases in HPRT were evident also in comparison with total supernate protein. Consistent with this behaviour in starvation is the fact that administration of the artificial glucocorticoid triamcinolone was of no consequence to enzyme levels (data not shown).

Table 1. Hypoxanthine phosphoribosyltransferase activity in various tissues of the rat. Tissues from 1 to 4 adult ACI/N rats (180–270 g) were pooled, minced finely, homogenized in duplicate in 0·15 M KCl with a glass–Teflon homogenizer, and centrifuged at 100,000 × g for 30 min. Supernates were assayed by the standard procedure, as described in the text. Marrow was aspirated from the femur. Activity in intestinal mucosa was obscured by a PRPP-independent reaction and could not be estimated. Values in parentheses are percent of liver value

Tissue	Enzyme activity	
	(units/g tissue)	(units/g protein)
Liver	0·455 (100)	5·14 (100)
Bone marrow	0·340 (74·7)	6·13 (119)
Heart muscle	0·233 (51·3)	3·78 (73·7)
Spleen	0·211 (46·5)	2·56 (50·0)
Lung	0·148 (32·5)	1·97 (38·3)
Testis	0·126 (27·7)	2·57 (50·0)
Kidney cortex	0·125 (27·5)	1·74 (33·8)
Thymus	0·076 (16·7)	1·40 (27·3)
Skeletal muscle	0·063 (13·9)	1·12 (21·8)
Brain	0·058 (12·7)	1·82 (35·4)

Table 2. Hypoxanthine phosphoribosyltransferase activity in developing rat liver. Wistar rats were killed at various ages, and liver supernates prepared and assayed for enzyme activity by the standard procedures described in the text. Cellularity, defined as millions of cells per g of fresh tissue, was determined as described [18]. *One-day and six-day groups consisted of livers pooled from 29 and 6 animals, respectively, and assayed in quadruplicate; average values ± s.e. in other groups comprise 4 or 5 animals assayed individually. Ages of the final 5 groups were estimated from normal growth curves. Values in parentheses are percentages of adult.*

Age (days)	Body weight (g)	Liver weight (g)	Cellularity	Enzyme activity (milliunits)		
				per g tissue	per million cells	per liver
1	7·07	0·255 (2·9)	610 (263)	342 (63)	0·562 (24)	87·2 (1·8)
6	13·9	0·329 (3·7)	540 (233)	371 (68)	0·687 (29)	122 (2·5)
18	33·0	0·738 (8·3)	440* (190)	543 ± 28 (99)	1·23 (53)	400 ± 17 (8·2)
26	57·8	2·38 (27)	404 ± 12 (174)	647 ± 14 (119)	1·61 ± 0·06 (68)	1540 ± 50 (32)
30	87·2	3·40 (38)	350 ± 5 (151)	741 ± 8 (136)	2·11 ± 0·02 (90)	2520 ± 110 (52)
32	94·3	4·05 (45)	350 ± 4 (151)	763 ± 5 (140)	2·17 ± 0·02 (92)	3090 ± 80 (63)
40	153	5·85 (66)	302 ± 5 (130)	756 ± 14 (139)	2·51 ± 0·04 (106)	4420 ± 300 (91)
adult	234	8·93 (100)	232 ± 3 (100)	546 ± 5 (100)	2·35 ± 0·05 (100)	4870 ± 385 (100)

*Value is interpolated.

Table 3. Hypoxanthine phosphoribosyltransferase activity in livers from starved rats. Male Wistar rats were kept without food for the period indicated, after which liver supernates were prepared and assayed by the standard procedures described in the text. In experiment I, groups were selected with an average initial body wt of 186 g; in experiment II, of 213 g. Each group contained four animals, and results are given as the mean ± s.e. of the mean, with percentage of control in parentheses

Days without food	Final wt		Enzyme activity	
	Body	Liver		
	(g)	(g)	(units/g tissue)	(units/g protein)
Expt. I				
0	209	8·55	0·698 ± 0·012 (100)	8·43 ± 0·23 (100)
1	169	5·57	0·616 ± 0·011 (88)	6·77 ± 0·30 (80)
3	147	4·20	0·582 ± 0·040 (83)	7·96 ± 0·12* (94)
Expt. II				
0	234	8·93	0·546 ± 0·005 (100)	6·38 ± 0·07 (100)
6	132	3·28	0·229 ± 0·002 (42)	2·56 ± 0·05 (40)

*Not significantly different from zero-day control, $P > 0·05$; all other values differ from their controls.

HPRT activities in hepatomas and regenerating liver

Table 4 presents enzyme activities measured in a series of experimental hepatomas encompassing a range of growth rates, as characterized by average transplantation intervals of 16 days to 1 year. Viewed statistically, nearly all values lay outside the normal range, but in no consistent pattern relative to growth rate.

Regenerating liver has a growth rate more rapid than that of the fast-growing hepatomas: the mass doubling time for regenerating liver is *ca.* 2 days [22], for hepatoma 3924A, 5·5 days [23]. HPRT activity of neither the 24 hr regenerating liver nor of liver from sham-operated rats was different from that in normal liver.

DISCUSSION

Assay system

We believe the assay method employed here has an advantage of simplicity compared with other methods currently in use [4, 24]. There is, however, a basic inconsistency in results not easily explained. Thus, our normal rat liver value of 0·53 units/g tissue exceeds that of Murray's [5], 0·077 units/g tissue, by factor 7. Other laboratories have reported values in human liver [25] and mouse liver [26] comparable to Murray's though Krenitsky has measured 1 unit/g of liver tissue from rhesus monkey [27].

Slight differences in reaction conditions used by different laboratories, and the low temperature coefficient we observe, are insufficient to account for this discrepancy. So too is the difference in centrifugal fractions employed by Murray and us. Although interference from pelleted material is substantial, this interference is nearly as effectively removed at $20,000 \times g$ for 20 min, as prescribed by Murray [5], as at $100,000 \times g$ for 30 min. Noteworthy in this regard is the activity of various rat tissues. Liver is ranked first by us and third by Murray, and that is the only difference in our rankings, so that in both rank and absolute activity we agree more closely on non-hepatic tissues than on liver.

Kinetic properties

Curves of initial reaction velocity vs substrate concentration with tissue supernates indicate half-maximal velocities are attained at about 3 μM hypoxanthine and 5μM PRPP. Excepting the anomalous behavior of the

Table 4. Hypoxanthine phosphoribosyltransferase activity in hepatomas of various growth rate and in regenerating liver. Tumors were removed from the number of rats indicated and pooled; normal livers from control rats of the same strain and age were prepared at the same time. Values in parentheses are percentages of this individual control. Hepatomas 3924A and 3683 were carried in ACI/N rats, all others in Buffalo rats. Homogenates and supernates were prepared from 4 separate samples and assayed by the standard method. Methods of tissue preparation, enzyme assay, cell counting and hepatectomy are described in the text. Tumor growth rate is given as the average time in months between transplants. The s.e. of the mean of quadruplicate supernates from the same tissue mince averaged 2·8% of the activity per g tissue

Tissue	Number of rats	Transplant generation	Growth rate (months)	Enzyme activity (units/g tissue)	Enzyme activity (units/g protein)
Normal liver					
Average ± s.d.	8			0·528 ± 0·065	6·23 ± 0·9
Hepatoma					
16	2	90	12·0	0·953 (164)	9·74 (191)
20	3	5	12·0	1·05 (164)	11·1 (189)
66	2	7	10·2	0·843 (160)	11·4 (185)
8999	1	18	6·5	0·363 (65)	5·95* (85)
7777	1	109	1·0	0·195 (39)	4·15 (61)
3924A	1	321	0·94	0·322 (73)	7·80* (102)
$9618A_2$	3	67	0·57	0·197 (40)	2·73 (52)
3683	1	523	0·53	0·750 (154)	9·90 (164)
Regenerating liver (ACI/N)					
Sham operated ± s.e.	4			0·471 ± 0·024	4·89 ± 0·38
24-hr ± s.e.	4			0·439 ± 0·025	5·09 ± 0·39

*These values lie within two s.d. of the average for normal liver.

enzyme from liver with respect to hypoxanthine, the $S_{0.5}$ values may be considered Michaelis constants. The values for hypoxanthine compare with those reported for HPRT from various sources: 9·9 μM for human erythrocyte [28] 18 μM for yeast [29], 11 μM for Ehrlich ascites cells [4]. The values for PRPP appear lower than those found with enzymes from other species: *ca.* 25 μM for Ehrlich ascites cells [6], 28 μM for mouse liver [7], 42 μM for yeast [29], 240 μM for human erythrocyte [28].

With respect to [PRPP], [$MgCl_2$] and pH the kinetic behavior of the enzymes from liver and hepatoma 3924A are similar. At this time we do not regard the differences between liver and tumor enzymes with respect to hypoxanthine sufficient to justify conclusion of different isozymes in these two tissues. The matter requires more thorough kinetic investigation of purified enzymes.

Relation of HPRT to growth rate

The data presented here provide no evidence that elevated HPRT activity is prerequisite to accelerated growth. Thus we find no obvious correlation between activities of normal rat tissues and the rates of proliferation of those tissues. Unlike the mouse, where HPRT activity in developing liver decreases by nearly half from two days of age to adulthood [7], the growing rat attains adult levels first at 18 days, though it surpasses these by 40% at 32 days of age before returning to them.

In regenerating liver, 24 hr after hepatectomy, when RNA synthesis has been maximal for some hours and DNA synthesis is at its peak [22], HPRT activity remains unchanged. Activity in a series of experimental hepatomas appears correlated negatively, if at all, with hepatoma growth rate.

The lack of positive correlation with tumor growth rate sets the purine salvage enzyme, HPRT, apart from several enzymes of pyrimidine nucleotide synthesis. Thymidine kinase [30], carbamyl-aspartate transferase [31], and dihydroorotase [31] all increase with increasing growth rate in the Morris spectrum of hepatomas. These elevations of enzyme activities presumably confer an advantage on tumor cell proliferation [32], and are accompanied by elevated incorporation of thymidine into DNA [33].

Relation of purine salvage to purine synthesis de novo and purine degradation

Based on the RNA and DNA content of hepatoma 3924A [34, 35] and its volume doubling-time [23], one may calculate that one gram of this tumor accumulates, net, 5·8 nmoles of nucleic acid residue per min. The accumulation of purine residues in nucleic acid of this hepatoma is thus some 10^2 less than the V_{max} of HPRT. The contribution of purine salvage to nucleotide synthesis under physiological conditions, and its possible modulation to meet the demands of growth, are open questions. The present results indicate that change of enzyme level is not a crucial mechanism for regulation of hypoxanthine phosphoribosylation.

At the substrate level, HPRT competes for PRPP with the amidophosphoribosyltransferase (E.C. 2.4.2.14), first enzyme of purine synthesis *de novo*, and for hypoxanthine with xanthine oxidase. All of these enzymes are in the cytosol. HPRT has an advantage of affinity over amidophosphoribosyltransferase: HPRT attains half maximal velocity at 5 μM PRPP (this paper), while $S_{0.5}$ values for the amidophosphoribosyltransferase partially purified from rat liver have been reported as 86 μM [36] and as about 500 μM (M. Tsuda, personal communication). The concentration of PRPP in rat liver has not been reported, however, Lalanne and Henderson [37] have recently measured it in freeze-clamped mouse liver; it is approximately 8 μM (24–52 nmoles/g protein). A steady-state concentration at this level would confer a crucial advantage on HPRT in comparison with amidophosphoribosyltransferase, and would, in effect, give precedence to purine salvage.

Competition with xanthine oxidase for hypoxanthine is more evenly matched. As measured here, the $S_{0.5}$ of HPRT with respect to hypoxanthine is about 3 μM; K_m of rat liver xanthine oxidase is 2–3 μM [38]. Tissue contents of hypoxanthine are, apparently, quite small. Deuticke *et al.* [39] could not detect hypoxanthine ($\leqq$ 10 μM) in rapidly frozen rat liver, nor could Busch *et al.* [40] in rabbit liver. A concentration of 38 μM hypoxanthine in human plasma has been estimated by Orsulak and coworkers [41]. Analogy to adenine would indicate very rapid turnover of such pools as might exist. Using perfused rat liver, for example, Pritchard *et al.* [42] recover no [^{14}C]adenine as free base 8 min after a pulse of this compound. Rapid turnover is consistent with the maximal velocities and low K_m's observed for the hypoxanthine-metabolizing enzymes. The activity of xanthine oxidase in the rat liver is generally estimated at about 200 milliunits/g of tissue [38, 43–45], comparable to that found here for HPRT.

One may expect thus, that the availability of hypoxanthine (and presumably of guanine) limits synthesis of IMP (or guanine monophosphate (GMP)) via HPRT, and that the balance between HPRT and xanthine oxidase levels determines the fate of hypoxanthine. In fact, xanthine oxidase activity in Novikoff hepatoma is greatly reduced in comparison with normal liver [43].

Acknowledgements—The author gratefully acknowledges the collaboration of Harold P. Morris, Department of Biochemistry, Howard University, who supplied the tumor-bearing rats, and of George Weber, of this laboratory, who excised tumors and performed hepatectomies. Thanks are due Aldo S. Bagnara and Robert C. Jackson for their helpful criticism of the manuscript.

REFERENCES

1. T. A. Krenitsky, R. Papaioannou and G. B. Elion, Human hypoxanthine phosphoribosyltransferase I. purification, properties, and specificity. *J. biol. Chem.* **244,** 1263 (1969).
2. B. Bakay and W. L. Nyhan, The separation of adenine and hypoxanthine-guanine phosphoribosyltransferase isoenzymes by disc gel electrophoresis. *Biochem. Genet.* **5,** 81 (1971).
3. J. G. Flaks, M. J. Erwin and J. M. Buchanan, Biosynthesis of purines XVI. the synthesis of adenosine 5′-phosphate and 5-amino-4-imidazolecarboxamide ribotide by a nucleotide pyrophosphorylase. *J. biol. Chem.* **228,** 201 (1957).
4. M. J. Atkinson and A. W. Murray, Inhibition of purine phosphoribosyltransferases of Ehrlich ascites-tumour cells by 6-mercaptopurine. *Biochem. J.* **94,** 64 (1965).
5. A. W. Murray, Purine phosphoribosyltransferase activities in rat and mouse tissues and in Ehrlich ascites-tumour cells. *Biochem. J.* **100,** 664 (1966).
6. A. W. Murray, Inhibition of purine phosphoribosyltransferases from Ehrlich ascites-tumour cells by purine nucleotides. *Biochem. J.* **100,** 671 (1966).

7. A. W. MURRAY, The activities and kinetic properties of purine phosphoribosyltransferases in developing mouse liver. *Biochem. J.* **104,** 675 (1967).
8. A. W. MURRAY, The biological significance of purine salvage. *Ann. Rev. Biochem.* **40,** 811 (1971).
9. K. O. RAIVIO and J. E. SEEGMILLER, The role of phosphoribosyltransferases in purine metabolism. *Curr. Top. cell. Regul.* **2,** 201 (1970).
10. M. LESCH and W. L. NYHAN, A familial disorder of uric acid metabolism and central nervous system function. *Amer. J. Med.* **36,** 561 (1964).
11. J. E. SEEGMILLER, Molecular aspects of the Lesch–Nyhan syndrome. *Biochimie* **54,** 703 (1972).
12. A. W. MURRAY, D. C. ELLIOTT and M. R. ATKINSON, Nucleotide synthesis from performed purines in mammalian cells: Regulatory mechanisms and biological significance. *Prog. nucleic Acid Res. mol. Biol.* **10,** 87 (1970).
13. A. KORNBERG, I. LIEBERMAN and E. S. SIMMS, Enzymatic synthesis and properties of 5-phosphoribosylpyrophosphate. *J. biol. Chem.* **215,** 389 (1955.)
14. R. M. C. DAWSON, D. C. ELLIOTT, W. H. ELLIOTT and K. M. JONES, *Data for Biochemical Research*, Oxford University Press, New York and Oxford (1969).
15. H. P. MORRIS and B. P. WAGNER, Induction and transplantation of rat hepatomas with different growth rate (including "minimal deviation" hepatomas). *Meth. Cancer Res.* **4,** 125 (1968).
16. G. M. HIGGINS and R. M. ANDERSON, Experimental pathology of the liver I. restoration of the liver of the white rat following partial surgical removal. *Arch. Path.* **12,** 186 (1931).
17. GEIGY PHARMACEUTICALS, *Scientific Tables.* (Edited by K. DIEM) Geigy Pharmaceuticals, Ardsley, New York (1962).
18. G. WEBER and A. CANTERO, Studies on hormonal factors influencing hepatic glucose-6-phosphatase. *Endocrinology* **61,** 701 (1957).
19. C. S. SONG and O. BODANSKY, Subcellular localization and properties of 5'-nucleotidase in rat liver. *J. biol. Chem.* **242,** 694 (1967).
20. K. RANDERATH and E. RANDERATH, Ion-exchange chromatography of nucleotides on poly(ethyleneimine)-cellulose thin layers. *J. Chromatogr.* **16,** 111 (1964).
21. R. E. A. GADD and J. F. HENDERSON, The role of magnesium ion in the adenine phosphoribosyltransferase reaction. *Canad. J. Biochem.* **48,** 302 (1970).
22. N. L. R. BUCHER and R. A. MALT, *Regeneration of Liver and Kidney.* Little, Brown, Boston (1971).
23. W. B. LOONEY, A. A. MAYO, M. Y. JANNERS, J. G. MELLON, P. ALLEN, D. SALAK and H. P. MORRIS, Cell proliferation and tumor growth in hepatomas 3924A. *Cancer Res.* **31,** 821 (1971).
24. W. GUTENSOHN and G. GUROFF, Rapid assay for purine phosphoribosyltransferases, *Analyt. Biochem.* **47,** 132 (1972).
25. F. M. ROSENBLOOM, W. N. KELLEY, J. MILLER, J. F. HENDERSON and J. E. SEEGMILLER, Inherited disorder of purine metabolism. *J. Amer. med. Assoc.* **202,** 175 (1967).
26. M. E. BALIS, Aspects of purine metabolism. *Fed. Proc.* **27,** 1067 (1968).
27. T. A. KRENITSKY, Tissue distribution of purine ribosyl- and phosphoribosyltransferases in the Rhesus monkey. *Biochim. Biophys. Acta (Amst.)* **179,** 506 (1969).
28. J. F. HENDERSON, L. W. BROX, W. N. KELLEY, F. M. ROSENBLOOM and J. E. SEEGMILLER, Kinetics of hypoxanthine-guanine phosphoribosyltransferase. *J. biol. Chem.* **243,** 2514 (1968).
29. R. L. MILLER and A. L. BIEBER, Substrate binding specificity and properties of inosine monophosphate: pyrophosphate phosphoribosyltransferase (EC 2.4.2.8) from brewers yeast. *Biochemistry* **8,** 603 (1969).
30. T. W. SNEIDER, V. R. POTTER and H. P. MORRIS, Enzymes of thymidine triphosphate synthesis in selected Morris hepatomas. *Cancer Res.* **29,** 40 (1969).
31. M. J. SWEENEY, D. H. HOFFMAN and G. A. POORE, Enzymes of pyrimidine biosynthesis. *Adv. Enzyme Regul.* **9,** 51 (1971).
32. G. WEBER, Molecular correlation concept: ordered pattern of gene expression in neoplasia. *Gann Monograph* **13,** 47 (1972).
33. J. A. FERDINANDUS, H. P. MORRIS and G. WEBER, Behavior of opposing pathways of thymidine utilization in differentiating, regenerating and neoplastic liver. *Cancer Res.* **31,** 550 (1971).
34. G. WEBER, R. L. SINGHAL and S. K. SRIVASTAVA, Regulation of RNA metabolism and amino acid level in hepatomas of different growth rate. *Adv. Enzyme Regul.* **3,** 369 (1965).

35. M. A. Lea, H. P. Morris and G. Weber, Comparative biochemistry of hepatomas VI. thymidine incorporation into DNA as a measure of hepatoma growth rate. *Cancer Res.* **26,** 465 (1966).
36. C. T. Caskey, D. M. Ashton and J. B. Wyngaarden, The enzymology of feedback inhibition of glutamine phosphoribosylpyrophosphate amidotransferase by purine ribonucleotides. *J. biol. Chem.* **239,** 2570 (1964).
37. M. Lalanne and J. F. Henderson, Determination of 5-phosphoribosyl-1-pyrophosphate in mouse liver. *Anal. Biochem.* **62,** 121 (1974).
38. H. G. Markley, L. A. Faillace and E. Mezey, Xanthine oxidase activity in rat brain. *Biochim. Biophys. Acta (Amst.)* **309,** 23 (1973).
39. B. Deuticke, E. Gerlach and R. Dierkesmann, Abbau freier Nukleotide in Herz, Skeletmuskel, Gehirn und Leber der Ratte bei Sauerstoffmangel. *Pflügers Arch. ges. Physiol.* **292,** 239 (1966).
40. E. W. Busch, I. M. Von Borcke and B. Martinez, Abbauwege und Abbaumuster der Purinnucleotide in Herz-, Leber-, und Nierengewebe von Kaninchen nach Kreislaufstillstand. *Biochim. Biophys. Acta (Amst.)* **166,** 547 (1968).
41. P. J. Orsulak, W. Haab and M. D. Appleton, Quantitative estimation of uric acid, xanthine, and hypoxanthine in plasma using thin-layer chromatography. *Anal. Biochem.* **23,** 156 (1968).
42. J. B. Pritchard, F. Chavez-Peon and R. D. Berlin, Purines: supply by liver to tissues. *Amer. J. Physiol.* **219,** 1263 (1970).
43. G. De Lamirande, C. Allard and A. Cantero, Purine-metabolizing enzymes in normal rat liver and Novikoff hepatoma. *Cancer Res.* **18,** 952 (1958).
44. C. Wu and J. M. Bauer, Catabolism of xanthine and uracil in tumor-bearing rats. *Cancer Res.* **22,** 1239 (1962).
45. W. E. Knox, *Enzyme Patterns in Fetal, Adult and Neoplastic Rat Tissues.* Karger, Basel and New York (1972).

Europ. J. Cancer Vol. 11, pp. 473–478. Pergamon Press 1975. Printed in Great Britain

Tumor Induction Studies with *n*-Butyl- and *n*-Propylhydrazine Hydrochlorides in Swiss Mice*

DONALD NAGEL†, HIDESUKE SHIMIZU‡ and BELA TOTH†§
†*The Eppley Institute for Research in Cancer, University of Nebraska Medical Center, 42nd Street and Dewey Avenue, Omaha, NB* 68105, *U.S.A.*,
‡*Jikei University School of Medicine, Department of Public Health, 3-chome, Nishi-shinbashi, Minato-Ku, Tokyo, Japan*

Abstract—*Solutions of* 0·0125% n-*butylhydrazine hydrochloride* (*BH*) *and* 0·025% n-*propylhydrazine hydrochloride* (*PH*) *were given separately and continuously in the drinking water of* 6-*week-old, randomly-bred Swiss albino mice for the remainder of their lives. The lung tumor incidence was* 53% *in BH-treated and* 49% *in PH-treated animals, while the corresponding incidence in the untreated controls was* 22%. *Histopathological examinations of lung tumors revealed the characteristic appearances of adenomas and adenocarcinomas. The treatments had no apparent effect on the development of other types of neoplasms.*

The study demonstrates for the first time the tumorigenicity of these 2 *chemicals. Because of widespread use of substituted hydrazines in the environment, their possible significance is discussed.*

INTRODUCTION

AFTER the demonstration of the tumorigenicity of hydrazine sulfate in mice [1], a number of studies were directed in various cancer research laboratories toward revealing the possible tumor-inducing abilities of the different substituents. This work received particular attention because many synthetic hydrazine derivatives were being used for various purposes in industry, agriculture, and even in medicine. As of today, in addition to the base compound hydrazine, there are 19 derivatives known to induce tumors in animals [2–9] and indeed, very few of those studies were found to be non-tumorigenic [10].

In this laboratory, investigations with a series of hydrazine derivatives are under way in 2 species: Swiss mice and Syrian golden hamsters. With each compound, a toxicity study was first performed separately in both species. Afterwards, the chemicals were given at the maximum tolerated dose in drinking water daily for life.

The present investigation shows for the first time the tumor-inducing capabilities of *n*-butylhydrazine hydrochloride and *n*-propylhydrazine hydrochloride administered orally for life to randomly-bred Swiss albino mice.

MATERIAL AND METHODS

Swiss albino mice from the colony randomly bred by this laboratory since 1951 were used. They were housed in plastic cages with granular cellulose bedding, randomized, separated according to sex in groups of ten, and given Wayne lab-blox diet in regular pellets (Allied Mills, Inc., Chicago, Illinois) and tap water or the test solutions *ad libitum* as described below:

The chemicals used were *n*-butylhydrazine

Accepted 25 March 1975.

*This investigation was supported by Public Health Service Contract 43-E-68-959 from the National Cancer Institute, NIH, USA.

§Recipient of USPHS Research Career Development Award KO-Ca 42,522 from the National Cancer Institute, NIH, USA.

hydrochloride (BH), Fig. 1(a) MW: 124.60, MP:> 250°, and *n*-propylhydrazine hydrochloride (PH), Fig. 1(b) MW: 74.15, MP:> 300°. BH was obtained from City Chemical Corporation, New York, N.Y. as the oxalate salt. After recrystallization from ethanol, gas chromatography of the free base indicated 97 % purity. The BH oxalate was converted to the hydrochloride as described below for PH.

(a) $CH_3—CH_2—CH_2—NH—NH_2 \cdot HCl$
(b) $CH_3—CH_2—CH_2—CH_2—NH—NH_2 \cdot HCl$

Fig. 1 *Chemical structures of* (*a*) n-*butylhydrazine hydrochloride;* (*b*) n-*propylhydrazine hydrochoride.*

Propylhydrazine oxalate was synthesized as follows: With vigorous stirring, 24·6 g of *n*-propylbromide was added dropwise to 100 g of hydrazine hydrate at 40°C. After stirring for 4 hr the resultant solution was extracted continuously with ether for 20 hr. The ether was removed by distillation and the residue poured into 35 g of oxalic acid in 400 ml of 95 % ethanol. The resultant oxalate salt was filtered and recrystalized from ethanol (m.p. 175°, lit 175° [11]. Yield was 23·0 g (70 %). The purity was greater than 98 % by gas chromatography.

The above oxalate was added to a cold solution containing an equal wt of sodium hydroxide and 400 ml of water. The solution was distilled to near dryness. An aliquot of the distillate was titrated with 0·1 N HCl to a methyl red endpoint and the concentration of *n*-propylhydrazine was calculated. The distillate was acidified with concentrated HCl to a pH of 4·5 and the concentration of *n*-propylhydrazine adjusted to 5 %. The solution was stored in a dark bottle under refrigeration.

Toxicity studies were carried out with both chemicals prior to the chronic experiments. Five dose levels of BH and PH, such as 0·1, 0·05, 0·025, 0·0125 and 0·00625 %, were administered in the drinking water daily for 35 days to Swiss mice. By taking into account four parameters: survival rates, body weights, chemical consumption figures and histological changes, the 0·0125 % in the BH-treated and the 0·025 % in the PH-treated were found to be suitable for the lifelong treatments. This toxicity technique was developed in this laboratory [12]. The 0·0125 % BH and the 0·025 % PH solutions which were used for the chronic experiments were analyzed by gas chromatography after 48 hr standing at room temperature and found to contain greater than 98 and 97 % of the original compounds unchanged, respectively.

Table 1 *Treatments and survival rates in* n-*butylhydrazine HCL* (*BH*)—*and* n-*propylhydrazine HCL* (*PH*)—*treated and control Swiss mice*

GROUP	TREATMENT	INITIAL NO. AND SEX	NO. OF SURVIVORS (AGE IN WEEKS)													
			10	20	30	40	50	60	70	80	90	100	110	120	130	140
1	0.0125% BH in drinking water daily for life	50 ♀	50	48	47	46	44	39	32	26	15	8	3	3	-	
		50 ♂	47	30	26	23	22	18	14	10	5	3	-			
2	0.025% PH in drinking water daily for life	50 ♀	45	43	33	28	25	21	13	4	2	1	-			
		50 ♂	48	43	18	16	15	10	5	2	1	-				
3	Untreated Control	100 ♀	100	100	97	95	91	88	81	71	57	36	14	5	1	-
		100 ♂	100	100	96	93	88	83	74	65	48	27	12	7	1	-

The solutions were prepared thrice weekly, and the total consumption of water containing BH and PH was measured at the same intervals during the treatment period. The solutions were contained in brown bottles because of the possible light sensitivity of the chemicals. The chronic experimental groups and the controls were the following:

Group 1. BH was dissolved in the drinking water as a 0·0125 % solution and was given for life to 50 female and 50 male mice which were 6 weeks (45 days) old at the beginning of the experiment. The average daily water consumption containing BH per animal was 10·8 ml

for the females and 16·8 ml for the males. Therefore, the average daily intake of BH was 1·3 mg for a female and 2·1 mg for a male.

Group 2. PH was dissolved in drinking water as a 0·025 % solution and was given for the life span of 50 female and 50 male mice which were 6 weeks (47 days) old at the beginning of the experiment. The average daily water consumption containing PH per animal was 12·0 ml for the females and 11·7 ml for the males. Therefore, the average daily intake of PH was 3·0 mg for a female and 2·9 mg for a male.

Group 3. As an untreated control, 100 female and 100 male mice were kept and observed from weaning time (5 weeks of age).

The experimental and control animals were checked carefully and weighted at weekly intervals and the gross pathological changes were recorded. The animals were either allowed to die or were killed with ether when found in poor condition. Complete necropsies were performed on all animals. All organs were examined macroscopically and were fixed in 10 % buffered formalin. Histological studies were done on the liver, spleen, kidney, bladder, thyroid, heart, pancreas, testis, brain, nasal turbinale and at least four lobes of the lungs of each mouse as well as on those organs showing gross pathological changes. Sections from these tissues were stained routinely with hematoxylin and eosin.

RESULTS

The survival rates after weaning at ten-week intervals are recorded in Table 1. It is clear that the treatments with both compounds significantly shortened the survival time when compared with the untreated controls.

The number, percentage of animals with tumors and their ages at death with each tumor type (latent periods) are summarized in Table 2. In the following, the significant lesions are described in detail.

Lung tumors in BH-treated mice

Of the treated females, 34 (68 %) developed 151 such neoplasms. Out of these, 17 mice had 54 adenomas, 1 had 2 adenocarcinomas and 16 had 71 adenomas and 24 adenocarcinomas. The average age at death was 89 weeks; the first was found at the 52nd week and the last at the 120th week of age. In the treated males, 19 (39 %) developed 95 lung tumors. Out of these, 15 had 55 adenomas and 4 had 25 adenomas and 15 adenocarcinomas. The average age at death was 84 weeks; the first was observed at the 49th week and the last at the 102nd week of age.

Lung tumors in PH-treated mice

Of the treated females, 27 (54 %) developed 212 such neoplasms. Out of these, 15 had 87 adenomas, 1 mouse had 1 adenocarcinoma and 11 mice had 87 adenomas and 37 adenocarcinomas. The average age at death was 70 weeks; the first was found at the 26th week and the last at the 100th week of age. In the treated males, 22 (44 %) developed 158 lung tumors. Out of these, 14 mice had 64 adenomas, and 8 mice had 73 adenomas and 21 adenocarcinomas. The average age at death was 58 weeks; the first was observed at the 26th week and the last at the 71st week of age.

Macroscopically and histologically, these tumors appeared similar to those reported and described earlier in this laboratory [13, 14].

Other tumors

In a few instances other types of tumors were also observed which are noted in Table 2. Since only a few were found in each group, their appearances cannot be attributed to the treatment.

Tumors in untreated controls

The number and description of spontaneous tumors in the controls were published recently [14]. Their incidences are, however, included in Table 2 for comparison.

DISCUSSION

This investigation demonstrated for the first time the tumorigenicity of *n*-butyl- and *n*-propylhydrazine hydrochlorides. BH was administered separately as a 0·0125 % solution and PH, as a 0·024 % solution, in drinking water continuously for life to randomly-bred Swiss albino mice from 6 weeks of age. The treatments gave rise to 53 % and 49 % incidences of lung tumors, respectively, while the corresponding incidence in untreated controls was 22 %. The number of tumors of other sites in these mice did not increase. Histologically, the lung neoplasms exhibited the typical appearances of adenomas and adenocarcinomas.

The present study is part of a concentrated effort to reveal the possible roles which synthetic and naturally-occurring hydrazines may play in the causation of cancer. It is estimated that well over 100 synthetic hydrazines are found in the environment, and used in industry, agriculture and medicine. Furthermore, a recent observation revealed that 6 hydrazines

Table 2. Tumor distributions in n-*butylhydrazine HCL (BH)—and* n-*propylhydrazine HCL (PH)—treated and control Swiss mice*

GROUP	TREATMENT	EFFECTIVE NO. AND SEX	LUNG TUMORS			ANIMALS WITH: OTHER TUMORS+		
			NO.	%	AGE AT DEATH*			
1	0.0125% BH in drinking water daily for life	50 ♀	34	68	89 (52-120)	5 Malignant lymphomas (26,59,81, 96,120) 1 Angiosarcoma of ovary (91)	1 Angiosarcoma of subcutis (103) 1 Angioma of ovary (98)	1 Papilloma of forestomach (97) 1 Sebaceous glands carcinoma (77)
		49 ♂	19	39	84 (49-102)	4 Malignant lymphomas (59,81,95, 95) 1 Angiosarcoma of liver (100)		
2	0.025% PH in drinking water daily for life	50 ♀	27	54	70 (26-100)	2 Malignant lymphomas (8,69) 1 Papilloma of forestomach (54)	1 Fibrosarcoma, subcutaneous (49) 1 Adenoma of skin appendage (43)	
		50 ♂	22	44	58 (26-71)	2 Liver cell carcinomas (64,99) 1 Adrenocortical carcinoma (70)	1 Angiosarcoma of liver (83)	
3	Untreated Control	99 ♀	21	21	95 (60-122)	24 Malignant lymphomas (31,36,68, 69,71,79,80,80,90,94, 96,96,97,101,102,103,105,106,109,115,118,122,123,130) 2 Adenocarcinomas of breasts (73, 93) 2 Adenocarcinomas of ovaries (104,106) 1 Adenoma of thyroid (116) 1 Papilloma of skin (109)	1 Angioma of subcutis (114) 1 Malignant histiocytoma (58) 1 Fibrosarcoma, subcutaneous (44) 1 Papilloma of esophagus (130) 1 Leiomyosarcoma of uterus (81) 1 Adenoma of glandular stomach (115)	1 Papillary adenoma of ovary (103) 1 Angiosarcoma of liver (99) 1 Angioma of liver and ovary (97) 1 Angiosarcoma of ovary (130) 1 Angiosarcoma of liver, lung, ovary (122)
		99 ♂	23	23	92 (53-125)	12 Malignant lymphomas (40,57,76,88,93,94,95,97,102 107,116,126) 3 Angiomas of livers (65,98,105) 3 Angiosarcomas of livers (71,87,102) 1 Fibrosarcoma, subcutaneous (82) 1 Adenocarcinoma of duodenum (113)	1 Malignant histiocytoma (101) 1 Carcinoma of skin (122) 1 Adrenocortical adenoma (130) 1 Papilloma of esophagus (63) 1 Papilloma of forestomach (63)	

* Age in weeks (average and range)
\+ Age at death given in parentheses

occur naturally, mainly in tobacco and in some mushrooms [9]. In this laboratory, the following hydrazine derivatives were, for the first time, found to be tumorigenic in animals: methyl- [15], ethyl- [16], carbamyl- [17], 1-carbamyl-2-phenyl- [14], *n*-amyl- [18], allyl- [19]. 1-acetyl-2-isonicotinoyl- [20]. An additional reason for initiating these studies was to see whether a relationship exists between the chemical structures of these hydrazines and tumor development at specific organ sites. So far, 5 compounds have induced only lung tumors in mice. These are hydrazine [15], monomethyl- [15], 1-carbamyl-2-phenyl [14] and now *n*-propyl- and *n*-butyl-hydrazines. Six other compounds, namely: 1,2-dimethyl- [21], 1,1-dimethyl- [22], ethyl- [16], carbamyl- [17], *n*-amyl- [18] and allyl- [19] hydrazines, evoked vascular tumors in addition to lung tumors. As a matter of fact, the 1,1-dimethylhydrazine also produced kidney and liver tumors. Finally, benzoylhydrazine elicited the development of lung and lymphoreticular tissue neoplasms [23]. Therefore, it appears that the various substituents on the hydrazine molecule exert some sort of neoplastic organotropic effect. Further studies are in progress which may clarify this aspect of the study.

The *n*-butylhydrazine hydrochloride and the *n*-propylhydrazine hydrochloride as far as we know are not used for any specific purpose and were chosen for the present tumorigenesis study on the basis of reasoning that if the length of the alkyl chain is increased, tumor types could hopefully be modified. This expectation did not turn out to be correct, even though in earlier studies it was shown that by attaching an ethyl- instead of a methyl- group to the hydrazine molecule, blood vessel tumors were induced, in addition to lung tumors. Even more surprising was the fact that *n*-amylhydrazine with a longer alkyl chain produced tumor types identical to those found after ethylhydrazine treatment.

The various hydrazine derivatives were also shown to induce tumors in other species. The most notable which induced tumors in rats are: 1,2-dimethylhydrazine in intestines [3]; 1,2-diethylhydrazine in lymphoreticular and nerve tissues, liver and ethmoturbinals [24]; procarbazine in the breasts, lungs and blood vessels [25, 26]; 1-methyl-2-butylhydrazine in the intestines and bulbus olfactorious [8] and 1-methyl-2-benzylhydrazine in the central and peripheral nervous systems and bulbus olfactorious [8]. In the hamsters, monomethylhydrazine produced tumors of Kupffer cells and the cecum [27], while 1,2-dimethylhydrazine evoked tumors in the liver, stomach, intestines and blood vessels [28, 29].

Acknowledgements—The authors are thankful to Mrs. Marilyn Jepson for her assistance in the experiment.

REFERENCES

1. C. Biancifiori and R. Ribacchi, Pulmonary tumours in mice induced by oral isoniazid and its metabolites. *Nature (Lond.)* **194,** 488 (1962).
2. D. B. Clayson, C. Biancifiori, U. Milia and F. E. Giornelli-Santilli, The induction of pulmonary tumours in Balb/Cb/Se mice by derivatives of hydrazine. In *Lung Tumors in Animals.* (Edited by L. Severi) p. 869. Proceedings of the Quadrenniel Conference on Cancer, Perugia, Italy (1966).
3. H. Druckrey, R. Preussmann, F. Matzkies and S. Ivankovic, Selective Erzeugung von Darmkrebs bei Ratten durch 1,2-Dimethylhydrazin. *Naturwissenschaften* **54,** 285 (1967).
4. F. J. Roe, G. A. Grant and D. M. Millican, Carcinogenicity of hydrazine and 1,1-dimethylhydrazine for mouse lung. *Nature (Lond.)* **216,** 375 (1967).
5. M. G. Kelly, R. W. O'Gara, S. T. Yancey, K. Gadekar, C. Botkin and V. T. Oliverio, Comparative carcinogenicity of N-isopropyl-α-(2-methylhydrazino)-p-toluamide HCl (Procarbazine hydrochloride): its degradation products, other hydrazines and isonicotinic acid hydrazide. *J. nat. Cancer Inst.* **42,** 337 (1969).
6. J. R. M. Innes, B. M. Ulland, M. G. Valerio, L. Petrucelli, L. Fishbein, E. R. Hart, A. J. Pallotta, R. R. Bates, H. L. Falk, J. J. Gart, M. Klein, I. Mitchell and J. Peters, Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: a preliminary note. *J. nat. Cancer Inst.* **42,** 1101 (1969).
7. A. Schauer, T. Völlnagel and F. Wildanger, Cancerisierung des Rattendarmes durch 1,2-Dimethylhydrazin. *Z. Ges. Exp. Med.* **150,** 87 (1969).

8. H. Druckrey, Production of colonic carcinomas by 1,2-dialkyl hydrazines and azoxyalkanes. In *Carcinomas of the Colon and Antecedent Epithelium.* (Edited by W. J. Burdette) p. 269. Charles C. Thomas Springfield, Ill. (1970).
9. B. Toth, Concern with synthetic and naturally occurring hydrazines as possible cancer causative agents. *Cancer Res.* (Submitted for publication).
10. R. J. W. Cremlyn and F. J. C. Roe, A study of certain substituted sulfonohydrazides for carcinogenicity in mice. *Food cosm. Toxicol.* **9,** 319 (1971).
11. G. Gever and K. Hayes, Alkylhydrazines. *J. org. Chem.* **14,** 813 (1949).
12. B. Toth, A toxicity method with calcium cyclamate for chronic carcinogenesis experiments. *Tumori* **58,** 137 (1972).
13. B. Toth, P. N. Magee and P. Shubik, Carcinogenesis study with dimethylnitrosamine in orally treated adult and subcutaneously injected newborn Balb/c mice. *Cancer Res.* **24,** 1712 (1964).
14. B. Toth and H. Shimizu, 1-Carbamyl-2-phenylhydrazine tumorigenesis in Swiss mice. Morphology of lung adenomas. *J. nat. Cancer Inst.* **52,** 241 (1974).
15. B. Toth, Hydrazine, methylhydrazine and methylhydrazine sulfate carcinogenesis in Swiss mice. Failure of ammonium hydroxide to interfere in the development of tumours. *Int. J. Cancer* **9,** 109 (1972).
16. H. Shimizu, D. Nagel and B. Toth, Ethylhydrazine hydrochloride as a tumor inducer in mice. *Int. J. Cancer* **13,** 500 (1974).
17. B. Toth, H. Shimizu and J. Erickson, Carbamylhydrazine hydrochloride as a lung and blood vessel tumor inducer in Swiss mice. *Europ. J. Cancer,* **11,** 17 (1975).
18. H. Shimizu, D. Nagel and B. Toth, Tumor induction study with *n*-amylhydrazine hydrochloride in Swiss mice. *Brit. J. Cancer* In press (1975).
19. In preparation (1975).
20. B. Toth and H. Shimizu, Lung carcinogenesis with 1-acetyl-2-isonicotinoylhydrazine, the major metabolite of isoniazid. *Europ. J. Cancer* **9,** 285 (1973).
21. B. Toth and R. B. Wilson, Blood vessel tumorigenesis by 1,2-dimethylhydrazine dihydrochloride (symmetrical). Gross, light and electron microscopic descriptions. I. *Amer. J. Pathol.* **64,** 585 (1971).
22. B. Toth, 1,l-Dimethylhydrazine (unsymmetrical) carcinogenesis in mice. Light microscopic and ultrastructural studies on neoplastic blood vessels. *J. nat. Cancer Inst.* **50,** 181 (1973).
23. B. Toth, Benzoylhydrazine carcinogenesis in lungs and lymphoreticular tissues of Swiss mice. *Europ. J. Cancer* **8,** 341 (1972).
24. H. Druckrey, R. Preussmann, F. Matzkies and S. Ivankovic, Carcinogene Wirkung von 1,2-Diäthylhydrazin an Ratten. *Naturwissenschaften* **53,** 557 (1966).
25. M. G. Kelly, R. O'Gara, K. Gadekar, S. T. Yancey and V. T. Oliverio, Carcinogenic activity of a new antitumor agent, N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride (NSC–77213). *Cancer Chemoth. Rep.* **39,** 77 (1964).
26. M. G. Kelly, R. O'Gara, S. T. Yancey and C. Botkin, Induction of tumors in rats with procarbazine hydrochloride. *J. nat. Cancer Inst..* **40,** 1027 (1968).
27. B. Toth and H. Shimizu, Methylhydrazine tumorigenesis in Syrian golden hamsters and the morphology of malignant histiocytomas. *Cancer Res.* **33,** 2744 (1973).
28. H. Osswald and F. W. Krüger, Die cancerogene Wirkung von 1,2-Dimethylhydrazin beim Goldhamster. *Arzneimittel-Forsch.* **19,** 1891 (1969).
29. B. Toth, Tumorigenesis studies with 1,2-dimethylhydrazine dihydrochloride, hydrazine sulfate and isonicotinic acid in golden hamsters. *Cancer Res.* **32,** 804 (1972).

Europ. J. Cancer Vol. 11, pp. 479–482. Pergamon Press 1975. Printed in Great Britain

The Histochemical Demonstration of Arylsulphatase and Acid Hydrolases in Carcinoma of the Colon and the Breast

JOHAN KOUDSTAAL

Department of Pathology, "SSDZ", Stichting Samenwerking Delftse Ziekenhuizen, Reynier de Graefweg 7, Delft, Hollande

Abstract—*With membrane techniques the activities of the enzymes arylsulphatase, acid phosphatase, β-glucuronidase, 1-naphthol acetate esterase and β-galactosidase were studied in a number of human tumours of the colon and the breast.*

Most carcinomas of the breast, in contrast to carcinomas of the colon and rectum, showed a moderate to strong enzyme activity of the various hydrolytic enzymes with the exception of arylsulphatase. It seemed, through histochemical demonstration, that the enzyme arylsulphatase has no lysosomal localization.

INTRODUCTION

In a previous paper we described a membrane technique for the demonstration of the enzyme arylsulphatase (aryl-sulphate sulphohydrolase E.C. 3.1.6.1. [1]. The various iso-enzymes (A, B and C) cannot be differentiated under the usual histochemical conditions. Sulphatases A and B, readily solubilized, are found in lysosomes and sulphatase C was found to be localized in the endoplasmatic reticulum.

Several acid hydrolases can be visualized by membrane techniques developed by Meijer *et al.* [2, 3] and Lojda [4]. The membrane technique was introduced in histochemistry by McMillan [5] for the demonstration of lactate dehydrogenase.

There are good reasons to localize the acid hydrolytic enzymes in lysosomal organelles. The enzyme acid phosphatase is known to have a lysosomal distribution and therefore, in histochemical studies particularly, this enzyme is used as a marker for the localization of lysosomes.

In a previous paper we demonstrated by enzyme histochemical methods an increased activity of the enzyme acid phosphatase in mammary carcinomas and a decreased activity of colonic–rectal carcinomas [6]. Recently, carcinomas of the colon with increased activity of the enzyme arylsulphatase was shown [1].

With membrane techniques we tried to investigate the relationship between various acid hydrolases and arylsulphatase in the two mentioned tumours.

Accepted 12 March 1975.

MATERIAL AND METHODS

Human tissues obtained by surgical procedures were rapidly frozen. Thirty-nine carcinomas of the breast and 21 carcinomas of the colon and rectum were studied.

The membrane techniques for the enzymes acid phosphatase (E.C. 3.1.3.2.), β-glucuronidase (E.C. 3.2.1.31), 1-naphthol acetate esterase, (E.C. 3.1.1.1.) described by Meijer *et al.* [2 3], for the enzyme β-galactosidase (β-D-galactoside galactohydrolase E.C. 3.2.1.23) described by Lojda [4] and for the enzyme arylsulphatase (arylsulphate sulphohydrolase E.C. 3.1.6.1.) reported by Koudstaal [1] were used. Meijer used an azo-dye pararosanilin and Koudstaal, Fast Blue B. Staining was performed by the

simultaneous coupling of hexazotised pararosanilin with the released naphthol. Lojda developed and used an indogogenic method. In addition two methods were combined to visualize two enzymes in one section (arylsulphatase first and acid phosphatase afterwards).

RESULTS

In normal epithelial cells of the breast and the colon the precipitates of the coloured product of the various hydrolytic enzymes were seen mostly on the lumen side of the tubules Fig. 1, 2 and 3. The blue purple endproduct of the enzyme arylsulphatase (Fig. 4) is seen as a diffuse colour marked at the cell borders. Sometimes fine particles could be seen in the case of the acid hydrolases. Activity of the various enzymes in the tumour cells is summarized in Fig. 5.

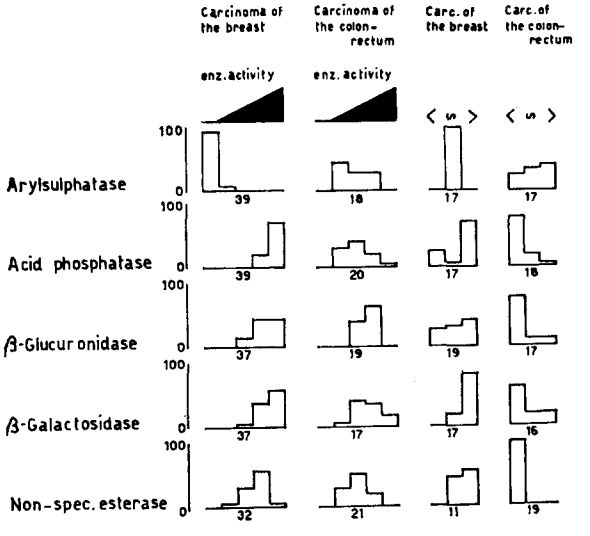


Fig. 5. In the left column, percentages of intensities of enzyme activity are shown of tumours of the colon, rectum and of the human breast. On the right side, the number of tumours are indicated with increased, decreased or normal enzyme activity compared with the enzyme activity of the neighbouring, non-tumorous epithelium.

A subjective judgment cannot be avoided. Normally there is no arylsulphatase enzyme activity in the human breast or in carcinomas of the breast but for a rare exception. Most carcinomas of the colon and rectum showed a slight to moderate activity of the mentioned enzyme.

Most carcinomas of the breast showed a moderate to strong enzyme activity of the various hydrolytic enzymes in contrast to the carcinomas of the colon and rectum. This phenomenon is more pronounced when the enzyme activities of the non-tumourous surrounding epithelial tissue is compared with the activity in the tumour tissue. It can be seen that the activity of the acid hydrolytic enzymes has increased in breast carcinomas and decreased in carcinomas of the colon and rectum in contrast to the behaviour of the enzyme arylsulphatase. This seemed to be a general rule. In an individual case, an acid hydrolytic enzyme (*e.g.*, β-glucuronidase Fig. 6) may show an increased enzyme activity where the activity of another acid hydrolytic enzyme (*e.g.*, esterase, Fig. 7) has decreased. The coloured products of the various acid hydrolytic enzymes were seen as fine and coarse particles roughly distributed in tumour cells and macrophages. No enzyme activity of arylsulphatase could be shown in macrophages. The other acid hydrolytic enzymes showed a high activity in these cells. In a combined method the high activity of acid phosphatase in macrophages in stroma surrounding coloncarcinoma could be shown as well as the moderate activity of arylsulphatase in the tumour cells (Fig. 8).

In the combined method the different localization of the various hydrolytic enzymes and arylsulphatase is clearly shown. It seems that the arylsulphatase is shown especially on the side of the cell membranes.

DISCUSSION

In experimental pathology various investigations are known about the activity of arylsulphatase and lysosomal enzymes. Goldfischer [7] visualized arylsulphatase activity in preparations suitable for light and electron microscopy with a Gomori-type medium with *p*-nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate) as a substrate. A veronal acetate buffer, pH 5·4 and 4·2 was used. From staining, it was noted that the two lysosomal arylsulphatases, types A and B, with different pH optima in the acid range are present in different cell types. More lysosomes are visualized in vascular endothelium and glial cells by the sulphatase method than by acid phosphatase procedure.

Using a nitrocatechol sulphate barium chloride medium, Corrin and Clark [8] localized lysosomal arylsulphatase at the electron microscopic level.

The distribution of arylsulphatase in the lung corresponded closely to that of acid phosphatase. Large amounts were found in



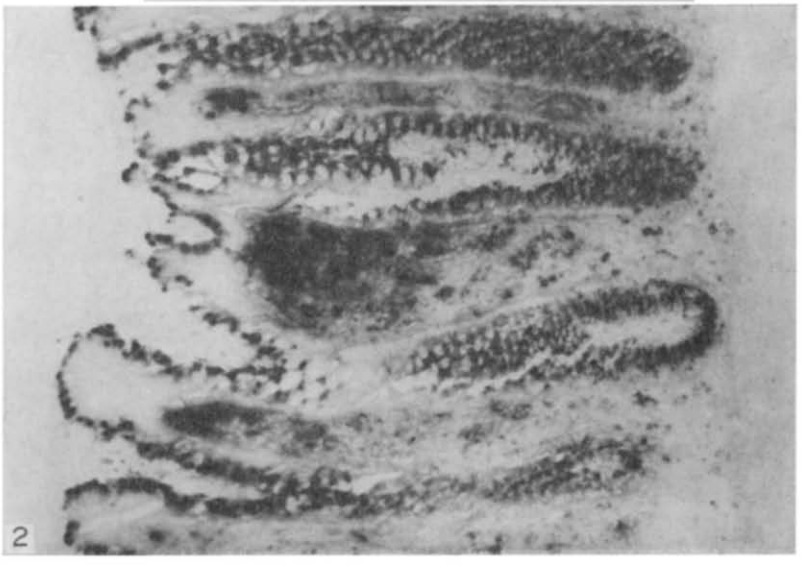


Fig. 1. *Enzyme activity of acid phosphatase in normal colon. High activity is seen, especially in the apical regions, in the epithelial cells and in macrophages in the underlying stroma.*

Fig. 2. *Enzyme activity of β-galactosidase in normal colon as crude precipitates (especially in the apical regions of the epithelial cells).*

(to face p. 480)

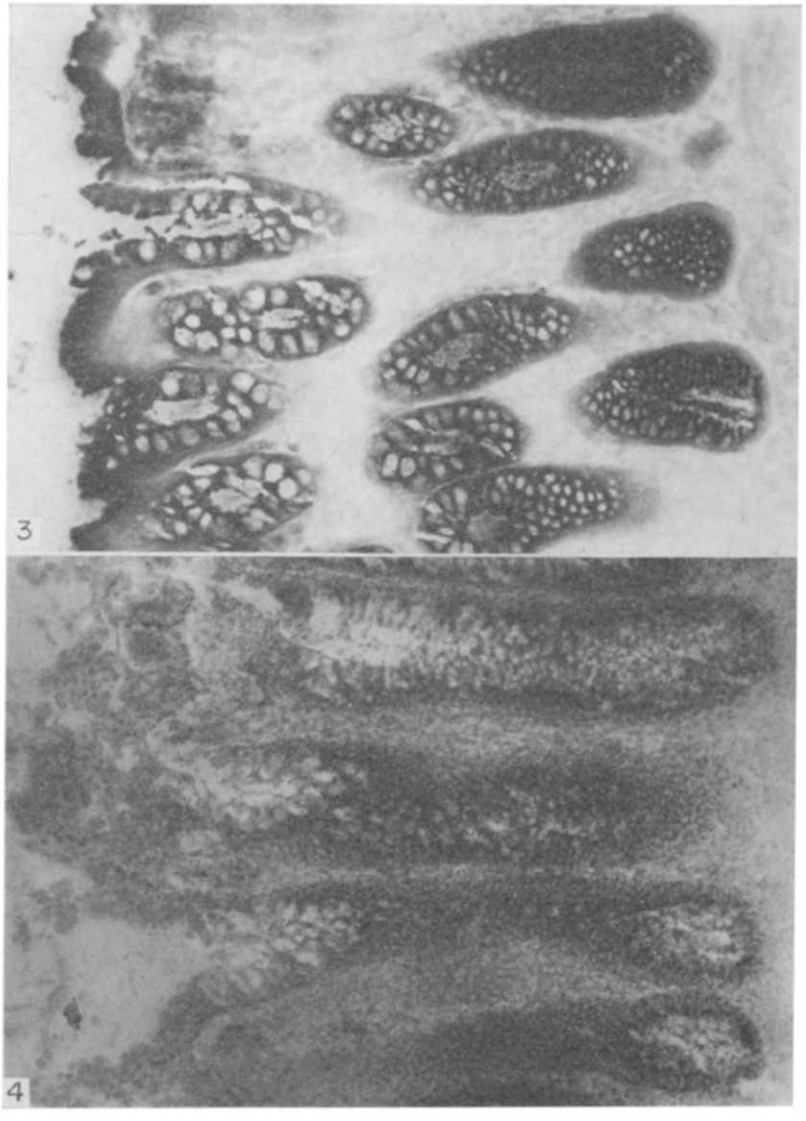


Fig. 3. *Enzyme activity of l-naphthol acetate esterase in normal colon. Diffuse activity is seen in all epithelial cells.*

Fig. 4. *Enzyme activity of arylsulphatase is shown especially in the epithelial cells of the crypts. No enzyme activity is present in macrophages.*

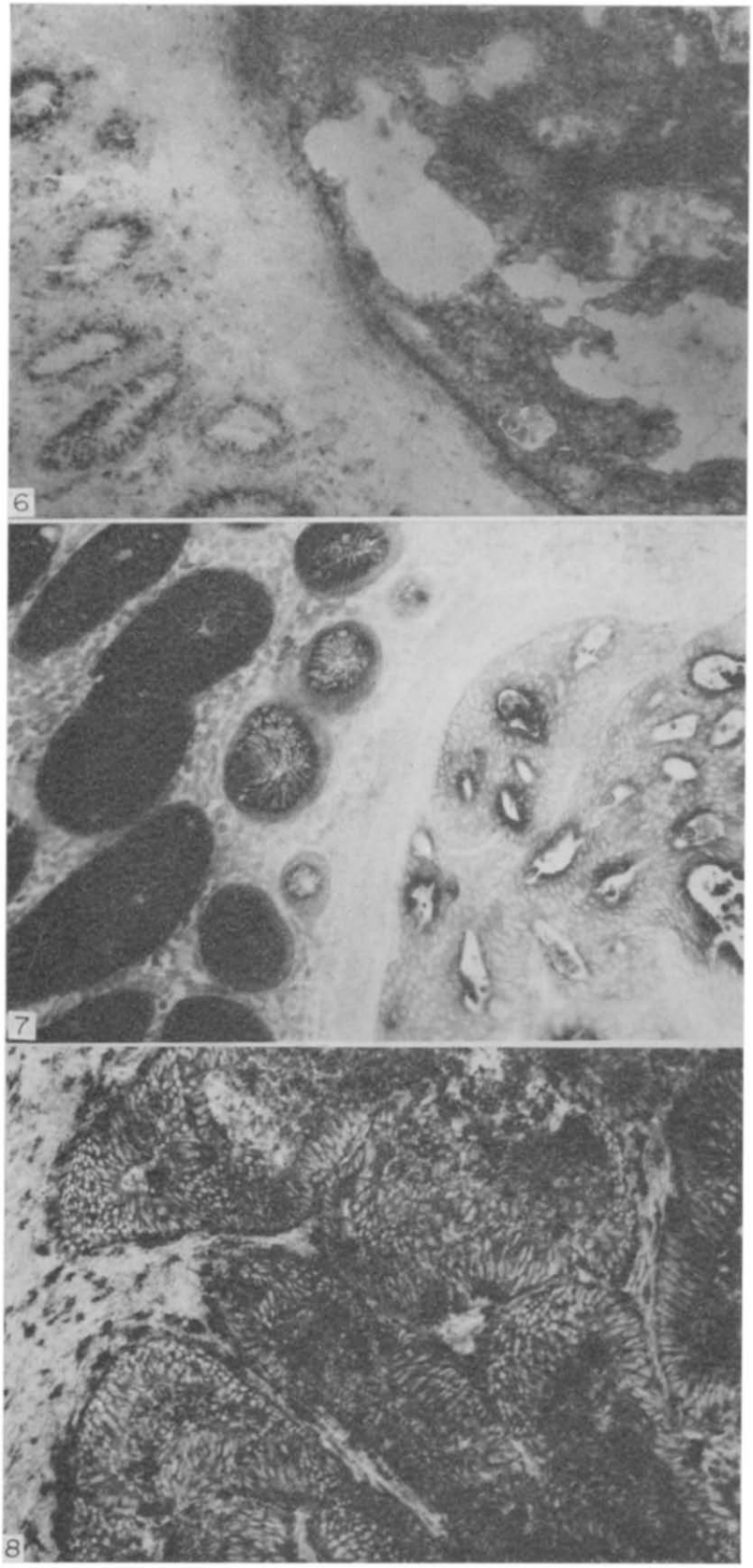


Fig. 6. *Enzyme activity of β-glucuronidase is shown in normal epithelium and tumour cells of the colon. The enzyme activity in cancer cells is increased in this case.*

Fig. 7. *Enzyme activity of l-naphthol acetate esterase is shown in normal epithelium and tumour cells of the colon in the same tumour as shown in Fig.* 6. *The enzyme activity in cancer cells is decreased.*

Fig. 8. *A combined method to show enzyme activity of acid phosphatase and arylsulphatase was applied in this case. Acid phosphatase activity in macrophages and arylsulphatase activity in tumour cells.*

alveolar macrophages and small quantities in the type II alveolar epithelial cells of the rat.

Pfeiffer [9] reported a cytochemical method permitting the electron-microscopical localization of both arylsulphatase and acid phosphatase within the same specimen by a double incubation. Results suggested that enzymical heterogenous lysosomes exist within the same cell.

Epithelial cells of the proximal convoluted tubule of the rat kidney were examined. *p*-Nitrocatechol sulphate and bariumchloride were used as a substrate.

Leznicki and Bleszynski [10] showed in neurons that the arylsulphatases are localized in lysosomes. They remarked that the activity of soluble arylsulphatase does not necessarily have to be localized in lysosomes only.

Finally Davidoff and Galabov [11] investigated arylsulphatase and β-glucuronidase in the central nervous system of the rat. Histochemistry revealed a similar distribution pattern for both enzymes in various parts of the central nervous system. Electronmicroscopically, the precipitates of the arylsulphatase reaction are preferentially localized in the periphery of lysosomes with a homogenous dense matrix and in the Golgi vesicles and vacuoles. The β-glucuronidase predominates in lysosomes with lipid components.

From the biochemical work of Rutenberg and Seligman [12] it is known that there are large differences in activity of arylsulphatase between different species of mammals. The organs of the hamster and the rat showed the highest activity. There was less activity in human and mouse tissues and very little to none in the dog, guinea pig and rabbit.

Thus, it seemed very difficult and dangerous to come to general conclusions from the results of experimental investigations about the enzyme arylsulphatase.

Regarding the activity of the enzyme arylsulphatase and lysosomal enzymes in human tissues, hardly any literature could be found. Boyland *et al.* [13] found biochemically that bladder cancer tissue contained more β-glucuronidase and sulphatase than the corresponding bladder tissue.

Hanefeld [14] studied in 17 neurinomas the distribution and activities of the hydrolytic enzymes β-D-glucuronidase, β-D-glucosidase, β-D-galactosidase and arylsulphatase. Highest enzyme activity was seen in neurinomas stuffed with lipid material.

From the experimental investigations we can conclude that there may be a close relationship between some arylsulphatases and lysosomal enzymes.

The membrane technique we used disclosed an inversed pattern between the enzyme arylsulphatase and the acid hydrolases. At the same time it was seen that the coloured end-products of the acid hydrolases generally differed in structure from the products of the arylsulphatase. The precipitates of arylsulphatase activity were especially localized in the neighbourhood of the cell membranes. So, roughly it seemed that through histochemical demonstration the enzyme arylsulphatase has no lysosomal localization. In order to solve this problem further, biochemical studies of human tumours, possibly combined with histochemistry at the electronmicroscopical level, are necessary.

REFERENCES

1. JOH. KOUDSTAAL, The cytochemical demonstration of arylsulphatase in human tumours. *Europ. J. Cancer.* In press (1975).
2. A. E. F. H. MEIJER, Semipermeable membranes for improving the histochemical demonstration of enzyme activities in tissue sections. I. Acid Phosphatase. *Histochemie* **30,** 31 (1972).
3. A. E. F. H. MEIJER and A. H. T. VLOEDMAN, Semipermeable membranes for improving the histochemical demonstration of enzyme activities in tissue sections. II. Non specific esterase and β-Glucuronidase. *Histochemie* **34,** 127 (1973).
4. A. LOJDA, Histochemical methods for acid β-galactosidase: techniques for semipermeable membranes. *Histochemie* **37,** 375 (1973).
5. P. J. MCMILLAN, Differential demonstration of muscle and heart type lactic dehydrogenase of rat liver, muscle and kidney. *J. Histochem. Cytochem.* **15,** 21 (1967).
6. JOH. KOUDSTAAL, B. MAKKINK and S. H. OVERDIEP, Enzyme histochemical pattern in human tumours—I. Hydrolytic enzymes in carcinoma of the colon and the breast. *Europ. J. Cancer* **11,** 111 (1975).

7. S. Goldfischer, The cytochemical demonstration of lysosomal arylsulphatase activity by light and electronmicroscopy. *J. Histochem. Cytochem* **13,** 520 (1965).
8. B. Corrin and A. E. Clark, Lysosomal aryl sulphatase in pulmonary alveolar cells. *Histochemie* **15,** 95 (1968).
9. U. Pfeiffer, Kombinierte electronenmikroskopische Darstellung der Arylsulfatase und der sauren Phosphatase in lysosomen des Nierentubules. *Histochemie* **17,** 284 (1969).
10. A. Leznicki and W. Bleszynski, Histochemical localization of the soluble arylsulphatase activities in rat brain. *Histochemie* **24,** 251 (1970).
11. M. Davidoff and G. Galabov, Licht- und elektronenmikroskopische Verteilung der Lysosomale Arylsulfatase und β-Glucuronidase. Aktivität im Zentralnervensystem der Ratte. *Histochemie* **35,** 341 (1973).
12. A. M. Rutenberg and A. M. Seligman, Coloric estimation of arylsulfatase. Enzyme kinetics and distribution of the enzyme in seven mammals and in tumours. *Arch. Biochem.* **60,** 198 (1956).
13. E. Boyland, D. M. Wallace and D. C. Williams, The activity of the enzymes sulphatase and β-glucuronidase in the urine, serum and bladder tissue. *Brit. J. Cancer.* **9,** 62 (1955).
14. F. Hanefeld, Nachweis und Verteilung der Enzyme: β-D-Glucuronidase, β-D-Galaktosidase, β-D-Glucosidase und Arylsulfatase in Neurinomen. *Acta neuropath. (Berl.)* **12,** 189 (1969).

Europ. J. Cancer Vol. 11, pp. 483–492. Pergamon Press 1975. Printed in Great Britain

Cytokinetics of Human Acute Leukemia Before and After Chemotherapy

GREGORY SARNA* MITSUHIRO OMINE,† and SEYMOUR PERRY‡
Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014, *U.S.A.*

Abstract—*In order to evaluate the relationship of relative and absolute blast size to blast proliferative activity, both in treated and untreated human acute leukemia, blasts from peripheral blood of patients with acute leukemia were labeled* in vitro *with tritiated thymidine and then separated as to size by the* 1 *g sucrose gradient technique for subsequent evaluation of thymidine uptake and DNA content. Results suggested:* (1) *Leukemic lymphoblasts were smaller and less variable in size than leukemic myeloblasts;* (2) *thymidine uptake was excluded from relatively small cells and concentrated in relatively large cells, with some medium cell uptake;* (3) *DNA content of blasts increased with cell size;* (4) *Cytosine arabinoside and 6-thioguanine in AML caused an early increase in thymidine uptake and a shift of the cell size distribution to larger cells followed by a rapid depletion of all blasts with preferential large cell loss. This is consistent with early recruitment of small cells into cycle and S-phase specific cell kill* (5) L-*asparaginase in ALL caused a rapid depletion of blasts with a preferential loss of both large and medium cells and with decreased thymidine uptake, consistent with cycle nonspecific kill plus a* G_1/S *block inhibiting recruitment. The data support a cytokinetic model of human acute leukemia in which relative cell size correlates with cell cycle stage. Changes in size distribution may reflect drug effect and may be of value in evaluating response to chemotherapy, both of proliferating and resting cells, and in planning and sequencing multiple drug chemotherapy.*

INTRODUCTION

CYTOKINETIC studies during the past decade have generally supported the concept that advanced human acute leukemia is a disease in which abnormal leukocytes accumulate and, as a group, have relatively low replicative activity. The proliferative activity within the group, however, seems to be related to cell size. Many studies have suggested that, in a given leukemic cell population, relatively large cells are actively proliferating and relatively small cells are slowly proliferating or possibly non-proliferating or "resting" cells [1]. Because these small cells may be non-cycling or slowly cycling, it is reasonable to suggest that they may preferentially survive treatment with anti-leukemic agents, the majority of which are either cellcycle phase specific or cycle related. Kinetic implications of absolute cell size have not been so well studied, although there is evidence to suggest that, at least in acute lymphocytic leukemia, the baseline tritiated thymidine (^{3}HTdR) labeling index may correlate with the magni-

Accepted 18 February 1975.

*Oncology Fellow, University of California at Los Angeles, formerly Staff Associate, Division of Cancer Treatment, NCI, NIH, Bethesda, MD.
†3rd Department of Internal Medicine, Gunma University School of Medicine, Japan.
‡Special Assistant to the Director, NIH, Bethesda, MD.
Reprint Address: Dr. Seymour Perry, National Institutes of Health, Building 1, Room 111, Bethesda, MD 20014.

Presented in part to the American Society of Hematology, Chicago, IL, December 4, 1973.

tude of cell size of the blast population as a whole [2].

The spontaneous AKR mouse leukemia has proven to be a useful model of human acute leukemia. Cytokinetic analysis of AKR lymphoblasts, using 1 g sucrose gradient techniques to separate and harvest cells according to size, have suggested a strong correlation between relative cell size and cell cycle stage and have elucidated the dynamics of active and resting cell proliferation and loss, both in the untreated state and as perturbed by various chemotherapeutic agents [3–5].

The present investigation was undertaken to study human acute leukemia with similar techniques, in order to assess the relationship of cell size, both relative and absolute, to cell cycle stage and to assess the effects of chemotherapy on cells in various phases of the cell cycle.

MATERIAL AND METHODS

Clinical subjects

A total of eight patients with active acute leukemia was studied in a total of fifteen experiments. Five patients (cases 1–5) had acute myelogenous leukemia (AML). Two patients (cases 6 and 7) had acute lymphocytic leukemia (ALL). One patient (case 8) had chronic myelogeneous leukemia (CML) of childhood in a blastic stage. Cases 1 and 6 were studied prior to and sequentially during chemotherapy. Details of diagnoses and status at the time of study are presented in Table 1. All patients were untreated except for case 4, studied 12 hr after receiving hydroxyurea.

Preparations and incubation of white blood cell (WBC) concentrates

White blood cell (WBC) concentrates were prepared either directly from peripheral blood or from WBC-rich plasma obtained via leukapheresis (NCI–IBM cell separator). Cells from either source were purified by dextrose/dextran/EDTA separation and by hypotonic lysis and were suspended in Hanks' BSS as described previously [3]. In case 3B, a simultaneous bone marrow aspirate was obtained, and a nucleated cell concentrate was prepared by the same method.

Purified WBC suspensions from all cases except case 2 were incubated for 30 min 37° in 20% fetal calf serum, Eagle's medium and 1 μCi/ml ^{3}HTdR (specific activity 6·7 Ci/mM, New England Nuclear, Boston, Mass.) for 30 min. The cells were then washed twice, resuspended in Hanks' BSS with 0·05% sucrose, and applied to the sedimentation chamber in known quantities ranging from 10^7 to 10^8 cells.

Separation of cells according to size

The cells were separated for approximately 4 hr at room temperature by the 1 g sucrose gradient technique, with separation occurring as a function of size [3, 6]. This technique has been shown in our laboratory to be consistently reproducible in duplicate runs both in the original studies with AKR leukemia and in the current work with human leukemic cells. Small cells sedimented more slowly than large cells and, when "pushed up" through the top of the chamber for harvest, appeared in the early, low number fractions. Large cells sedimented further and appeared in the later, high number fractions. Fractions were collected in 50 ml aliquots after an initial discard of 150–250 ml. Fraction one, by convention, was always the fraction following the first 150 ml. At least 30 fractions were collected. At the 2 hr point in the separation, the distance between the meniscus and the peak band of cells was used to calculate the peak sedimentation velocity (PSV).

Table 1. *Clinical characteristics of cases studied*

Diagnosis	Case	Age/Sex	WBC ($\times 10^3$/mm^3)	Blast %
AML	1	28 M	99·0	98
	2	28 F	16·9	95
	3	39 F	28·5	82
	4*	14 F	88·0	95
	5	17 M	95·0	83
ALL	6	20 M	85·2	82
	7	10 F	41·0	90
Blastic CML	8	11 F	43·0	90

Clinical characteristics, white blood counts and percentage blasts are shown for each patient at the time of initial study. *12 hr after hydroxyurea.

Assay of cell number, ^{3}HTdR uptake and labeling index (L.I.)

WBC count for each fraction was calculated from a 10 ml aliquot assayed by an electronic counting device (Coulter Electronics, Inc., Hieleah, Fla, or Particle Data, Inc., Elmhurst, IL). The remaining 40 ml of each fraction were centrifuged at 700 *g*, and the resultant pellet was resuspended in Hanks' BSS to a concentration approximating 10^6cells/ml. Sequential fractions with few cells were combined when appropriate. Slides from the original WBC preparation (post-incubation but preseparation) and from separated fractions or fraction pools were prepared for autoradiography, utilizing the cytocentrifuge (Shandon Scientific Co., Sewickley, PA). After Kodak AR-10 film was applied by the stripping technique, the autoradiographs were exposed at 4°C for approximately two weeks, then stained with Giemsa stain. Labeling indices were determined using a threshold of 5 grains/cell. Areas of high background activity or artifactual cell clumping were few and were not analyzed. Differential cell counts were performed, and total and labeled WBC counts in each fraction were corrected accordingly to total and labeled blasts per fraction. An aliquot of the original and of each fraction or fraction pool was digested and evaluated for 3HTdR uptake by liquid scintillation count (LSC) [3]. An aliquot of each cell suspension was counted by hemocytometer to determine more precisely cell concentration and to calculate cell number subjected to LSC.

Other studies

In case 8, an aliquot of the original post ^{3}HTdR incubation cell suspension was washed and resuspended at a concentration of $1{\cdot}8 \times 10^6$ cells/ml in NCTC medium with 20% fetal calf serum at 37°C for 18 hr of short term culture. No further ^{3}HTdR labeling was performed; viability post culture was 90% by trypan blue exclusion. Cells were then washed, resuspended, and applied to the sedimentation chamber.

In cases 2 and 5 relative DNA content was estimated in combined aliquots by the diphenylamine reaction according to the Dische technique [7].

RESULTS

Cell size distribution

Representative profiles for AML (case 1) and ALL (case 7) are shown in Fig. 1. Results of other cases will be seen in Fig. 2–6. The cases of AML studied had larger cells than the cases of ALL, as evidenced by the more rightward positions of both the entire cell size profile

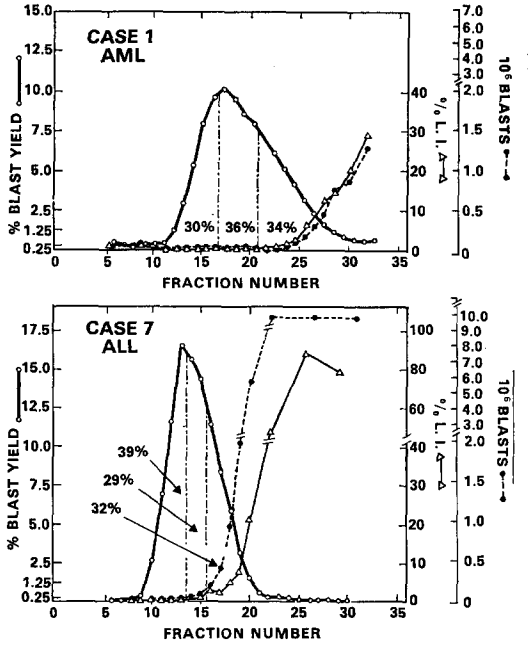


Fig. 1. Representative cytokinetic profiles for AML (case 1) and ALL (case 4). Fraction number, on the asymptote, increases with increasing blast size. Percentage of blast yield scaled on the left ordinate, and ^{3}HTdR uptake as measured by both labeling indices and liquid scintillation counts, scaled on the right ordinate, are shown for the various fractions. Vertical dotted lines separate blast yield into small, medium and large cell fractions, each consisting of approximately one third of the blast yield. Accurate percentage yield is denoted for each size grouping.

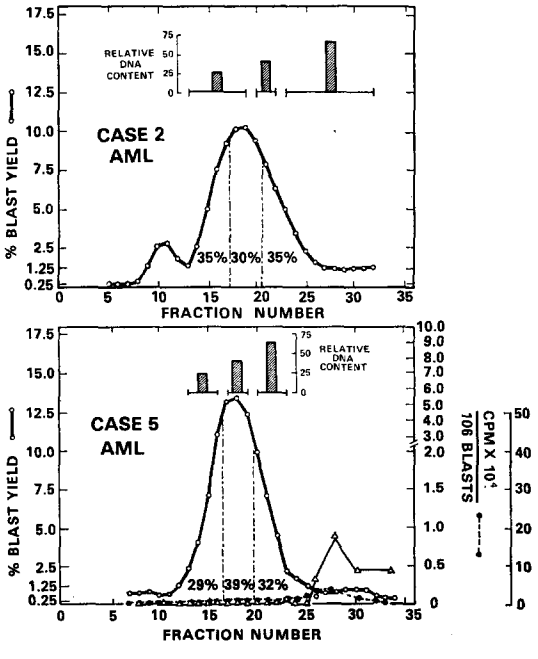


Fig. 2. Relative DNA content for pooled fractions in case 2 (AML) and case 5 (AML).

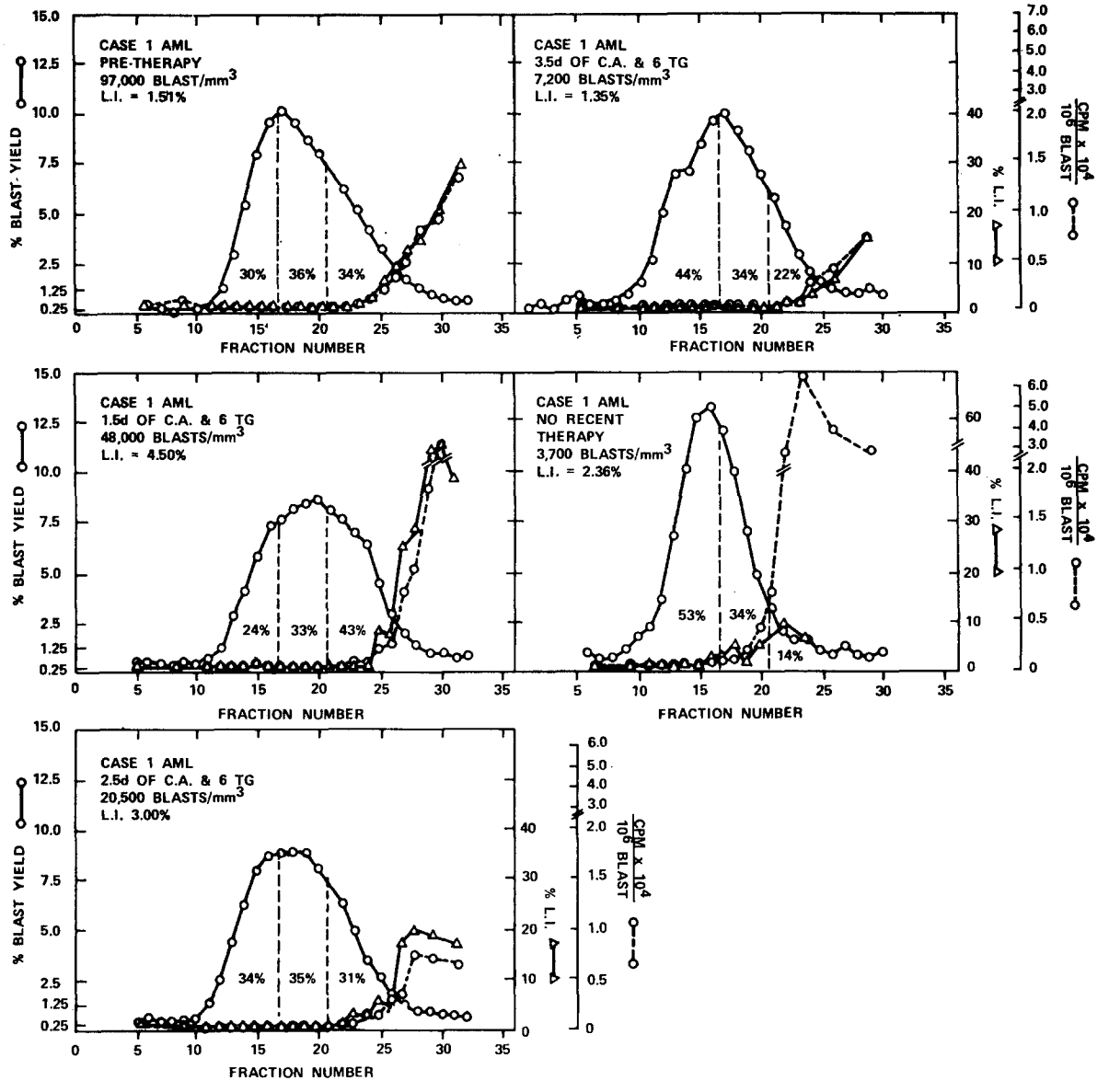


Fig. 3. Cytokinetic profiles before and sequentially during cytosine arabinoside (C.A.) and 6-thioguanine (6-TG) therapy for case 1 (AML). Total blast count and unseparated labeling indices are denoted for each study. Boundaries between small, medium and large cell fractions are determined from the pretherapy profile. Changes in the percentage blast yield in each of those fraction groups are denoted.

and the peak fraction in AML. The smallest blasts in each case were comparable in size, but AML was more variable and contained a population of blasts larger than the largest ALL cells. Table 2 compares, for each case, peak fraction position to peak sedimentation velocity and to modal cell diameter, as determined with an ocular micrometer. All three parameters were correlated strongly, with linear regression lines yielding correlation coefficients between peak position and PSV of 0·78 and between peak position and modal cell diameter of 0·83. Case 6 and case 7, with ALL, had the smallest cells by all three parameters.

Wide cell size distributions, defined by $< 85\%$ blast yield in the peak fraction ± 5 fractions, were found in blastic CML and in all cases of AML except case 4, studied 12 hr after hydroxyurea. That case, like both cases of ALL, had $> 85\%$ blast yield in the defined region and was considered to have a narrow cell size distribution. Cases 1 and 7, in Fig. 1, demonstrate the wide and narrow distributions, respectively.

To classify cells as to relative size within a given cell separation, the blast profile of each baseline study was divided into three parts of approximately equal yield. The segment on the

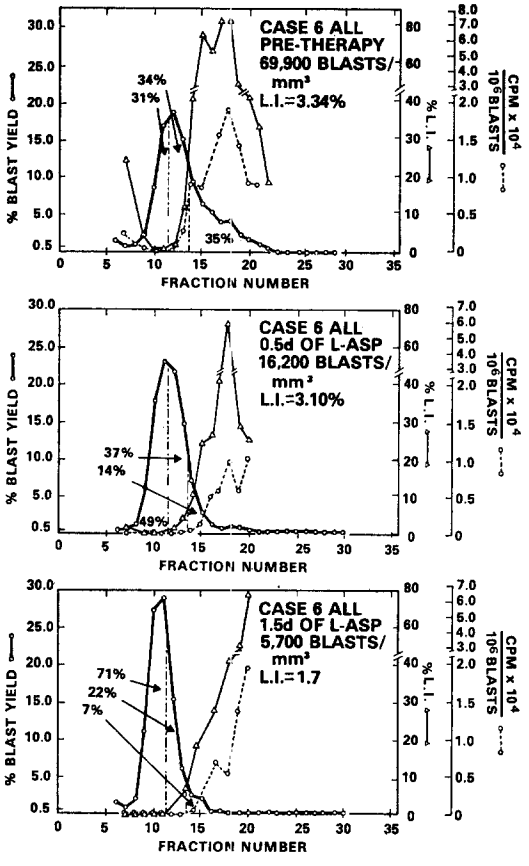


Fig. 4. *Cytokinetic studies before and sequentially during* L-*asparaginase* (L-*ASP*) *therapy in case* 6 (*ALL*).

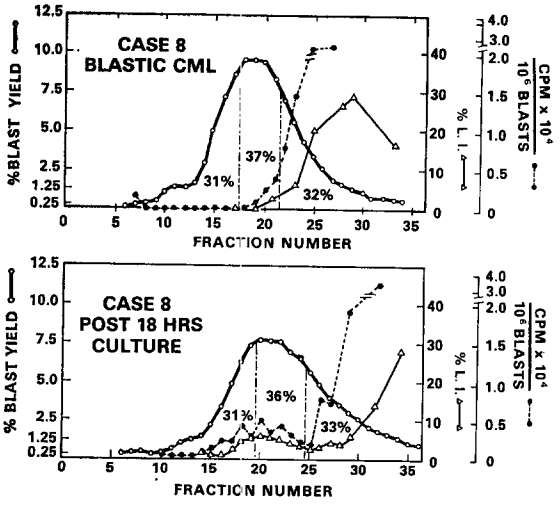


Fig. 5. *Cytokinetic profiles for case* 8 (*blastic CML*) *immediately after* ^{3}*HTdR incubation and after* 18 *hr of culture following* ^{3}*HTdR incubation.*

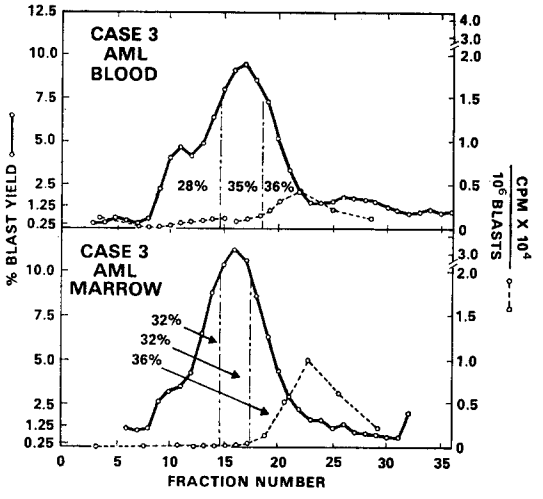


Fig. 6. *Simultaneous cytokinetic profiles from bone marrow and peripheral blood of case* 3 (*AML*). *Low level small cell* ^{3}*HTdR uptake seen in peripheral blood may be artifactual.*

far left contained the small cell fractions. The middle segment contained the medium cell fractions, and the segment on the right contained the large cell fractions. The boundaries of the fraction groups in baseline studies defined the small, medium and large cells in sequential chemotherapy studies, regardless of changes in cell size distribution. Percentage of total blast yield within each group is depicted for baseline and post chemotherapy profiles in each figure. By these definitions, the "large" cells of a narrow distribution, as in case 7, may be equivalent in size to the "small" or "medium" cells of a population with a wide distribution of cells of greater absolute size, as in case 1.

^{3}*HTdR uptake*

^{3}HTdR uptake, as measured by both labeling indices and scintillation counting, was essentially absent from small cell fractions and was concentrated in large cell fractions in all cases. In the narrow ALL distributions, and in blastic CML, uptake was also seen in medium sized cells. Figure 1 demonstrates the uptake curves in the representative cases; the relation of uptake to relative, rather than absolute, size is stressed. The curve obtained by liquid scintillation counting generally paralleled the labeling index curve, although the magnitude of scintillation counts associated with a given labeling index varied significantly. This was due to differences in the rate of ^{3}HTdR uptake, and thus grain counts per cell, and, as will be seen, to the presence of labeled myelocytes which contributed to the large cell scintillation

Table 2. Data relating to cell size

DX	Case	Peak fraction	Peak sed. Veloc. (mm/hr)	Modal cell dia (μm)
AML	1	17	9·5	15·0
	2	19	10·0	15·0
	3	17	8·4	11·5
	4*	14	8·0	12·5
	5	18	11·5	15·0
ALL	6	12	7·5	11·0
	7	13	6·5	10·0
Blastic CML	8	18	8·5	15·0

Cell size for each case is reflected by peak fraction, peak sedimentation velocity in the lg sucrose gradient density separation, and by modal cell diameter. *12 hr after hydroxyurea.

counting but not to the large blast labeling indices. Baseline labeling indices ranged from essentially 0 to 9·33%, with no relationship to absolute cell size or to morphology.

DNA content

DNA content, like ^{3}HTdR uptake, increased as a function of cell size. Relative DNA content of pooled cells from cases 2 and 5 can be seen in Fig. 2. Large cell DNA was slightly more than twice the small cell DNA, and medium cell DNA was at an intermediate value. In case 5, no appreciable ^{3}HTdR labeling was seen in medium sized cells of intermediate DNA content. This case had an unseparated baseline labeling index of essentially zero, with labeled cells appearing in separated fractions only in the largest cells. It appears that the bulk of the cells located in S phase by DNA content in this case were thus either "resting" or progressing very slowly through S phase.

Effects of therapy

Sequential cytokinetic studies were performed in cases 1 and 6. Case 1 is a patient with AML who was treated with cytosine arabinoside (Ara-C, 100 mg/M^2 bid) and 6-thioguanine (6-TG, 90 mg/M^2 bid) to hypoplasia. He failed to achieve a remission and received a second course of Ara-C and 6-TG when his disease recurred. He then became aplastic and died. Case 6 is a patient with ALL treated with L-asparaginase (15,000 i.u./M^2 qd or ~ 330 i.u./kg qd) as the initial therapy of a combination protocol. He was subsequently treated with vincristine, daunomycin and prednisone and rapidly achieved a complete remission.

Case 1 responded to therapy with a progressive decrease of peripheral blast count. Figure 3 shows the effects of therapy on sequential blast counts and cell profiles. After 1.5 days of Ara-C and 6-TG, the cell size distribution shifted rightward, toward larger cells. Relative large cell count increased from 34% to 43%, and relative small cell content decreased from 30% to 24%. The labeling indices and ^{3}HTdR uptake determined by LSC increased in the unseparated aliquots and showed a possible increase in the large cell fractions. At 2·5 days of therapy the size distribution shifted back toward baseline, and the ^{3}HTdR uptake decreased slightly from the previous day. At 3·5 days there was further preferential loss of the largest cells, along with decreased proliferative activity. When disease recurred, the cell distribution was narrower and shifted towards smaller cells, with low blast labeling indices but artifactually high LSC uptake/blast reflecting the low number of blasts and the presence of labeled myelocytes.

In case 6 (Fig. 4) there was a narrow grouping of cells in fractions 7–13 with a significant tail of cells in fractions 12–23. Labeling indices and liquid scintillation counts in large cells were high. Sequential studies at 0·5 and 1·5 days after inception of daily therapy revealed a rapid diminution in the number of blasts. The tail of large and medium sized cells markedly decreased with therapy, and the cell size distribution shifted toward small cells. Along with the preferential loss at 1·5 days of large and medium sized cells—cells of expected high proliferative activity—the labeling indices and liquid scintillation counts in the unseparated aliquot decreased. The remaining large cells continued to take up ^{3}HTdR avidly; however, no shift to large cells or increase in labeling was observed.

Short-term culture

Blast yield after short term (18 hr) culture of cells from case 8 (blastic CML) was 100% of the initially cultured aliquot, suggesting roughly steady state kinetics over that period. Blast profiles before and after culture are shown in Fig. 5. With incubation, the cell size distribution widened and shifted to the right. Liquid scintillation counts and labeling indices in the post culture study showed, in addition to the large cell ^{3}HTdR uptake seen before the 18 hr culture, a second peak of uptake in the medium cell range (fraction 17–24). The interpretation that these are daughter cells is supported by a mean grain count of 80 in fraction 18 and of > 120 in fraction 24. The relative increase in large cells upon culture reflects either relative loss of small (*e.g.*, by cell death or recruitment) or relative accumulation of large cells with decreased small cell formation. Proliferative capacity of large cells was not tested during the culture period, as only a pre-culture label was given.

Bone marrow and peripheral blood

Simultaneous profiles of bone marrow blasts and peripheral blood blasts from case 3 are seen in Fig. 6. The results are similar, except for low liquid scintillation counts, present in small cells of the blood but not of the marrow, associated with a modest early cell size peak, probably both artifactual secondary to fragmented blasts.

DISCUSSION

The present study reveals that there are both a greater absolute size and greater variability of blast sizes in AML than in ALL. This is consistent with previous data from larger series [8–9] in which morphological classification was based on cytochemical and/or other cytological features. This relationship may become to some extent self-fulfilling, as cell size may play an increased role in morphological classification of acute leukemia.

The cellular morphology and the various parameters reflecting absolute size of the leukemic population as a whole did not correlate with differences in 3HTdR labeling index in this study. These data are probably meaningful in AML, but are too limited to reflect the proliferative significance of size in ALL. Faille *et al.* noted a positive correlation between bone marrow labeling index and preponderance of macrolymphoblasts in ALL, but they did not describe such a relationship in AML [2]. Saunders *et al.* found a similar positive correlation in a group ofpatients with leukemia, the majority of whom had childhood ALL [10].

It is to be noted, however, that much of the literature dealing with cell size and acute leukemia is ambiguous as to the objective criteria for "largeness" and "smallness," both relative and absolute, and is subject to the inherent errors and limitations of cell size estimations from slide preparations, either prepared by hand or by cytocentrifuge.

It is clear, though, from the many *in vivo* studies which have been reported previously, that with a pulse label of ^{3}HTdR, labeling is initially concentrated in the relatively large blasts, and, with time, the relatively small blasts become labeled but with lower grain counts [11–16]. These cells are considered to be daughter cells, and they may be stimulated to transform into large cells [17]. There has been some evidence that the smallest of these cells may be "resting cells," or at least cells of low proliferative potential. Karle *et al.* noted that while the larger small cells in a case of ALL rapidly accumulated label after an *in vivo* pulse of ^{3}HTdR, the smallest cells were slow to accumulate label and were still unlabeled at 170 hr [18]. In continuous labeling experiments, Clarkson found a small but significant population of unlabeled small cells after approximately 3 weeks of continuous ^{3}HTdR [19].

The present studies support the concept of size-related proliferative capacity and are consistent with a cytokinetic model of human acute leukemia in which relative blast size correlates with cell cycle stage. The smallest cells appear neither to synthesize DNA nor to be the immediate offspring of dividing cells and may thus be candidates for either G_0 or G_1 status. The increased DNA content and ^{3}HTdR uptake in the medium and large cells, along with the concentration of daughter cells in the medium cell fractions, suggest that progress from active G_1 cells through the cell cycle may correlate with increasing cell size from the medium compartment onward.

The model is further supported by the cytokinetic response of acute leukemia to chemotherapy. Cytosine arabinoside and 6-thioguanine, which are S-phase specific in activity, [20–22] caused preferential depletion of large blast population and decreased labeling in the large cell compartment. Cell loss was not

limited to large cells, however; there was also a lesser depletion of the small and medium blast populations. Three factors may account for the small cell depletion: (1) natural loss of small cells by death, metastases or by transformation to medium or large cells at a normal rate, combined with a decrease in or cessation of small cell renewal from medium or large cells due to drug effect, (2) drug-induced small cell kill, perhaps due to 6-TG, and (3) recruitment, or enhanced transformation to medium or large cells, either directly drug-induced or secondary to decreased total leukemic cell burden.

Cell loss by death or metastasis is a significant factor in the kinetics of solid tumor growth [23]. The magnitude of this flux has been difficult to evaluate in human leukemia because of the problems in estimating total body tumor and doubling times. There is evidence, however, for appreciable cell loss, with preferential loss of small, noncycling cells in the AKR model [4] and of noncycling cells in Ehrlich ascites tumors [24].

An estimate of the magnitude of small cell loss in leukemia can be inferred from data reflecting small cell renewal, since in a steady state or in a growing population the rate of exit from the small cell compartment would be expected to be equal to or to be less than the small cell renewal rate. In case 8, under *in vitro* conditions, the smallest cells do not appear to be rapidly renewed from immediate daughter cells, but might have a significant renewal rate from older medium sized cells (or less likely from the daughters of very slowly cycling cells). The continuous infusion studies of Clarkson *et al.* indicate furthermore that 7–12% of the leukemic cells (small blasts) may lie dormant, without DNA synthesis, for 8–10 days [19, 25]. Such data suggest that a significant portion of the small cells is not renewed rapidly, and is thus not lost rapidly via death, metastases or transformation into larger cells.

This would imply, in the absence of documented significant cycle nonspecific kill secondary to Ara-C and 6-TG, that the synchronization evidenced in Case 1 by the increased labeling and increased cell size at 1·5 days is explained in part by enhanced transformation or recruitment of small cells, resulting in the secondary depletion of the small cell compartment. Persistence of significant numbers of medium cells is further evidence that recruitment did occur. Studies of Ernst *et al.* [22] have suggested that Ara-C does not block entry of G_1 or G_0 cells into cycle, and that, at least with low dose Ara-C, some cells may be arrested by Ara-C in early S-phase. The preferential preservation of medium cells may reflect the arrest of cells.

Synchronization with Ara-C has been proposed previously in human acute leukemia [26, 27]. Ernst *et al.* [22] did not find clear evidence for synchronization, but this was attributed to the low dose of Ara-C which was used in the study.

In case 6, L-asparaginase caused a loss of cells of all sizes, with a preferential loss of both medium and large cells. These findings are consistent with two modes of action for L-asparaginase, *e.g.*: one causing an early cycle non-specific blast lysis [28, 29] and the other causing a preferential depletion of cycling cells. The latter effect could be mediated by a G_1/S block, as postulated by Saunders [28] based on his data from patients receiving very large doses (2000 i.u./kg) of L-asparaginase, or by S phase destruction and inhibition without a G_1/S block, as postulated by Ernst [29], also based on data from patients receiving large doses (1000 i.u./kg). Pagliardi *et al.* [30], using lower doses of L-asparaginase (100–200 i.u./kg) did not find a consistent early cycle nonspecific lytic effect. Their data, however, favored an early S phase specific killing or slowing effect and a late G_1/S block.

In case 6, the active thymidine uptake in surviving large cells would favor relatively unimpaired DNA synthesis in cells escaping a G_1/S block and proceeding through the cell cycle. Unlike the data from Case 1, treated with Ara-C, there is no increase in labeling index or shift to large cells suggesting synchronization or recruitment, and there are few medium and large cells suggesting arrest or slowing in S phase. The small cell loss may thus be primarily due to cycle nonspecific cytolysis rather than to recruitment. Lack of recruitment would also support the concept of a G_1/S block, although it may be that transient synchronization or recruitment occurred prior to the first post L-asparaginase study.

The data in the present study were obtained utilizing peripheral, rather than bone marrow cells. While occasional patients have shown higher ^{3}HTdR labeling indices in peripheral blood than in bone marrow [30, 31], the majority have significantly greater marrow labeling [32–34]. This is consistent with a predominantly intramedullary proliferation of leukemic cells and subsequent marrow release of blasts which, at least in part, maintaia the capacity to synthesize DNA. It has been argued that peripheral blood blasts are "dead end" cells, on the basis of infrequent mitoses in peri-

pheral blood, half life studies of peripheral blasts heavily labeled with ^{3}HTdR, and autologous transfusion of uridine labeled cells [19]. The latter studies, however, were characterized by poor recovery of the uridine labeled cells, and half life studies did not account for transformation of heavily labeled cells into circulating or intramedullary cells with less label per cell due to division. Furthermore, evidence that peripheral blasts do recirculate to the marrow and divide is found in the studies of Tarocco *et al.* [35], of Killman *et al.* [36], and in unpublished observations of Rosen *et al.* [37], who used autotransfused chromium-51 labeled myeloblasts in patients with AML. The comparative study of bone marrow and peripheral blood in the present study reveals similarity of both size and labeling profiles.

Studies of peripheral blood cytokinetics, then, probably reflect but may not entirely define bone marrow kinetics. The study of peripheral blood offers the advantage of permitting multiple and easily obtainable samples. In a patient with relative high peripheral blast count, the monitoring of relative changes in cell size distribution with chemotherapy is a potentially useful tool in evaluating efficacy of treatment relative to the destruction of large proliferative cells and relative to the recruitment and destruction of smaller, slowly proliferating and resting cells. Such data may be useful in planning and modifying the sequence of combination chemotherapy.

Acknowledgements—The authors gratefully acknowledge the excellent technical assistance of Ms. Ada Brooks and the secretarial assistance of Mrs. Marilyn Leach. The authors also wish to express their appreciation to Dr. Brigid Leventhal and Dr. John Zeigler for allowing us to study patients under their care.

REFERENCES

1. S. Perry and R. C. Gallo, Physiology of human leukemic leucocytes: Kinetic and biochemical considerations. In *Regulation of Hematopoiesis*, (Edited by A. S. Gordon) Vol. 2. p. 127, Appleton-Century-Crofts, New York (1970).
2. A. Faille, Y. Najean and J. Bernard, Capacité de Prolifération des cellules leucemiques de la moelle humaine. *Nouv. Presse Med.* **2,** 889 (1973).
3. M. Omine and S. Perry, Use of cell separation at 1g for cytokinetic studies in spontaneous AKR leukemia. *J. nat. Cancer Inst.* **48,** 697 (1972).
4. M. Omine, G. P. Sarna and S. Perry, Composition of leukemic cell populations in AKR leukemia and effects of chemotherapy. *Europ. J. Cancer* **9,** 557 (1973).
5. M. Omine and S. Perry, Perturbation of leukemic cell population in AKR mice due to chemotherapy. *Cancer Res.* **33,** 2596 (1973).
6. E. A. Peterson and W. H. Evans, Separation of bone marrow cells by sedimentation at unit gravity. *Nature (London)* **214,** 824 (1967).
7. Z. Dische, Über einege neue charakteristiche Farbreaktionen der Thymonuklernsäure and eine Mikromethode zur Bestimmung derselben in tierischen Orgenen met Hilfe dieser Reaktionen. *Mikrochemie* **8,** 4 (1930).
8. J. Gauthier, P. Harel and C. Brosseau, Leukocyte mean corpuscular volume in the differential diagnosis of acute leukemias. *Canad. med. Ass. J.* **109,** 187 (1973).
9. F. G. J. Hayhoe, D. Quaglino and D. Doll, The cytology and cytoehemistry of acute leukaemias, a study of 140 cases. *Spec. Rep. Ser. med. Res. Coun. (Lond.)* **304,** (1964).
10. E. F. Saunders, B. C. Lampkin and A. M. Mauer, Variation of proliferative activity in leukemic cell populations of patients with acute leukemia. *J. clin. Invest.* **46,** 1356 (1967).
11. F. Gavosto, A. Pileri, C. Bachi and L. Pegoraro, Proliferation and maturation defect in acute leukaemia cells. *Nature (London)* **203,** 92 (1964).
12. F. Gavosto, A. Pileri, V. Gabutti and P. Masera, Non self maintaining kinetics of proliferating blasts in human acute leukemia. *Nature (London)* **216,** 188 (1967).
13. A. Todo, Proliferation and differentiation of hematopoietic cells in hematologic disorders. Part III. *In vivo* radioautographic study of leukemia including erythroleukemia. *Acta haemat. jap.* **31,** 947 (1968).
14. F. Gavosto, A. Pileri, V. Gabutti and P. Masera, Cell population kinetics in human acute leukemia. *Europ. J. Cancer* **3,** 301 (1967).

15. H. P. WAGNER, M. COTTIER and E. P. CRONKITE, Variability of proliferative patterns in acute lymphoid leukemia of children. *Blood* **39,** 176 (1972).
16. M. L. GREENBERG, M. B. CHARANA, E. B. CRONKITE, G. GIACOMELLI, K. R. RAI, L. M. SCHIFFER, P. A. STRYCKMANS and P. C. VINCENT, The generation time of human leukemic myeloblasts. *Lab. Invest.* **26,** 245 (1972).
17. V. GABUTTI, A. PILERI, R. P. TAROCCO, F. GAVOSTO and E. H. COOPER, Proliferative potential of out-of-cycle leukaemic cells. *Nature (London)* **224,** 375 (1969).
18. H. KARLE, P. ERNST and S. A. KILLMAN, Changing cytokinetic patterns of human leukaemic lymphoblasts during the course of the disease, studied *in vivo*. *Brit. J. Haemat.* **24,** 231 (1973).
19. B. D. CLARKSON, Review of recent studies of cellular proliferation in acute leukemia. In *Human Tumor Cell Kinetics* (Edited by S. PERRY) NCI Monograph #30, U.S. Government Printing Office, Washington, D.C. (1969).
20. M. KARON and S. SHIRAKAWA. The locus of action of 1-B-D-Arabinofuranosyl-cytosine in the cell cycle. *Cancer Res.* **29,** 687 (1969).
21. D. PRICE and J. TIMSON, 6-Thioguanine: antimitotic effect on human lymphocytes *in vitro* prevented by adenine. *Brit. J. Cancer* **25,** 106 (1971).
22. P. ERNST, A. FAILLE and S. A. KILLMANN, Perturbation of cell cycle of human leukaemic myeloblasts *in vivo* by cytosine arabinoside. *Scand. J. Haemat.* **10,** 209 (1973).
23. G. G. STEEL, Cell loss as a factor in the growth rate of human tumours. *Europ. J. Cancer* **3,** 381 (1967).
24. P. K. LALA, Evaluation of the mode of cell death in Ehrlich ascites tumor. *Cancer* **29,** 261 (1972).
25. B. CLARKSON, J. FRIED, A. STRIFE, Y. SAKAI, D. OTA and T. OHKITA, Studies of cellular proliferation in human leukemia. III. Behavior of leukemic cells in three adults with acute leukemia given continuous infusions of ^{3}H-Thymidine for 8 or 10 days. *Cancer* **25,** 1237 (1970).
26. B. C. LAMPKIN, T. NAGAO and A. M. MAUER, Synchronization and recruitment in acute leukemia. *J. clin. Invest.* **50,** 2204 (1971).
27. A. FAILLE, Y. NAJEAN and J. BERNARD, La cinétique des myéloblastes leucemiques hemains aprés cytosine-arabinoside. *Acta Haemat.* **49,** 6 (1973).
28. E. F. SAUNDERS, The effect of L-Asparaginase on the nucleic acid metabolism and cell cycle of human leukemia cells. *Blood* **39,** 575 (1972).
29. P. ERNST, Perturbation of generation cycle of human leukemic lymphoblasts *in vivo* by L-asparaginase. *Brit. J. Haemat.* **25,** 33 (1973).
30. G. L. PAGLIARDI, V. GABUTTI and F. GAVOSTO, Mechanism of action of L-Asparaginase on the cell cycle and growth in acute lymphoblastic leukemia. *Acta Haemat.* **50,** 257 (1973).
31. F. GAVOSTO, A. PILERI, A. PONZONE, P. MASERA, R. P. TAROCCO and V. GABUTTI, Different blast kinetics in acute myeloblastic and lymphoblastic leukaemia. *Acta Haemat.* **41,** 215 (1967).
32. A. M. MAUER and V. FISHER, Comparison of the proliferative capacity of acute leukaemia cells in bone marrow and blood. *Nature (London)* **193,** 1085 (1962).
33. S. A. KILLMANN, Proliferative activity of blast cells in leukemia and myelofibrosis. *Acta med. scand* **178,** 263 (1965).
34. M. D. FOADI, E. H. COOPER and R. M. HARDISTY, Proliferative activity of leukaemic cells at various stages of acute leukaemia of childhood. *Brit. J. Haemat.* **15,** 269 (1968).
35. R. A. TAROCCO, A. PILERI, A. PONZONE and F. GAVOSTO, Bone marrow return and division of circulating acute lymphoblastic leukaemia cells. *Acta Haemat.* **47,** 277 (1972).
36. S. A. KILLMANN, H. KARLE, P. ERNST and V. ANDERSEN, Return of human leukemia myeloblasts from blood marrow. *Acta med. scand.* **189,** 137 (1971).
37. P. ROSEN, H. S. EYRE, D. L. LILIAN and S. PERRY. Unpublished data.

Europ. J. Cancer Vol. 11, pp. 493–497. Pergamon Press 1975. Printed in Great Britain

Effects of the Carcinogen Urethane on Nuclear RNA Polymerase Activities

P. EKER

Norsk Hydro's Institute for Cancer Research, Department of Biochemistry, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway.

Abstract—*Intraperitoneal injection of male rats with a single dose of the carcinogen urethane inhibited the Mg^{2+}-dependent RNA polymerase activity of isolated lung nuclei by about 50%. The inhibition was maximal 1 hr after the injection. Twenty hours after administration, the Mg^{2+}-stimulated activity was again normal. The non-carcinogenic derivatives methylcarbamate and butylcarbamate had no effect on RNA polymerase activities of isolated lung nuclei. The inhibition of nuclear RNA synthesis by urethane was not due to increased hydrolysis of newly synthesized RNA, and the template activity of lung chromatin was not affected by the carcinogen.*

The activity of $Mn^{2+} + (NH_4)SO_4$-dependent RNA polymerase activity of isolated lung nuclei was not affected by urethane injection. The two RNA polymerase activities of liver nuclei were not inhibited by the carcinogen.

Mg^{2+}-stimulated nuclear RNA synthesis in vitro *was inhibited markedly by urethane at concentrations above 50 mM, whereas the $Mn^{2+} + (NH_4)_2SO_4$-dependent activity was only slightly affected.* In vitro *RNA synthesis catalyzed by* E. coli *RNA polymerase was inhibited also by high concentrations of the carcinogen.*

INTRODUCTION

URETHANE (ethyl carbamate) is a multipotential carcinogen which appears to be particularly useful for investigating the mechanism of carcinogenesis by chemical agents. Structurally, it is one of the simplest carcinogens; it is soluble in water and when injected into an animal, it is rapidly and evenly distributed throughout the body [1, 2]. Only minimal cell death occurs following exposure to urethane and accumulating evidence indicates that rapidly proliferating cells are more susceptible to its carcinogenic action than are resting cells [3–5].

In spite of extensive studies, the molecular mechanism by which urethane induces neoplasia has not been elucidated. A connection between urethane carcinogenesis and nucleic acid metabolism has been postulated [1, 2, 6] and recent data suggest that the synthesis of ribosomal RNA may be one of the primary metabolic alterations induced by the administration of urethane [7, 8].

In the present work the effect of urethane on RNA polymerase activities of nuclei isolated from liver and lung of male rats are investigated. The effect of the carcinogen on *in vitro* RNA synthesis with *E. coli* RNA polymerase are also studied.

MATERIAL AND METHODS

Male Wistar rats (250–300 g) were used. Nuclei were isolated as described by Zieve [9]. The final nuclear preparation contained about 75% of the DNA of the liver or lung homogenates. RNA polymerase activities of isolated nuclei were determined essentially as described by Zieve [9] except that ^{3}[H] ATP was used. The incubation mixture for the Mg^{2+}-activated RNA polymerase activity contained in a final volume of 250 μl, 50 mM Tris–HCl buffer, pH 8·5, 5 mM $MgCl_2$, 0·6

Accepted 25 March 1975.

mM GTP, CTP, and UTP, 0·04 mM ^{3}[H] ATP, and 50 μl of nuclear suspension containing about 80 μg of DNA. Incubation was for 10 min at 37°. The incubation mixture for the $Mn^{2+} + (NH_4)_2SO_4$-activated RNA polymerase contained, in a final volume of 250 μl, 50 μl Tris–HCl buffer, pH 7·5, 4 mM $MnCl_2$, 0·4 M $(NH_4)_2SO_4$, 0·6 mM GTP, CTP, and UTP, 0·04 mM ^{3}[H] ATP, and 25 μl of nuclear suspension. Incubation was for 10 min at 37°. RNA polymerase from *E. coli* was prepared by the method of Burgess [10]. The final purification step was the glycerol gradient centrifugation method. The specific activity of the enzyme, as defined by Burgess [10], was 390 units per mg protein. RNA polymerase activity was measured according to Burgess [10], except that the concentration of potassium phosphate in the assay mixture was 0·6 mM. RNase activity was measured according to Utsunomiya and Roth [11]. Chromatin was isolated as described by Maruzige and Bonner [12]. DNA was prepared by the method of Marmur [13], as modified by Gillespie and Spiegelman [14]. DNA suspension in Tris buffer ph 7·9, containing 1 mM NaCl was denatured by heating at 90° for 10 min, and then immediately cooling in an ice bath. DNA was determined by the method of Ceriotti [15], and protein by the method of Lowry *et al.* [16].

Adenosine-8-^{3}[H]-5′-triphosphate was purchased from the Radiochemical Centre, Amersham, England. Urethane, calf thymus DNA, and all other chemicals used in the present work were obtained from Sigma.

RESULTS AND DISCUSSION

Widnell and Tata [17] have shown that intact nuclei incubated in the presence of Mg^{2+} synthesize ribosomes-like RNA while nuclei incubated in the presence of $Mn^{2+} + (NH_4)_2SO_4$ synthesize DNA-like RNA. Recent data suggest that the Mg^{2+}-stimulated activity reflects polymerase I, which is located in the nucleolus and is responsible for the synthesis of ribosomal RNA. The $Mn^{2+} + (NH_4)_2SO_4$-dependent activity probably reflects polymerase II which is located in the nucleoplasme and is involved in the synthesis of DNA-like RNA [9].

Figure 1 shows the effect of urethane administration on the two RNA polymerase activities of nuclei isolated from liver and lung. It appears that a single i.p. injection of 1 mg/g body wt of the carcinogen inhibited the Mg^{2+}-dependent RNA polymerase activity of intact lung nuclei by about 50%. The inhibition reached its maximal value within 1 hr after injection of urethane and was reversible. Thus, nuclei isolated from the lungs of rats 20 hr after administration of the carcinogen had normal levels of Mg^{2+}-stimulated RNA polymerase activity (Fig. 1). The $Mn^{2+} + (NH_4)_2SO_4$-dependent activity of lung nuclei was not affected by urethane.

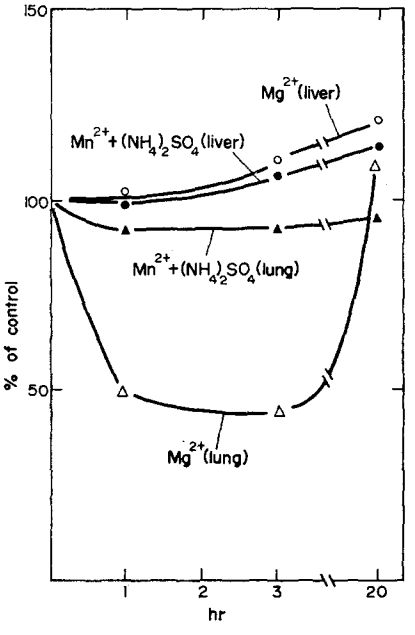


Fig. 1. Effect of urethane injection on nuclear RNA polymerase activities. Urethane was dissolved in 0·9% NaCl-solution (300 mg/ml) and injected i.p. at a level of 1 mg/g of body wt. Control animals received an equal volume of 0·9% NaCl solution. At the times indicated, the livers and lungs from 3 controls and 3 carcinogen-treated animals were pooled and the nuclei isolated. The Mg^{2+}-dependent and $Mn^{2+} + (NH_4)_2SO_4$-dependent nuclear RNA polymerase activities were measured as described earlier. The data represent the mean value from 3 experiments. The results obtained did not differ by more than ±15%. The average control value for liver nuclei was 0·54 mμmole ^{3}[H] ATP incorporated per mg DNA for Mg^{2+}-dependent activity, and 1·89 mμmole ^{3}[H] ATP incorporated per mg DNA for $Mn^{2+} + (NH_4)_2SO_4$-dependent activity. The corresponding values for lung nuclei were 0·43 mμmole and 1·32 mμmole.

It can also be seen from Fig. 1 that the two RNA polymerase activities of nuclei isolated from rat liver were not inhibited by urethane. In fact, a small stimulation (10–20%) in the activities was observed after 20 hr. The failure of urethane to inhibit the Mg^{2+}-stimulated RNA polymerase activity of liver nuclei is of interest in view of the fact that livers of adult rats are virtually resistant to the carcinogenic action of urethane [18, 19]. On the other hand, urethane induces lung tumors in newborn as well as in adult rats [18, 20]. These data

suggest that a correlation may exist between the inhibition of nuclear RNA synthesis and the carcinogenic action of urethane. Further support for this view was obtained in experiments with the non-carcinogenic derivatives methylcarbamate and butylcarbamate. In separate experiments it was found that these compounds, when administered i.p. to male rats, failed to inhibit significantly RNA polymerase activities of lung nuclei. A similar correlation between hepatic carcinogenicity and inhibition of liver RNA polymerase activities has been reported in the case of N-hydroxy-2-fluorenylacetamide and N-acetoxy-2-fluorenylacetamide [9].

The inhibition of RNA synthesis in lung nuclei from rats treated with urethane may be due to several possible factors such as decrease in the template activity of chromatin, or to an increased activity of nuclear ribonuclease activity, resulting in an increased rate of degradation of synthesized RNA. In the present study the template activity of chromatin isolated from lung nuclei was measured by using *E. coli* RNA polymerase. From the results in Table 1, it appears that the template

Table 1. *Effect of urethane on template capacity of chromatin*

Treatment*	Incorporation of AMP
	cpm/10 μg of chromatin DNA
Control	1286 ($\pm$ 146)
Urethane	1303 ($\pm$ 132)

*Rats were treated with urethane as described in the legend to Fig. 1. After 3 hr, the lung chromatin was isolated and the template activity measured as described earlier. Values are mean results from 3 experiments ($\pm$ s.d.).

property of chromatin isolated from urethane-treated rats was identical with that of normal chromatin. Furthermore, in separate experiments it was found that RNase activity of isolated nuclei was not affected by urethane-treatment. These data suggest that the inhibition of RNA synthesis of isolated lung nuclei by urethane is due to inactivation of the Mg^{2+}-stimulated RNA polymerase. It should be emphasized, however, that several other possibilities exist such as decreased permeability of the nuclear membrane, or changes in the levels of activators or inhibitors of RNA polymerase.

In view of the pronounced inhibition of the Mg^{2+}-dependent RNA polymerase activity of lung nuclei by the *in vivo* administration of urethane, it was of interest to determine whether the carcinogen could inhibit nuclear RNA synthesis *in vitro*. Interestingly, in accordance with the results from the *in vivo* experiments, the Mg^{2+}-dependent RNA polymerase activity was markedly inhibited by urethane, whereas the $Mn^{2+} + (NH_4)_2$-stimulated activity was only slightly affected (Fig. 2). The

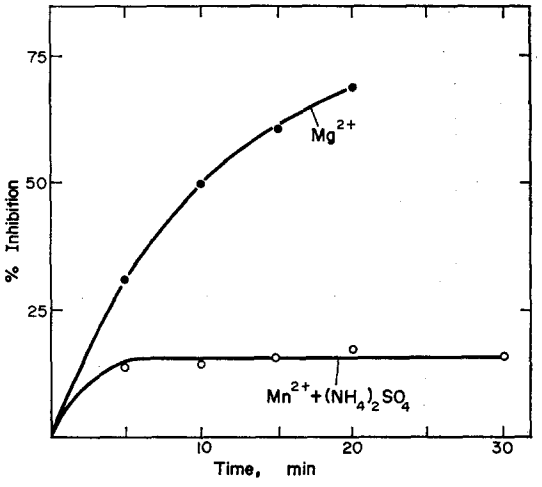


Fig. 2. *Effect of urethane on nuclear RNA synthesis* in vitro. *Urethane* (0·2 *M*) *was added to the reaction mixture and at the times indicated, the* Mg^{2+}*-dependent and* $Mn^{2+} + (NH_4)_2SO_4$*-dependent RNA polymerase activities of intact nuclei isolated from the lungs of normal rats were determined as described earlier. The results represent the average from* 3 *experiments. The control values were* 0·38 *and* 1·19 *m*μ*mole* $^3[H]$ *ATP incorporated for* Mg^{2+}*-dependent and* $Mn^{2+} + (NH_4)_2SO_4$*-dependent activity, respectively.*

extent of inhibition of the Mg^{2+}-activated polymerase increased with the time of incubation. Microscopic examinations of the nuclei showed that they were not disrupted by the urethane treatment. It should be noted that rather high concentrations of urethane were necessary to obtain a significant inhibition of nuclear RNA synthesis in the present experiments. The lowest concentration of the carcinogen found to inhibit the Mg^{2+}-dependent polymerase activity of isolated lung nuclei after 20 min incubation was about 50 mM. This concentration of urethane is considerably higher than the maximal concentration (about 10 mM) which can be tolerated *in vivo*.

Experiments were also carried out to determine whether urethane could affect *in vitro* RNA synthesis catalyzed by bacterial RNA polymerase. The results in Fig. 3 show that RNA transcription with *E. coli* RNA polymerase was markedly inhibited by the carcinogen both when rat liver DNA and when

chromatin were used as templates. As was found in the *in vitro* experiments with isolated nuclei, rather high concentrations of urethane were necessary to obtain a significant effect. At concentrations above 25 mM a linear relationship exists between the amount of urethane added to the incubation mixture and the extent of inhibition of RNA synthesis. Similar results were obtained when calf thymus DNA or denatured rat liver DNA were used as template in the RNA polymerase system.

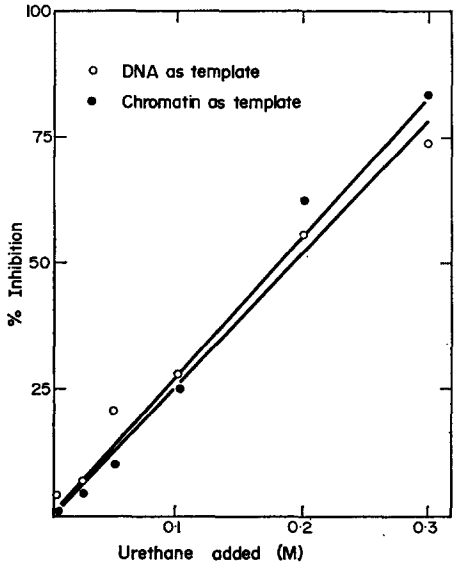


Fig. 3. *Effect of urethane on* in vitro *RNA synthesis with* E. coli *RNA polymerase. Urethane was added to the reaction mixture at the concentrations indicated, and RNA synthesis was measured as described earlier, using DNA* (10 μg) *or chromatin* (10 μg *chromatin DNA*) *as template and* 5 *units of polymerase. The control assay incorporated* 0·93 *mμmole of* ^{3}[*H*] *ATP with DNA as template, and* 0·21 *mumole of* ^{3}[*H*] *ATP with chromatin as template.*

Accumulating evidence suggests that metabolic alterations in RNA synthesis, particularly ribosomal RNA synthesis, may possibly be fundamental to carcinogenesis induced by urethane [2, 7, 8]. The finding in the present study that the inhibition of Mg^{2+}-dependent nuclear RNA polymerase activity by urethane appears to be correlated with its carcinogenic activity is consistent with this view. There is, however, no direct experimental evidence that inhibition of RNA polymerase by urethane plays any role in the carcinogenic action of the compound. Future work on this problem must probably involve studies on the effect of urethane on the different purified RNA polymerases of mammalian tissues. Such work is in progress in our laboratory.

Acknowledgements—The author is indebted to Professor A. Pihl for valuable discussions. The skilful technical assistance of Mrs. M. Svaeren and Mrs. B. Tuhus is gratefully acknowledged.

REFERENCES

1. S. S. Mirvish, The carcinogenic action and metabolism of urethan and N-Hydroxyurethan. In *Advances in Cancer Research* (Edited by A. Haddow and S. Weinhouse) Vol. II, p. 1. Academic Press, New York (1968).
2. K. Williams and R. Nery, Aspects of the mechanism of urethane carcinogenesis. *Xenobiotica* **1,** 545 (1971).
3. C. F. Hollander and P. Bentvelzen, Enhancement of urethan induction of hepatomas in mice by prior partial hepatectomy. *J. natl. Cancer Inst.* **41,** 1303 (1968).
4. I. N. Chernozemski and G. P. Warwick, Liver regeneration and induction of hepatomas in B6AF$_1$ mice by urethan. *Cancer Res.* **30,** 2685 (1970).
5. M. Lane, A. Liebelt, J. Calvert and R. A. Liebelt, Effect of partial hepatectomy on tumor incidence in BALB/c mice treated with urethan. *Cancer Res.* **30,** 1812 (1970).
6. A. M. Kaye and N. Trainin, Urethan carcinogenesis and nucleic acid metabolism: Factors influencing lung adenoma induction. *Cancer Res.* **26,** 2206 (1966).
7. R. I. Glazer, Inhibition of the synthesis of nuclear RNA by urethan in regenerating liver. *Biochem. biophys. Res. Commun.* **53,** 780 (1973).
8. R. I. Glazer, Inhibition by urethan of the synthesis of free and membrane-bound ribosomal RNA in regenerating liver. *Cancer Res.* **33,** 1759 (1973).
9. F. J. Zieve, Inhibition of rat liver ribonucleic acid polymerase by the carcinogen N-hydroxy-2-fluorenylacetamide. *J. biol. Chem.* **247,** 5987 (1972).
10. R. R. Burgess, A new method for the large scale purification of *Escherichia coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase. *J. biol. Chem.* **244,** 6160 (1969).
11. T. Utsunomiya and J. S. Roth, Studies on the function of intracellular ribonucleases. V. Ribonuclease activity in ribosomes and polysomes prepared from rat liver and hepatomas. *J. cell. Biol.* **29,** 395 (1966).

12. K. MARUSHIGE and J. BONNER, Template properties of liver chromatin. *J. molec. Biol.* **15,** 160 (1966).
13. J. MARMUR, A Procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. molec. Biol.* **3,** 208 (1961).
14. D. GILLESPIE and S. SPIEGELMAN, A quantitative assay for DNA–RNA hybrids with DNA immobilized on a membrane. *J. molec. Biol.* **12,** 829 (1965).
15. G. CERIOTTI, A microchemical determination of desoxyribonucleic acid. *J. biol. Chem.* **198,** 297 (1952).
16. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
17. C. C. WIDNELL and J. R. TATA, Studies on the stimulation by ammonium sulphate of the DNA-dependent RNA polymerase of isolated rat-liver nuclei. *Biochim. biophys. Acta (Amst.)* **123,** 478 (1966).
18. M. F. GUYER and P. E. CLAUS, Tumor of the lung in rats following injections of urethane (ethyl carbamate). *Cancer Res.* **7,** 342 (1947).
19. V. R. CHOUDARI KOMMINENI, M. GREENBLATT, N. MIHAILOVICH and S. D. VESSELINOVITCH, The significance of perinatal age periods and the dose of urethan on the tumor profile in the MRC rat. *Cancer Res.* **30,** 2552 (1970).
20. A. ROSIN, Early changes in the lungs of rats treated with urethane (ethyl carbamate). *Cancer Res.* **9,** 583 (1949).

Europ. J. Cancer Vol. 11, pp. 499–507. Pergamon Press 1975. Printed in Great Britain

Factors Influencing the Estimation of Oestrogen Receptors in Human Malignant Breast Tumours*

HANNELORE BRAUNSBERG
Department of Chemical Pathology, St. Mary's Hospital Medical School, London W2 1PG, Great Britain.

Abstract—*Estimates of concentrations of binding sites* (P_0) *and dissociation constants* (K_d) *in extracts of human breast tumours had coefficients of variation of* 16 *and* 29%. *The sensitivity for* P_0 *was about* 1 *fmole per mg wet tissue. Samples taken from different areas of a tumour or from two tumours in the same breast showed significant differences in some cases. Freezing of cytosol preparations can cause deterioration. Oestrogen antagonists increased* K_d *without affecting values of* P_0, *consistent with competition for oestrogen by binding to receptor with high dissociation constant. Sulphydryl reagents added to tissue supernatants increased* P_0 *in a few cases, and in one case exposed binding sites in a receptor-negative preparation. The presence of low-affinity binding components can lead to erroneous estimates of* P_0 *and* K_d *of high-affinity receptors.*

INTRODUCTION

IT HAS been shown that some human malignant breast tumours concentrate oestrogen *in vivo* [1–3] and that this property can be demonstrated *in vitro* by equilibrating tumour slices [4] or cytosol preparations [5, 6] with tritium-labelled oestradiol of high specific activity. Several *in vitro* techniques have been developed for detecting, or estimating the concentration of, oestrogen-binding components in tumour samples, and this work has gained importance following the claim that the presence of receptors is related to response to endocrine therapy [6, 7].

Further work has been done with tissue slices [8, 9] while others [10–18] have preferred cytosol preparations. Three groups [7, 13, 18] have compared results for slices and cytosol preparations and there appears to be fairly good agreement.

Accepted 12 March 1975.
*Supported by the Cancer Research Campaign.

When tissue supernatant preparations are used, the separation of free from bound oestradiol is an important step. This has been effected by Sephadex gel filtration [19, 20], adsorption by dextran-coated charcoal [5, 10–13], density gradient centrifugation [6] and agar gel electrophoresis [7, 14].

A major difficulty arises from the fact that in addition to one or more high-affinity oestrogen binding components, breast tumour and other tissues contain macromolecules which bind oestrogen more loosely. To distinguish between so-called "specific" and "non-specific" binding Jensen and co-workers [6] introduced the use of oestrogen antagonists and these have been employed by some workers [7, 12, 15, 17]. Others have discriminated between the two types of binding by using excess oestradiol, believed to saturate the high-affinity, low capacity component(s) but not the large capacity "non-specific" component(s) [8, 11, 13]; by making use of the fact that the "specific" components are heat-labile, while the non-specific ones are

not [7, 14]; or by dissociation of the low-affinity complex in the presence of charcoal [10].

Evidence has been obtained that binding of oestradiol *in vitro* to target tissues [21, 22] or to tissue extracts [22] may involve sulphydryl groups of the binding components. A preliminary study with extracts from human breast tumour tissue has shown that binding can be enhanced by treatment with sulphydryl reagents [23] and this has been confirmed by another study [24].

A simple method [10] for the determination of oestrogen binding components in extracts prepared from 100 mg tissue and capable of estimating both the concentration of binding sites (P_0) and the dissociation constant (K_d) of the high-affinity complex has been examined in the present paper. A study has been made of some factors which influence the parameters estimated.

MATERIAL AND METHODS

[6, 7-^{3}H] oestradiol-17β (40 Ci/mmole, Radiochemical Centre, Amersham, Bucks) was purified once a month by paper chromatography in the following system: petroleum ether, benzene, methanol, water (33:17:40:10), and stored at 4° in benzene containing 10% methanol. Aqueous solutions of radioactive oestradiol were made up freshly every two weeks and stored at 4°C. Tumours were analysed on the day of operation except where otherwise stated. The method of Feherty *et al.* [10] was followed carefully, 0·25M sucrose being used for homogenisation. Tissue extracts were equilibrated at 30° for 20 instead of 30 min [10]. This did not affect values of P_o and K_d.

Oestrogen antagonists and sulphydryl reagents were added in 100 μl aqueous solution to 1 ml supernatant preparations (or equivalent proportions) to give the final concentrations stated. Controls consisted of 1 ml supernatant solutions plus 100 μl water. Tamoxifen (ICI 46474) was added in solutions containing bovine serum albumin to give a final albumin concentration of 1% in the incubation mixtures [25]. Controls for these tests contained 1% albumin in the incubation mixture and blanks were prepared with 1% albumin in sucrose medium. Pre-incubations with sulphydryl reagents were carried out in an ice-bath for 10 min.

RESULTS

Variability of estimates of binding parameters

To estimate assay precision, tumour samples were chopped finely, the pieces thoroughly mixed and two portions analysed separately. The results obtained by two analyses are shown in Table 1. The estimated standard deviations [26] for P_0 and K_d were 0·8 and 1·6 respectively, and the corresponding coefficients of variation (CV) were 16 and 29%. In order to arrive at an estimate of sensitivity, the standard deviation for P_0 was recalculated omitting tumours 3, 4 and 12, which had high figures. This gave $2{\cdot}0 \pm 0{\cdot}4$ (mean $\pm$ S.D.) and a CV of 20%. Although these are crude estimates because of the small number of duplicates, they can be used to interpret data on the variability within tumours and between discrete tumours found in a patient.

Table 1. *Precision of estimates of* P_0 *and* K_d

Tumour	Results of duplicate analyses* P_0	K_d	Analyst
1	2·2, 2·3	4·9, 5·7	A
2	3·0, 3·9	5·8, 5·8	A
3	20, 22	5·8, 8·0	A
4	11, 9·7	2·8, 3·0	A
5	1·8, 2·5	5·1, 4·8	A, B
6	1·3, 1·3	5·0, 5·2	B
7†	2·3, 2·4	5·0, 5·3	B
8†	1·9, 1·5	3·5, 3·0	B
9†	2·4, 2·7	5·6, 5·8	B
10	1·4, 1·7	3·3, 3·5	B
11	0·5, 0·8	7·8, 12·6	B
12‡	9·4, 9·9	5·7, 6·2	A
13	N.D., N.D.	— —	B

*P_0 concentration of binding sites (fmol per mg wet weight); K_d = dissociation constant (nM $\times 10^{-1}$).
†Analysed after deep-freezing tumour.
‡Analysed after keeping tumour in sucrose at 4° overnight.
N.D. = No binding detected.

Table 2 shows results for large tumours, from which widely spaced samples of similar naked-eye appearance were taken. While some tumours appear to be homogeneous, others showed significant differences for both P_0 and K_d.

Very different results may also be obtained for pairs of separate tumours taken from the same breast. Four patients gave the following results: P_0 83 and 15, 26 and 12, 4 and 0·7, 2·0 and 1·1 fmol per mg. wet weight with K_d 31 and 7·5, 10 and 6·9, 1·9 and 6·6, 5·6 and 7·5 nM $\times 10^{-1}$, respectively.

Table 2. Variability of P_0* *and* K_d* *within tumours*

Tumour	Sample	P_0	K_d
A	1	5·3	8·1
	2	8·2	9·9
B	1	8·5	9·3
	2	3·5	22
C	1	2·3	5·3
	2	1·4	4·7
D	1	4·8	6·4
	2	5·2	7·0
E	1	19	11
	2	30	13
F	1	3·0	5·8
	2	3·9	5·8
G	1	N.D.†	—
	2	N.D.	—
H	1	N.D.	—
	2	N.D.	—

*P_0 = concentration of binding sites (fmol per mg wet weight); K_d = dissociation constant ($nM \times 10^{-1}$).
†N.D. = none detected.

Effect of freezing cytosol preparations

Since the effect of freezing tissues on estimates of binding parameters did not appear to have been studied in detail, cytosol preparations were made on the day of operation, or in a few cases only, after storage at 4°C overnight. Freezing of supernatant preparations may lead to deterioration (Table 3). Binding was completely lost in one case while significant changes in P_0 or K_d or both were seen in 6 out of 10 tumours. The length of storage did not appear to be related to the effect. Deep-freezing of supernatant preparations was avoided in all subsequent work.

Table 3. Effect on binding of storage of supernatant preparations at −15°, thawed at 4°

Tumour	Initial Results P_0*	Initial Results K_d*	Time Stored (Days)	Results after Storage P_0*	Results after Storage K_d*
14	5·4	7·0	1	N.D.	—
15	83	31	1	37	19
16	15	7·5	2	13	6·1
17	21	10	3	10	5·6
18	4·5	2·4	3	3·2	3·2
2	3·4	5·8	3	2·7	5·3
19	2·4	3·6	5	2·2	3·2
20	8·1	3·9	5	7·4	3·9
21	5·1	6·1	14	2·4	5·6
22	3·0	5·4	21	1·3	3·5
23	2·0	1·9	28	3·2	4·6
24	9·8	3·4	30	6·8	2·9

*P_0 = concentration of binding sites (fM per mg wet weight); K_d = dissociation constant ($nM \times 10^{-1}$); N.D. = no binding detected.

Anomalous results

In spite of the 10-min incubation with dextran-coated charcoal at 30° [10], anomalous Scatchard plots were obtained occasionally. Some assays gave curved plots for which no good straight line could be fitted. These preparations are classed as "positive", but estimates of P_0 and K_d would be unreliable. In our series of 82 receptor-positive tumours, 10 such samples were found. Figure 1 shows an example of a very flat curve with high binding, indicating the presence of residual low-affinity binding which may well hide a low concentration of a high-affinity component such as that shown.

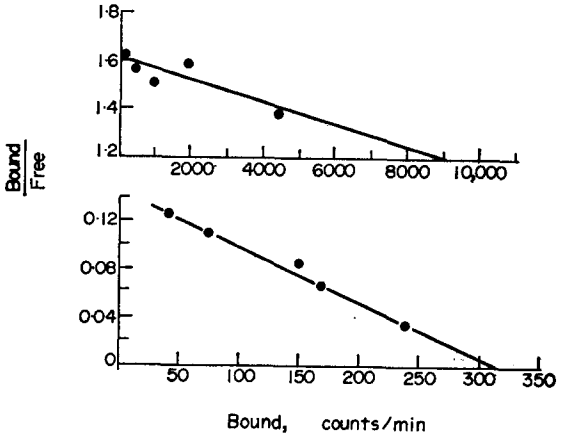


Fig. 1. Evidence of low affinity binding (top) after charcoal incubation which could hide a low concentration of high-affinity receptor (bottom) Ascissae: tritium (counts per min) Ordinates: bound/free radioactivity.
Top: P_0 = 143 *fmol per mg wet wt*
K_d = 4·4 *nM*
Bottom: P_0 = 1·4 *fmol per mg wet wt*
K_d = 0·47 *nM.*

Values of binding parameters

Figure 2 shows frequency distributions for values of K_d and P_0 for preparations from 60 primary and 12 metastatic tumours which gave linear Scatchard plots.

Estimated values of P_0 and K_d may or may not differ significantly in primary and metastatic tumours removed at the same time. Values for P_0 were 20 and 36, 2·1 and 2·6, 6·8 and 22 fmol per mg. wet tissue with K_d 6·0 and 3·9, 3·3 and 2·2, 2·3 and 5·3 $nM \times 10^{-1}$

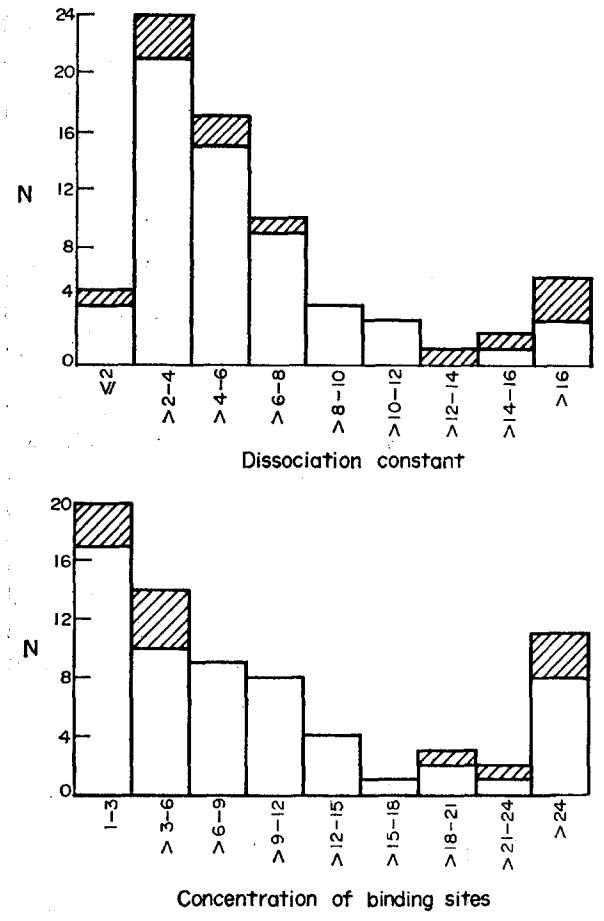


Fig. 2. Frequency distribution of K_d *(dissociation constants nM × 10, top) and* P_0 *(concentrations of binding sites, fmoles per mg wet wt, bottom). Hatched columns show figures for metastatic tumours.*

in primary and metastatic tissues, respectively, taken from three patients.

Effect of sulphydryl reagents

Preliminary experiments indicated that pre-incubation of tissue cytosols with sulphydryl reagents may increase binding in some cases [23]. The significance of these results was, however, difficult to assess in the absence of sufficient data on the precision of the assay. Further investigation has shown some significant changes (Table 4). Mercaptoethanol produced significant increases in P_0 in four and reductions of a similar order in two of nine receptor-positive tumours. In none of three receptor-negative tumours did it induce binding. Dithiothreitol increased P_0 in two and decreased P_0 in two of nine receptor-positive tumours. It induced binding in one of six cases where none was detectable in its absence.

Effect of oestrogen antagonists

Table 5 shows the results of an investigation of the effect of CI628 (Parke–Davis) on

Table 4. Effect of preincubation with mercaptoethanol (EtSH) or dithiothreitol (DTT)

Patient	Control		EtSH (0·1M)		DTT (0·1M)	
	P_0*	K_d*	P_0	K_d	P_0	K_d
A	2·0	3·2	—	—	2·2	2·7
B(1)	37	19	—	—	36	26
(2)	13	6·1	—	—	9·4	5·2
C	3·5	4·3	—	—	7·2	6·6
D	2·4	5·6	—	—	2·8	4·5
E	N.D.*	—	—	—	1·0	7·7
F†	101	70	111	87	84	78
G‡	2·6	2·6	3·3	2·6	3·9	3·6
H	14	5·2	15	4·0	14	4·8
I	11	5·7	11	6·3	11	6·3
J	4·6	16	6·8	4·3	—	—
K	15	6·0	21	5·5	—	—
L	16	6·9	23	7·1	—	—
M	21	18	16	14	—	—
N‡	18	7·8	13	7·4	—	—

*See footnote to Table 3.
†Metastasis.
‡Supernatant preparation stored at −15°, 3 days.

Table 5. Effect of CI628 (Parke-Davis) and preincubation with dithiothreitol (0·1M)

Patient	Concentration of CI628 (μM)	Control		Dithiothreitol	
		P_0*	K_d*	P_0	K_d
A	0	2·0	3·2	2·2	2·7
	2	2·5	40·5	1·2	14
B(1)	0	37	19	36	26
	2	37	60	N.D.	—
(2)	0	13	6·1	—	—
	0·2	12	21	—	—
C	0	3·5	4·3	7·2	6·6
	0·2	3·2	12	9·8	25
	2	N.D.*	—	N.D.	—
	20	N.D.	—	N.D.	—
D	0	2·4	5·6	2·8	4·5
	0·2	2·8	23	2·6	13
	2	N.D.	—	N.D.	—
O	0	10	5·6	—	—
	0·4	6·5	7·2	—	—
	0·8	6·5	11	—	—
P	0	1·3	3·5	—	—
	0·2	1·1	11	—	—
Q	0	2·7	4·6	—	—
	0·1	2·5	4·6	—	—
	0·2	3·1	7·3	—	—
	0·4	4·4	18	—	—
	0·8	3·2	21	—	—
R	0	5·7	2·9	—	—
	0·4	6·4	12	—	—
	0·8	7·4	22	—	—
S	0	N.D.	—	1·0	7·7
	0·2	—	—	1·0	10

*See footnote to Table 3.

estimates of P_0 and K_d for preparations from 9 tumours. The antagonist increased K_d without causing marked changes in P_0, and its effect increased with concentration. The Scatchard plot remained linear (Fig. 3).

Tumours differed in their sensitivity to CI628 (Table 5). Thus a concentration of 0·2 μM produced a marked change in tumour preparations from patients B (tumour 2), C, D and P, but had less effect on that from patient Q. A concentration of 0·4 μM had a relatively small effect on the tumour preparation from patient O, but markedly altered K_d in preparations from patients *Q* and *R*. Even a concentration of 2 μM abolished binding in

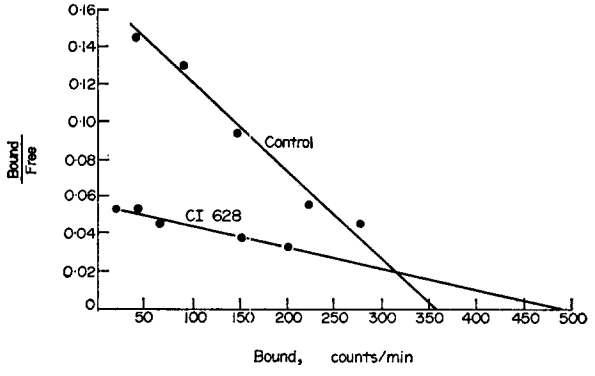


Fig. 3. Scatchard plots for a tumour cytosol in the absence and presence of an oestrogen antagonist.
Control: P_0 = 1·6 *fmoles per mg wet wt*
K_d = 0·45 *nM*
CI628: P_0 = 2·1 *fmoles per mg wet wt*
K_d = 1·9 *nM.*

only 2 out of 3 preparations tested. It is of interest that the effect on one tumour preparation containing excess low-affinity binding sites (patient B, tumour 1) was similar to that on high-affinity components.

The effect of CI628 on preparations which had been preincubated with dithiothreitol was also examined (Table 5). In two cases (patients A and D), CI628 was less effective than in the absence of dithiothreitol pretreatment, while in one case (patient C) CI628 produced a greater increase in K_d after dithiothreitol treatment. At a concentration of 0·2 μM, CI628 possibly had a slight effect on the binding induced by dithiothreitol (patient S).

Table 6 shows results of a smaller series of experiments with nafoxidine (U11,100A, Upjohn) and tamoxifen (ICI 46474, Imperial Chemical Industries). Nafoxidine produced effects similar to those of CI628 (Table 5). Albumin was added at a concentration of 1% to avoid adsorption of ICI 46,474 to glassware [25]. Comparison of results in the absence of inhibitor under the last four columns indicates

Table 6. Effect of inhibitors nafoxidine (U11,100A; Upjohn) and tamoxifen (ICI 46,474; Imperial Chemical Industries) on binding parameters

Patient	Concentration of inhibitor (μM)	Nafoxidine P_0*	Nafoxidine K_d*	Tamoxifen P_0	Tamoxifen K_d
O	0	10	5·6	13	18
	1	6·9	10	12	19
Q	0	2·7	4·6	8·4	20
	0·2	2·5	4·4	6·8	14
	0·4	2·8	6·3	—	—
R	0	5·7	2·9	10	26
	1	11·5	30	7·6	28
	2	7·4	27	—	—

*See footnote to Table 3.

that the presence of albumin markedly altered oestrogen binding. Albumin blanks showed that almost all the albumin–oestrogen complex was dissociated by incubation with charcoal according to Feherty *et al.* [10]. In the presence of albumin, tamoxifen had little effect on binding parameters in the tumours studied.

DISCUSSION

The method of Feherty *et al.* [10] has been stated to have a sensitivity for binding sites of 0·1 fmoles per mg wet tissue. On the basis of our estimate of precision at low concentrations, accurate estimates would not be possible below 1 fmole/mg wet tissue. The estimates of dissociation constants were somewhat less precise than those of concentrations of binding sites and differences of 0·3 nM (3×10^{-10}M) would not be significant.

The larger tumours varied in homogeneity and one appeared to contain high-affinity receptors in one region and low-affinity components in another. It may be difficult to classify such a tumour, if only the high-affinity material is of clinical interest. Findings such as this make it imperative to sample several tumour areas whenever possible. A difference in properties of two distinct malignant tumours removed from the same breast has also been found.

The results presented here support data obtained by other workers [10, 13, 27] that deep freezing supernatant preparations lead to decreased binding. But this was not so in all cases and in only 1 of 10 preparations examined was binding activity lost.

It has been reported that freezing of tumours prior to preparation of cytosols may lead to

reduction of binding [13] although evidence has been produced (for one tumour only) that it had no effect [28]. One group [15] has reported that no change occurred after freezing tissues up to 2 months at −86°, but it is not clear whether the fresh tissue was examined for comparison. The mode of freezing may be of importance and a detailed study remains to be made. Some workers [11, 15–17, 29] have used frozen tissues, but in this laboratory, tumours were analysed on the day of collection whenever possible and only in a few cases were cytosol preparations stored at 4° overnight prior to analysis.

The results in the presence and absence of albumin (Table 6) indicate that large concentrations of low-affinity binding material can alter the estimates of high-affinity binding parameters, even when the low-affinity complex is dissociated completely by charcoal incubation. Reduction of oestradiol binding to the "8S receptor" of rat uterine cytosol by albumin has also been demonstrated [15]. The presence of excessive amounts of "low-affinity" binding can thus lead to overestimates of K_d and to rejection of a receptor-positive tumour on the basis of its dissociation constant if no other criteria of specificity are used. On the other hand "false positives" could result if no distinction is made on the basis of K_d values. If accurate values of receptor binding parameters are considered important in relation to clinical findings, then low- as well as high-affinity components must be estimated and corrections made if necessary [30].

Primary tumours and axillary metastases removed at the same time behaved in a similar manner, though concentrations of binding sites tended to be higher in the metastases. Leclercq *et al.* [16] found a discrepancy in only one out of nine comparisons, with the primary—but not the metastasis—exhibiting binding properties. Other workers [17, 28] have found that concentrations of binding sites may differ in primary and metastatic tumours. The general agreement, as far as presence of binding sites is concerned, between primary tumours and their metastases appears to contradict the finding made by the majority of workers that the incidence of "positive" tumours is greater for primary than for metastatic lesions (Table 7). A possible explanation may be that metastases removed a long time after mastectomy no longer contain "receptors" while those removed at the time of mastectomy behave like the primary tumour. This would imply a progression towards loss of binding sites as the disease advances.

Most workers have found a clear separation into "positive" and "negative" tumours difficult. When sensitive quantitative estimates of binding are used, classification may be made according to magnitude of both P_0 and K_d. This is what we have attempted on the basis of frequency distributions. However, for reasons already discussed, erroneous estimates of P_0 and K_d and mis-classification could result if high concentrations of low-affinity components are present.

While some workers find that approximately 50% of primary tumours contain receptors [7, 8, 11, 13, 15, 16, 18, 27] others (including the present study) find a proportion nearer 70% [10, 17, 29, 31] (Table 7). This difference could be due to variations in patient material, or to technique. In most clinical studies, response to endocrine treatment has been obtained in 30% or, at most, 50% of cases, with 50 to 70% unresponsive. Findings of only 30% "receptor-negative" tumours would indicate that a high proportion of patients with "receptor-positive" tumours may not respond. Thus the use of such assays for predicting response would seem to be less promising than can be expected from earlier reports [6, 7, 18] and, if assay technique has affected results, this would need further study.

The oestrogen antagonists CI628 and U11, 100A increased K_d without materially affecting P_0. This effect is compatible with competitive binding of low affinity (equation 3, Appendix). On the other hand, a direct effect on the receptor, such as alteration of its configuration and properties [33] cannot be excluded. The exact mode of action of these compounds remains to be investigated.

The effects of CI628 and U11,100A depend on their concentration and some tumour preparations showed greater sensitivity than others. There appeared to be no relation between the concentration of binding sites and the magnitude of antagonist effect. A concentration of 0·2 μM CI628 may not suffice to reduce binding in all antagonist-sensitive tumour preparations and inhibition may even be incomplete at a concentration of2μM.

Tamofixen, which has been shown to cause regression of human breast carcinoma, [34 35] had no marked effect on oestrogen binding by the three preparations examined. However, it is possible that the addition of albumin [25] obscured small changes in the parameters studied.

The two sulphydryl reagents examined altered binding in some cases. In one "negative"

Table 7. Incidence of specific oestrogen receptors in primary and metastatic tumours*

Reference	Preparation†	Primary tumours				Metastases			
		+	±	−	*N*	+	±	−	*N*
[7]	S	41	0	59	164	35	0	65	20
[8]	S	45	0	55	31				
[10]	C	70	4	26	53				
[11]	C	45	0	55	43	67	0	33	21
[13]	S	28	0	72	14				
	C	44	0	56	36‡				
[15]	C	39	13	48	75				
[16]	C	56	0	44	77	37	0	63	65
		(51)§	(5)§	44	77	(29)§	(8)§	63	65
[17]	C	73	0	27	251	75	0	25	8
[18]	S+C	48	7	45	117	32	7	61	56
[27]	C	52	0	48	69				
[29]	C	63	0	27	27	79	0	21	14
[31]	C	69	0	31	26	50	0	50	32
[32]	C					46	0	54	37
Present work	C	70	6¶	24	91	50	18¶	32	23

*Incidence (%) of positive (+), doubtful (±) or negative (−) binding tumours. N = number reported.
†S = tissue slices; C = cytosol preparations.
‡Data on fresh cytosol preparations only (frozen tumours and frozen supernatants gave a lower incidence).
§Recalculated after defining tumours with $K_d > 20$ as ±.
¶Tumours with $K_d > 1{\cdot}2$ nM, or with $P_0 > 16$ fmoles/mg wet wt and $K_d > 1{\cdot}0$ nM.

cytosol, dithiothreitol induced binding, which was modified by CI628 to a small extent (Table 5). This component would thus be classed as "specific" by some workers. In two other experiments, CI628 produced a smaller change in K_d after pre-incubation with dithiothreitol than without this treatment, but the reverse was true in another case. These results suggest that dithiothreitol can make more oestrogen binding sites available while altering the quantitative effects of oestrogen antagonists. Since it had little effect on the dissociation constant it does not appear to act on competitors or on low-affinity binding components.

McGuire and de la Garza [24] have reported consistent and marked increases in concentrations of binding sites when frozen tumours were homogenised in the presence of dithiothreitol. They have postulated that this substance "protects" the receptor, and have shown that on sucrose gradient sedimentation, the "8S" peak is increased. Our results are far less dramatic than theirs, but there are considerable differences in analytical procedure. Our work was on fresh, not frozen, tumours and dithiothreitol was added after preparation of the cytosol. Further investigation is necessary to elucidate the mode of action of sulphydryl reagents on oestrogen binding components.

Much work is still needed on methods used for receptor assay. The effect and conditions of freezing tumours and supernatant preparations, of different concentrations of antagonists, of low-affinity components present and of sulphydryl reagents remain to be investigated in detail. These factors may have to be taken into account when clinical response is compared with the results of receptor studies.

Acknowledgements—I wish to thank Mr. C. W. Jamieson and Mr. A. E. Carter for providing tumour material for this study, Professor V. H. T. James for his interest in this work and the Cancer Research Campaign for financial support.

APPENDIX

Competition of inhibitor for binding sites

It can be shown that, if equilibrium is established in the presence of two competing substances (steroid and inhibitor) binding to receptor, the Scatchard equation is modified to:

$$\frac{[B]}{[F]} = \frac{P_0}{K_d} - \frac{[B]}{K_d} - \frac{C[B]}{K_i[F] + K_d[B]} \qquad (1)$$

where $[B]$, $[F]$ are concentrations of bound and free steroid, P_0 is the total receptor concentration, C is the total concentration of

inhibitor and K_d, K_i are dissociation constants for the complexes with steroid and inhibitor respectively.

If K_i and K_d are of the same order, then a plot of $\frac{[B]}{[F]}$ against $[B]$ should no longer be linear.

If K_i is very small compared to K_d, then

$$\frac{C[B]}{K_i[F]+K_d[B]} \rightarrow \frac{C}{K_d}$$

$$\frac{[B]}{[F} \approx \frac{P_0-C}{K_d} - \frac{[B]}{K_d} \qquad (2)$$

This is the situation when the inhibitor is "irreversibly" bound to receptor, reducing the estimate of P_0 but leaving K_d unchanged. Lastly, if K_i is very large compared to K_d, then

$$\frac{[B]}{[F]} \approx \frac{K_i}{K_i+C}\left(\frac{P_0}{K_d} - \frac{[B]}{K_d}\right) \qquad (3)$$

Thus the plot of $[B]/[F]$ against $[B]$ would be linear, giving an apparent value for K_d (estimated from the slope) equal to

$$K_d\left(1+\frac{C}{K_i}\right)$$

and leaving P_0 unchanged.

REFERENCES

1. P. J. Folca, R. F. Glascock and W. T. Irvine, Studies with tritium-labelled hexoestrol in advanced breast cancer. *Lancet* **2,** 796 (1961).
2. W. H. Pearlman, R. de Hertogh, K. R. Laumas, J. A. Brueggemann and M. R. J. Pearlman, Metabolism and tissue uptake of steroid sex hormones in patients with advanced carcinoma of the breast and in normal rats. In *Steroid Dynamics* Edited by G. Pincus, T. Nakao and J. F. Tait p. 159. New York (1966).
3. H. Braunsberg, W. T. Irvine and V. H. T. James, Comparison of steroid hormone concentrations in human tissues including breast cancer. *Brit. J. Cancer* **21,** 714 (1967).
4. S. Sander, The *in vitro* uptake of oestradiol in biopsies from 25 breast cancer patients. *Acta path. microbiol. scand.* **74,** 301 (1968).
5. S. G. Korenman and B. A. Dukes, Specific estrogen binding by the cytoplasm of human breast carcinoma. *J. clin. Endocr.* **30,** 639 (1970).
6. E. V. Jensen, G. E. Block, S. Smith, K. Kyser and E. R. de Sombre, Estrogen receptors and breast cancer response to adrenalectomy. Prediction of response in breast cancer therapy. *Nat. Cancer Inst. Monogr.* **34,** 55 (1971).
7. H. Maass, B. Engel, H. Hohmeister, F. Lehmann and G. Trams, Estrogen receptors in human breast cancer tissue. *Amer. J. Obstet. Gynec.* **113,** 377 (1972).
8. H. Johansson, L. Terenius and L. Thorén, The binding of estradiol-17β to human breast cancers and other tissues *in vitro*. *Cancer Res.* **30,** 692 (1970).
9. F. James, V. H. T. James, A. E. Carter and W. T. Irvine, A comparison of *in vivo* and *in vitro* uptake of oestradiol by human breast tumours and the relationship to steroid excretion. *Cancer Res.* **31,** 1268 (1971).
10. P. Feherty, G. Farrer-Brown and A. E. Kellie, Oestradiol receptors in carcinoma and benign disease of the breast: An *in vitro* assay. *Brit. J. Cancer* **25,** 697 (1971).
11. W. L. McGuire, Estrogen receptors in human breast cancer. *J. clin. Invest.* **52,** 73 (1973).
12. B. S. Leung, L. C. Manaugh and D. C. Wood, Estradiol receptors in benign and malignant disease. *Clin. chim. Acta* **46,** 69 (1973).
13. R. Hähnel, E. Twaddle and A. B. Vivian, Estrogen receptors in human breast cancer. 2. *In vitro* binding of estradiol by benign and malignant tumours. *Steroids* **18,** 681 (1971).
14. R. K. Wagner, Characterization and assay of steroid hormone receptors and steroid-binding serum proteins by agar-gel electrophoresis at low temperature. *Hoppe-Seylers Z. physiol. Chem.* **353,** 1235 (1972).
15. J. L. Wittliff, R. Hilf, W. F. Brooks, E. D. Savlov, T. C. Hall and R. A. Orlando, Specific estrogen-binding capacity of the cytoplasmic receptor in normal and neoplastic breast tissues of humans. *Cancer Res.* **32,** 1983 (1972).

16. G. LECLERCQ, J. C. HEUSON, R. SCHOENFELD, W. H. MATTHEIEM and H. J. TAGNON, Estrogen receptors in human breast cancer. *Europ. J. Cancer* **9,** 665 (1973).
17. U. SPAEREN, S. OLSNES, I. BRENNHOVD, J. EFSKIND and A. PIHL, Content of estrogen receptors in human breast cancers. *Europ. J. Cancer* **9,** 353 (1973).
18. E. V. JENSEN, G. E. BLOCK, S. SMITH and E. R. DE SOMBRE, Hormonal dependency of breast cancer. *Rec. Results in Cancer Res.* (Edited by G. MATHE) Vol. 42, p. 55. Springer-Verlag, Heidelberg (1973).
19. G. A. PUCA and F. BRESCIANI, Receptor molecule for oestrogens from rat uterus. *Nature (Lond.)* **218,** 967 (1968).
20. V. C. GODEFROI and S. C. BROOKS, Improved gel-filtration method for analysis of estrogen receptor binding. *Analyt. Biochem.* **51,** 335 (1973).
21. L. TERENIUS, SH-Groups essential for oestrogen uptake and retention in the mouse uterus. *Molec. Pharmacol.* **3,** 423 (1967).
22. E. V. JENSEN, D. J. HURST, E. R. DE SOMBRE and P. W. JUNGBLUT, Sulfhydryl groups and estradiol-receptor interaction. *Science* **158,** 385 (1967).
23. H. BRAUNSBERG, E. KILLEN and S. G. RICHARDSON, The effects of thiols on oestradiol binding by components of human malignant breast tumours. *Biochem. J.* **130,** 38P (1972).
24. W. L. MCGUIRE and M. DE LA GARZA, Improved sensitivity in the measurement of estrogen receptor in human breast cancer. *J. clin. Endocr.* **37,** 986 (1973).
25. J. SKIDMORE, A. L. WALPOLE and J. WOODBURN, Effect of some triphenylethylenes on oestradiol binding *in vitro* to macromolecules from uterus and anterior pituitary. *J. Endocr.* **52,** 289 (1972).
26. W. J. YOUDEN, *Statistical Methods for Chemists*, p. 16. Wiley, New York (1951).
27. R. HÄHNEL and E. TWADDLE, Estimation of the association constant of the estrogen receptor complex in human breast cancer. *Cancer Res.* **33,** 559 (1973).
28. E. V. JENSEN, G. E. BLOCK, S. SMITH, K. KYSER and E. R. DE SOMBRE, Estrogen receptors and hormone dependency. In *Estrogen Target Tissues and Neoplasia* (Edited by T. L. DAO) p. 23. Chicago (1972).
29. C. KORSTEN and J. P. PERSIJN, A simple assay for specific oestrogen binding capacity in human mammary tumours. *Z. klin. Chem.* **10,** 502 (1972).
30. H. BRAUNSBERG, The determination of high-affinity oestrogen receptors in human breast tumours: critique of a method approved by the EORTC Breast Cancer Cooperative Group. *Europ. J. Cancer* **11,** 101 (1975).
31. B. S. LEUNG, W. S. FLETCHER, T. D. LINDELL, D. C. WOOD and W. W. KRIPPAEHNE, Predictability of response to endocrine ablation in advanced breast carcinoma *Arch. Surg.* **106,** 515 (1973).
32. E. ENGELSMAN, J. P. PERSIJN, C. B. KORSTEN and F. J. CLETON, Oestrogen receptor in human breast cancer tissue and response to endocrine therapy. *Brit. med. J.* **2,** 750 (1973).
33. R. HÄHNEL, E. TWADDLE and T. RATAJCZAK, The influence of synthetic anti-oestrogens on the binding of tritiated estradiol-17β by cytosols of human uterus and human breast carcinoma. *J. Steroid Biochem.* **4,** 687 (1973).
34. M. P. COLE, C. T. A. JONES and I. D. H. TODD, A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Brit. J. Cancer* **25,** 270 (1971).
35. H. W. C. WARD, Anti-oestrogen therapy of breast cancer: a trial of tamoxifen at two dose levels. *Brit. med. J.* **1,** 13 (1973).

Europ. J. Cancer Vol. 11, pp. 509–515. Pergamon Press 1975. Printed in Great Britain

Biochemical Changes in GR Mouse Mammary Tissue During Hormonal Tumor Induction*

J. L. DAEHNFELDT, M. SCHÜLEIN and P. BRIAND,
with the technical assistance of LISBETH HUUSOM, JANNE KROGSGAARD PETERSEN, BIRGITTE RASK, MARIANNE HANSEN and ULLA HAERSLEV
The Fibiger Laboratory,† Ndr. Frihavnsgade 70, 2100 *København Ø, Denmark*

Abstract—*Treatment of spayed* 10–12 *weeks old virgin GR mice with estrone and progesterone led to mammary tumor development after about* 12 *weeks.*

Pronounced tissue changes, however, were observed during the first 3 *weeks after the start of hormonal treatment. The total weight of the glands more than doubled during this period while the protein/DNA ratio decreased. Glucose-6-phosphate dehydrogenase (G-6-PDH) activity rose by* 25% *during the first* 2 *weeks of hormonal treatment calculated on a protein basis, but fell again to the initial value during the remainder of the induction period. Lactose synthetase (LS) A-protein activity and B-protein content rose to a steadily high level after* 2 *weeks of treatment. The non-blocked cytoplasmic estradiol binding capacity decreased after hormonal treatment of spayed virgins.*

In the induced tumors, the RNA/DNA ratio was unchanged and the protein/DNA ratio decreased by 25% *compared to non-tumorous mammary tissue from the same mice. The activity of G-6-PDH increased by* 50% *calculated on a protein basis. The increased LS A-protein activity remained unchanged, while LS B-protein disappeared. There was* 10 *times as much non-blocked cytoplasmic estradiol receptor protein as in the non-tumorous tissue from the same animals.*

The hormone-induced versus tumor-characteristic changes are discussed.

INTRODUCTION

THE DEVELOPMENT of mouse mammary carcinomas is known in many cases to depend on a complex of factors including mouse mammary tumor virus (MMTV) and various hormones. In GR mice carriers of MMTV, tumors can be induced by 2 steroid hormones, estrone and progesterone. In the original host and during the first few transplant generations, the growth of hormone-induced mammary carcinomas usually depends on continuous hormonal stimulation. However, autonomy develops eventually and the tumors are then able to grow without any hormonal support.

Comparative studies of hormone-induced mouse mammary tumors and normal virgin mouse mammary glands have been carried out previously in this laboratory [1, 2]. These studies included investigations of the hormone inducible enzymes glucose-6-phosphate dehydrogenase (G-6-PDH) and lactose synthetase (LS) A and B protein. In hormone-dependent tumors, an increase in LS A-protein activity and a disappearance of LS B-protein were seen; G-6-PDH activity remained unchanged as compared to the virgin mammary gland.

Accepted 12 March 1975.

*This investigation was supported by grants from Mrs. Agathe Neye, the Daell Foundation, and the Danish National Science Foundation grants 512–2707 and 512–3718.

†Sponsored by the Danish Cancer Society.

Estrogen receptor protein has been found to be lower in autonomous tumors than in both virgin [3] and lactating mammary glands [4] as well as in pregnancy-dependent [5] and hormone-dependent tumors [6] but comparisons between normal glands and hormone-dependent tumors are still missing.

From the studies referred to above it is not possible in all cases to make a clear distinction between hormone-induced changes and tumor-dependent changes. In our previous studies, hormone treatment was without any influence on the G-6-PDH activity in autonomous tumors, but otherwise no information seems available about the hormonal effects on these tumors.

The purpose of the present investigation was to elucidate the changes in G-6-PDH and LS activity as well as in hormone receptor capacity which may occur during hormonal induction of GR mouse mammary tumors, and to compare these parameters in non-tumorous and tumorous tissue from the same animals, *i.e.* under the same hormonal influence.

MATERIAL AND METHODS

Chemicals

Progesterone and estrone were generously supplied by Leo Pharmaceuticals, Ballerup, Denmark. Bovine α-lactalbumin was purchased from BDH, Pool, U.K. ATP, tris, calf thymus DNA, yeast RNA, bovine serum albumin, and UDP-galactose were purchased from Sigma, St. Louis, U.S.A. All remaining enzymes, substrates and cofactors were obtained from Boehringer, Mannheim, West Germany. UDP-(14C)-galactose, 3H-progesterone and 3H-estradiol were purchased from the Radiochemical Center, Amersham, U.K. The remaining chemicals were of analytic grade.

Animals

Mice of the inbred GR/A strain [7] were obtained originally from Mühlbock and have been bred in our animal colony since 1971. Mammary tumors were induced as described by Briand and Daehnfeldt [1]. Ten- to 12-week-old virgins were spayed and treated with estrone and progesterone. Estrone was administered in the drinking water in a concentration of 0·5 μg/ml. In addition, the animals received a weekly dose of 5–10 mg of progesterone injected s.c. as pellets. Macroscopic tumors were detectable after 8–12 weeks of treatment. The tumors were usually hormone-dependent, *i.e.*, they grew after transplantation to hormone-treated and not when transplanted to non-treated, castrated males.

Tissue preparation

The animals were killed by cervical dislocation. The mammary tumors as well as all non-tumorous mammary tissue were removed and immediately cooled on crushed ice until homogenization. The tissue was pulverized with a micro-dismembrator (Braun, Melsungen, West Germany) in a Teflon container cooled in liquid nitrogen. The powder was suspended meticulously in a 1:3 proportion in appropriate buffers. For LS determinations, the buffer used contained Tris–HCl 20 mM, pH 7·4, $MgCl_2$ 10 mM, and β-mercaptoethanol 1 mM. For G-6-PDH and receptor protein determinations, the buffer contained Tris–HCl 10 mM, pH 7·4, EDTA 1·5 mM, and NaN_3 1·0 mM. Particle-free supernatants were prepared by centrifugation at 4°C for 1 hr at 100.000 ***g***. The supernatants were divided into suitable aliquots and stored at −80°C. Enzymatic activities and receptor protein content were found to be stable for 4 weeks under these conditions.

RNA, DNA, and protein determinations

The pulverized tissue was washed 3 times by centrifugation and resuspension in 10 vol. acetone at −20°C and twice in 10 vol. peroxide-free ether at −20°C. The acetone powder was vacuum dried in an exiccator over silica gel and then weighed.

The method of Schmidt and Thannhauser [8] was used to separate acid soluble compounds, RNA, DNA, and protein. RNA was determined by Schneider's method [9] using yeast RNA as standard. DNA was determined by Burton's method [10] using calf thymus DNA as standard. Protein was determined by the method of Lowry *et al.* [11] using bovine serum albumin as standard.

Enzyme assays

Enzymatic activity of LS A-protein (galactosyl transferase) was assayed as described previously [2, 12] using UDP-(14C)-galactose as the precursor. The incubation was at 37°C for 10 min with an excess of bovine α-lactalbumin.

The amount of LS B-protein (α-lactalbumin) was measured with excess bovine LS A-protein [2].

Enzymatic activity of G-6-PDH was determined spectrophotometrically at 25°C according to the principle of Glock and McLean [13] as described by Briand and Daehnfeldt [1] using a final volume of 1·0 ml. Reaction conditions were such as to give a constant rate of NADP reduction over a period of 4–5 min.

Cytoplasmic high affinity steroid receptor protein determinations

The content of non-blocked high affinity estradiol receptor protein was determined by the charcoal adsorption technique [14] modified as described for rat uterine tissue [15]. Particle-free supernatants were incubated in a final volume of 80 µl with concentrations of 3H-estradiol, ranging from $7{\cdot}2 \times 10^{-9}$ to $2{\cdot}9 \times 10^{-10}$M.

High affinity progesterone receptor protein was assayed under identical conditions using the same concentration range of 3H-progesterone.

The distribution of the data obtained was not normal. Therefore, statistical evaluation was performed with the non-parametric Wilcoxon's signed rank test [16].

RESULTS

Effect of hormonal treatment on wet weight, RNA/DNA, and protein/DNA ratios

Administration of estrone and progesterone to spayed virgin GR mice was followed by a significant increase of the total wet wt of the mammary glands to about the double of the original wt (Table 1). No further increase was observed before development of macroscopic tumors at about 8 weeks of treatment. After this point the wt was not recorded, since the occurrence of tumors in some animals and not in others would give no meaning to the average wt value. The RNA/DNA ratio remained constant during the entire induction period. The RNA/DNA ratio decreased after 3 weeks of hormonal treatment, and further treatment did not change this ratio.

The tumors showed the same RNA/DNA ratio as non-tumorous tissue from the same animal, but the protein/DNA ratio was significantly lower ($P < 0{\cdot}01$).

Effect of hormonal treatment on LS-activity

After 2 weeks of continued treatment, the LS A-protein activity had increased 3 times (Table 2). The high activity was constant during the induction period and was also found in the tumorous tissue.

After 2 weeks, the LS B-protein content increased above the initial value. This level was maintained during the induction period, but the development of tumorous tissue was accompanied by a decrease in LS B-protein to undetectable levels.

Effect of hormonal treatment on G-6-PDH activity

After 2 weeks of hormonal treatment, the median value of G-6-PDH activity in mammary tissue was 25% higher ($P < 0{\cdot}01$) than initially measured on a protein basis (Table 3). After 3 weeks, the G-6-PDH activity decreased again. In the tumorous tissue, the activity was about 50% higher, measured on a protein basis, than the activity of the non-tumorous tissue from the same animal ($P = 0{\cdot}01$) and about 15% higher than the initial value ($P = 0{\cdot}02$–$0{\cdot}05$).

Effect of hormonal treatment on the content of high-affinity receptor proteins

Non-blocked estradiol receptor protein was demonstrated in untreated non-tumorous mam-

Table 1. *The wet weight of the* 10 *non-tumorous mammary glands, and the RNA/DNA, protein/DNA ratio of the non-tumorous and tumorous tissue after estrone and progesterone administration to spayed GR mice*

Tissue	Weeks of hormonal treatment	mg wet weight			RNA/DNA ratio			Protein/DNA ratio		
		Median value	Range	n	Median value	Range	n	Median value	Range	n
Non-tumorous*	0	410	(380- 440)	6	0,54	(0.42-0.65)	6	24	(20-30)	6
-'-	1	520	(340- 540)	8						
-'-	3	860	(710-1120)	8	0.50	(0.44-0.50)	3	15	(15-16)	3
-'-	5	990	(820-1290)	8						
-'-	8	820	(470-1120)	8	0.54	(0.52-0.67)	3	17	(17-18)	3
-'-	14				0.54	(0.40-0.61)	6	18	(15-20)	6
Tumorous	9-14				0.52	(0.44-0.54)	6	13	(11-13)	6

*Results obtained 6–8 days after spaying.

Comparisons: Protein/DNA ratio: 0 to 14 weeks: $P = 0{\cdot}01$
14 non-tumorous to tumorous: $P < 0{\cdot}01$

Table 2. LS A-protein activity and LS B-protein content in non-tumorous and tumorous mammary tissue after estrone and progesterone administration to spayed GR mice

Tissue	Weeks of hormonal treatment	LS A-protein activity Median values	LS A-protein activity Range	LS B-protein content Median values	LS B-protein content Range	n
Non-tumorous*	0	61	40- 80	3.5	2.3- 5.5	6
-'-	2	195	162-232	7.7	5.7-12.0	10
-'-	5	160	101-287	5.5	4.4-19.7	10
-'-	8	215	110-342	10.0	7.5-27.0	9
Tumorous	9-14	171	135-181	<2		8

*Results obtained 6–8 days after spaying.

Activity of LS A-protein was measured with excess (100 μg) bovine LS B-protein and expressed as picomole lactose formed per min per mg protein. LS B-protein was measured with excess bovine LS A-protein. The median values were expressed as μg bovine LS B-protein calculated from a standard curve and measured per mg protein.

mary tissue (Table 3) as well as in mammary tissue during the induction period. After 3 weeks of treatment, the values dropped below the starting levels. A significant increase was seen in tumorous tissue when compared to treated and untreated non-tumorous tissue ($P < 0{\cdot}01$). High affinity progesterone receptor protein was below the detectable limit (1×10^{-14} mol progesterone bound/mg supernatant protein). Even the non-specific binding of progesterone to other proteins was found to be very low compared to the nonspecific binding of estradiol, which is on the order of 2–5% of the total hormone added.

DISCUSSION

Recent years have brought an increased understanding of the biochemical effects of steroid hormones. The influence of steroids on nucleic acid and protein biosynthesis has been amply documented [17–19]. However, the biochemical implication of steroid hormones in cancerogenesis is not understood.

The administration of estrone and progesterone to spayed 10- to 12-week-old virgin GR mice results in the formation of hormone-dependent mammary tumors after 14 weeks of treatment. Long before this, however, various temporary or permanent changes may be found in the mammary gland tissue.

Maximal wt increase in the non-tumorous phase was reached already after 3–5 weeks of treatment. At the same time a significant decrease in the protein/DNA ratio was observed. Since ovarian hormones are known to induce mammary cell proliferation *in vitro* [20] and macromolecular synthesis *in vivo* [21] it seems

Table 3. G-6-PDH activity and non-blocked binding capacity of high affinity estradiol receptors in non-tumorous and tumorous mammary tissue after estrone and progesterone administration to spayed GR mice

Tissue	Weeks of hormonal treatment	G-6-PDH activity Median values	G-6-PDH activity Range	n	Estradiol binding capacity Median values	Estradiol binding capacity Range	n
Non-tumorous *	0	41	32-50	19	3.1	2.1- 4.7	6
-'-	1	40	28-58	10	3.7	2.7- 4.2	6
-'-	2	50	44-61	8			
-'-	3	35	32-42	8	1.5	<1.0- 3.1	6
-'-	5	38	30-64	14	<1.0	<1.0- 3.2	5
-'-	8	35	22-50	18	1.7	<1.0- 4.2	6
-'-	14	30	22-48	7	<1.0	<1.0- 1.6	5
Tumorous	14	47	33-82	12	9.4	7.1-12.6	8

*Results obtained 6–8 days after spaying.

G-6-PDH activity was expressed as mU/mg protein and non-blocked high affinity estradiol binding capacity as 10^{-14} mol estradiol bound per mg protein. The values are corrected for non-specific binding.

Comparisons: G-6-PDH

0 to 2 weeks: $P < 0{\cdot}01$
0 to 14 weeks non-tumorous: $P > 0{\cdot}1$
0 to tumorous: $0{\cdot}02 < P < 0{\cdot}05$
2 weeks to 14 weeks non-tumorous: $P < 0{\cdot}01$
14 weeks non-tumorous to tumorous: $P = 0{\cdot}01$
Estradiol binding capacity 0 to tumorous: $P < 0{\cdot}01$
14 weeks non-tumorous to tumorous: $P < 0{\cdot}01$

reasonable to consider both of these changes as manifestations of increased cellularity.

The rise in G-6-PDH and LS A-protein could be explained theoretically by a change in the cellular composition of the tissue due to epithelial proliferation without simultaneous proliferation of fat cells. As evidence against this explanation it may be pointed out that the activity of G-6-PDH increased only temporarily and thus did not follow the increase in wet wt.

Hormone induction of these enzymes is another possibility to be considered. G-6-PDH as well as LS A- and B-proteins have been shown to be under hormonal regulation [2, 22–24].

We have reported previously non-detectable levels of LS B-protein after 4 days of estrone and progesterone treatment [2]. The high LS B-protein content observed in the present study after 2 weeks of the same treatment probably means that estrone induction may override progesterone inhibition.

The secondary decrease in G-6-PDH activity could be explained theoretically by alveolar degeneration caused by excessive steroid treatment. However, if this were the case a similar decrease in LS A- and B-protein might have been expected. Furthermore, no signs of alveolar degeneration could be seen in histological sections throughout the induction period.

The absence or low content of non-blocked receptor protein after treatment beyond 3 weeks may be explained by the high concentrations of estrone/estradiol. Under identical hormonal conditions, we have observed previously that the non-blocked receptor content of hormone-dependent transplanted GR mammary tumors is only 25% of the total receptor protein present [25].

The content of estradiol receptors in the non-tumorous mammary gland was too low to allow a determination of total binding capacity in the present investigation. However, we believe that the low content of estradiol receptors is not explained simply by blocked receptor sites, since initial receptor content was maintained even after one week of hormonal treatment. Furthermore, the receptor content was found to be 10 times higher in the tumorous tissue than in the non-tumorous tissue of the same animal in spite of continued hormone treatment.

No progesterone receptor protein could be demonstrated in mammary glands of ovariectomized virgin mice. Also, during the tumor induction period as well as in tumor tissue no progesterone receptors were found. However, in this case the explanation may be the blocking effect of the progesterone injected into the animals.

The occurrence of tumors was accompanied by a significant drop in protein/DNA ratio compared to the non-involved mammary tissue from the same animal. This possibly reflects an increased cellularity in the tumor tissue. The RNA/DNA ratio remained unchanged compared to the ratio during the entire induction period and compared to the non-tumorous mammary tissue. This, however, does not exclude qualitative changes in RNA species.

After 14 weeks of treatment the activity of G-6-PDH in tumor-bearing animals was low in non-tumorous gland tissue while the tumors showed higher values. The explanation for this may be increased cellularity in the tumors, as evidenced by the decrease in protein/DNA ratio. The high G-6-PDH activity found in tumor tissue is lower than previously reported for these tumors [1], but the results are not directly comparable, since the preparation of the tissue is not identical.

Increased activities of G-6-PDH measured on a protein basis have been found in spontaneous mouse mammary tumors compared to virgin mammary glands by Bhide [26], whereas Kopelovich *et al.* [27] reported decreased activities. This discrepancy might possibly be explained by our previous finding [1] that hormone-dependent tumors have a higher G-6-PDH activity than hormone-independent tumors.

The activity of LS A-protein found in the tumor was the same as in non-tumorous mammary tissue from the same animal. Therefore, that the increase in LS A-protein is a simple response to hormone treatment unrelated to tumor development cannot be discounted. On the other hand, that the increased level of LS A-protein activity remains elevated in hormone-dependent tumors even if hormone treatment is discontinued [2] may be an argument against this interpretation.

The LS B-protein content showed a striking difference between the elevated values of non-tumorous hormone stimulated glands and the undetectable values in mammary tumors from the same animal.

In spite of continuous hormone treatment, the non-blocked estradiol receptor protein increased in the tumors, indicating a change in regulation of the receptor protein synthesis.

In conclusion, the present experiments have shown that high estradiol receptor content and undetectable levels of LS B-protein were

characteristic changes in the hormone induced GR mouse mammary tumors. The changes in G-6-PDH activity could be explained by changes in cellularity. Whether the increase in LS A-protein early in the tumor induction period is an indication of a pre-malignant change in the mammary tissue cannot be answered by the present investigations.

REFERENCES

1. P. Briand and J. L. Daehnfeldt, Enzyme patterns of glucose catabolism in hormone-dependent and -independent mammary tumours of GR mice. *Europ. J. Cancer* **9,** 763 (1973).
2. M. Schülein, J. L. Daehnfeldt and P. Briand, Lactose synthetase induction by hormones in normal and tumorous GR mouse mammary tissue. *Int. J. Cancer* **14,** 372 (1974).
3. G. A. Puca and F. Bresciani, Decrease to complete loss of ability to bind estradiol by a soluble macromolecular fraction of C3H mammary tumors. *Europ. J. Cancer* **3,** 475 (1968).
4. J. E. Richards, G. Shyamaly and S. Nandi, Estrogen receptor in normal and neoplastic mouse mammary tissues. *Cancer Res.* **34,** 2764 (1974).
5. L. Terenius, Parallelism between oestrogen binding capacity and hormone responsiveness of mammary tumours in GR/A mice. *Europ. J. Cancer* **8,** 55 (1972).
6. M. Sluyser and R. Van Nie, Estrogen receptor content and hormone-responsive growth of mouse mammary tumors. *Cancer Res.* **34,** 3253 (1974).
7. O. Mühlbock, Note on a new inbred mouse-strain GR/A. *Europ. J. Cancer* **1,** 123 (1965).
8. G. Schmidt and S. J. Thannhauser, A method for determination of DNA, RNA and phosphoproteins in animal tissue. *J. biol. Chem.* **161,** 83 (1945).
9. W. C. Schneider, Determination of nucleic acids in the tissues by pentose analyses. In *Methods in Enzymology.* (Edited by S. P. Colowick and N. O. Kaplan) Vol. 3, p. 680. Academic Press, New York (1957).
10. K. Burton, A study of conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315 (1956).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, Protein measurement with the Folin Phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
12. K. Brew, T. C. Vanaman, and R. L. Hill, The role of α-lactalbumin and the A protein in lactose synthetase: A unique mechanism for the control of a biological reaction. *Proc. nat. Acad. Sci. (Wash.)* **59,** 491 (1968).
13. G. E. Glock and P. McLean, Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. *Biochem. J.* **55,** 400 (1953).
14. J. Mester, D. M. Robertson, P. Feherty, and A. E. Kellie, Determination of high affinity oestrogen receptor sites in uterine supernatant preparations. *Biochem. J.* **120,** 831 (1970).
15. J. L. Daehnfeldt, Endogenously blocked high affinity estradiol receptors in the immature and mature rat uterus. *Proc. Soc. exp. Biol. Med. (N.Y.)* **146,** 159 (1974).
16. *Ciba-Geigy Scientific Tables.* (Edited by K. Diem and C. Lentner), Ciba-Geigy, Basel (1970).
17. G. C. Mueller, B. Vonderhaar, U. H. Kim, and M. Le Mahiu, Estrogen action: An inroad to cell biology. In *Recent Progress in Hormone Research.* (Edited by E. B. Astwood) Vol. 28, p. 1. Academic Press, New York (1972).
18. E. V. Jensen and E. R. DeSombre, Mechanism of action of the female sex hormones. In *Annual Review of Biochemistry.* (Edited by E. E. Snell) Vol. 41, p. 203. Palo Alto (1972).
19. E. V. Jensen and E. R. DeSombre, Estrogen receptor interaction. *Science* **182** 126 (1973).
20. R. R. Ichinose and S. Nandi, Influence of hormones on lobulo-alveolar differentiation of mouse mammary gland *in vitro. J. Endocr.* **35,** 331 (1966).
21. M. R. Banerjee and F. M. Rogers, Stimulation of the synthesis of macromolecules by ovarian hormones during early development of the mouse mammary gland. *J. Endocr.* **49,** 39 (1971).

22. A. Bonsignore and A. DeFlora, Regulatory properties of glucose-6-phosphate dehydrogenase, In *Current Topics in Cellular Regulation.* (Edited by B. L. Horecker) Vol. 6, p. 21. Academic Press, New York (1972).
23. R. W. Turkington, K. Brew, V. Vanaman, and R. L. Hill, The hormonal control of lactose synthetase in the developing mouse mammary gland. *J. biol. Chem.* **243,** 3382 (1968).
24. R. Palmiter, Hormonal induction and regulation of lactose synthetase in mouse mammary gland. *Biochem. J.* **113,** 409 (1969).
25. J. L. Daehnfeldt, unpublished data.
26. S. V. Bhide, Arginase and glucose-6-phosphate dehydrogenase activities in spontaneous mammary carcinogenesis. *Brit. J. Cancer* **25,** 182 (1971).
27. L. Kopelovich, S. Abraham, H. McGrath, K. B. DeOme, and I. L. Chaikoff, Metabolic characteristics of a naturally occurring preneoplastic tissue, I. Glycolytic enzyme activities of hyperplastic alveolar nodule outgrowths and adenocarcinomas of mouse mammary gland. *Cancer Res.* **26,** 1534 (1966).

Europ. J. Cancer Vol. 11, pp. 517–519. Pergamon Press 1975. Printed in Great Britain

Inactivation of Mammalian DNA Methylase Activities by N-Methyl-N′-nitro-N-nitrosoguanidine*

DUSAN DRAHOVSKY and ADOLF WACKER

Abteilung für Therapeutische Biochemie Zentrum der Biologischen Chemie Universität Frankfurt a M.

THE DESCRIPTIONS of the biological actions of nitrosoguanidines are often confined to a modification of nucleic acids, especially to formation of 7-methylguanidines in the DNA molecule. There are reports however, indicating that the extent of DNA guanine alkylation is not always proportional to the frequency of bacterial mutations [1] or the rate of tumor formations [2]. These discrepancies have motivated studies on interaction of nitrosoguanidines with other cellular macromolecules, particularly with proteins. It has been shown, that guanidino groups and methyl groups of nitrosomethylguanidines modify lysine and arginine residues of histones and other proteins [3]. Mammalian DNA polymerase was found to be inactivated by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) due to modification of thiol groups in the active center of the enzyme molecule [4]. In this paper we report an inhibition of rat liver DNA methylase activity by MNNG. This inhibition is caused by an irreversible inactivation of DNA methylase molecules by the carcinogen.

The DNA methylase used in these experiments was isolated from livers of male Wistar rats weighing about 250 g, by precipitation at 60% ammonium sulphate saturation according to the method of Morris and Pih [5]. The enzyme activity was assayed using S-adenosyl-L-methionine-methyl-^{3}H as donor of methyl groups and *Escherichia coli* B DNA, as a methyl group acceptor. The assay conditions were essentially the same as previously used [6] and are described in the text of Table 1. The specific activity of the enzyme preparation was about 30 pmoles of incorporated methyl

Table 1. *Inactivation of rat liver DNA methylase by N-methyl-N′-nitro-N-nitrosoguanidine in the absence of methylation reaction*

Enzyme	DNA	Rate of methylation reaction (pmoles CH_3 incorporated)
Rat liver	*E. coli* B	9·3 ± 0·7
Rat liver (MNNG treated)	*E. coli* B	0·4 ± 0·1
Rat liver	*E. coli* B (MNNG treated)	9·4 ± 0·9

Three to four mg of enzyme proteins (Lowry) and 0·6 mg of *Escherichia coli* B DNA, respectively, were incubated with MNNG at a final concentration of 2×10^{-3}M for 5 min at 37°C. Unbound carcinogen was removed by dialysis against 0·05 M Tris–HCl pH 7·0, 0·02 M EDTA, and 0·0001 M dithioerythrit for 4 hr at 0°C. The control enzyme preparation was treated in a similar manner, including the incubation at 37°C for 5 min without carcinogen. The rate of methylation was measured in an assay system containing the following in a final volume of 0·25 ml: 10 μmoles of Tris–HCl, pH 7·0, 0·5 μCi of S-adenosyl-L-methioninemethyl-^{3}H(4·51 Ci. mmol^{-1} New England Nuclear), 20 μg of *Escherichia coli* B DNA and 0·2 mg of enzyme protein. Incubation time was 20 min at 37°C. The product of enzymatic reaction was isolated as described elsewhere [6]. The methyl acceptor DNA used in all experiments was isolated from bacterium *Escherichia coli* B according to the procedure of Marmur [7].

Accepted 27 February 1975.

*This work was supported by grant Dr 104/2 from the Deutsche Forschungsgemeinschaft.

groups per mg proteins in 30 min at 37°C, and the product of the enzyme reaction was chromatographically identified as DNA 5-methyl cytosine.

Addition of MNNG to the assay system inhibited DNA methylase activity in a dose-dependent manner (Fig. 1), whereby the initial rate of the enzyme reaction declined to about 50% at a concentration of MNNG of 5×10^{-4}M. In an attempt to characterize the reaction responsible for this inhibition we simultaneously pre-treated the methyl acceptor DNA and the enzyme with 2×10^{-3}M MNNG for 5 min at 37°C. The unbound carcinogen was removed by an extensive dialysis against buffer containing 0·05 M Tris. HCl, 0·02 M EDTA and 0·0001 M dithioerythrit at pH 7·0

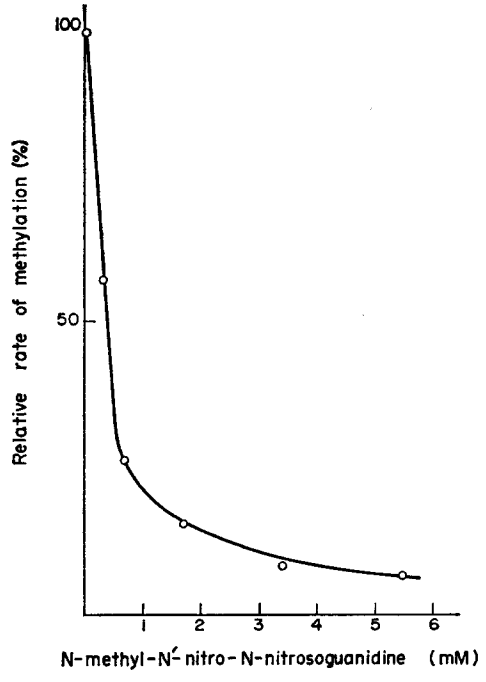


Fig. 1. Effect of N-methyl-N'-nitro-N-nitrosoguanidine on the rate of reaction of rat liver DNA methylase. The carcinogen was added to the assay system at 0°C. Standard assay for DNA methylase activity was performed at 37°C for 20 min.

and 2°C. The enzyme and the methyl acceptor DNA were then tested in the standard methylation assay (Table 1). The pre-treatment of the methyl acceptor DNA under the above conditions did not change its ability to serve as a substrate in the methylation assay. Since rat liver DNA methylase does not bind and methylate *Escherichia coli* DNA which contains 7-methylguanines [8], it is unlikely that this treatment with MNNG alters the structure of the methyl acceptor DNA to an extent that would interfere with the binding reaction. The pre-treatment of DNA methylase with MNNG under the identical conditions caused a complete inactivation of the enzyme. The removal of MNNG from the system by dialysis did not result in recovery of the DNA methylase activity, indicating that the carcinogen induced some permanent changes in the structure of the enzyme molecule.

So far, the only possibility of locating these changes in the molecule of DNA methylase is to compare the sensitivity of free and DNA-bound DNA methylase toward MNNG. In previous experiments we showed that rat liver DNA methylase is bound to methyl acceptor DNA during the methylation reaction and scans the helix for methylation sites without being detached between individual methyl group transfers [6, 9]. One can assume, therefore, that during this process the DNA-binding

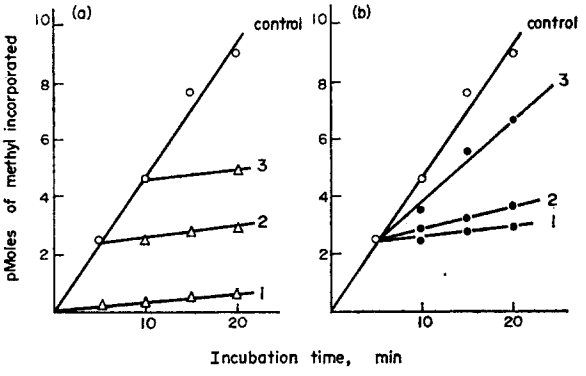


Fig. 2. Effect of N-methyl-N'-nitro-N-nitrosoguanidine on free and DNA-bound rat liver DNA methylase. (a) The carcinogen at a final concentration of $5 \times 10^{-3}M$ was added to the assay system either at 0°C, before the enzyme could have interacted with DNA (curve 1), or, at 5 and 10 min after the initiation of the methylation reaction, respectively (curves 2 and 3). (b) The carcinogen was added to the assay system at final concentrations of $5 \times 10^{-3}M$, $1 \times 10^{-3}M$ and $0{\cdot}5 \times 10^{-3}M$ (curves 1, 2 and 3 respectively), at the 5th minute after the initiation of the methylation reaction. The assay conditions were the same as described in the text to Table 1.

site of the DNA methylase molecule is occupied by the DNA helix and thus protected from being modified by the carcinogen. To test this possibility, MNNG was added to the assay system either at 0°C, that is, before the enzyme reaction started, or, at different times and in different doses after initiation of the reaction at 37°C (Fig. 2(a), 2(b)). The preincubation of the enzyme with DNA at 37°C leads to the formation of a stable complex [9]; however, the DNA methylase in this complex is sensitive toward MNNG to about the same extent as the free unbound enzyme. These results may indicate that a modification of protein structure in the DNA-binding site of the DNA methylase molecule is unlikely to account for the inactivation effect of the carcinogen on the enzyme.

No difference in sensitivity to MNNG by DNA methylating enzymes extracted from different tissues was observed. So far, DNA methylating enzymes from calf thymus, mouse liver and Ehrlich ascites tumor have been tested in this respect (article in preparation).

The biological function of 5-methylcytosines in mammalian DNA is unknown and therefore a discussion about possible consequences of inactivation of DNA methylating enzymes in cellular metabolism may not be relevant at present. However, if compared to the effect of MNNG on DNA polymerase which consequently may lead to a general inhibition of the replication process, the inactivation of DNA methylase activities may lead to different distribution of 5-methylcytosines in DNA molecule. Thus, the primary structure of chromosomal DNA in the diploid genome would differ from that of the haploid one as a consequence of DNA methylase inactivation. A possibility that such an alteration of primary structure of chromosomal DNA may modulate the consequences of exposure of mammalian cells to nitrosoguanidines is also to be taken into consideration.

REFERENCES

1. B. Singer, H. Frankel-Conrat, J. Greenberg and A. M. Michelson, Reaction of nitrosoguanidine (N-Methyl-N'-Nitro-N-nitrosoguanidine) with tobacco mosaic virus and its RNA. *Science* **160,** 1235 (1968).
2. P. F. Swann and P. N. Magee, Nitrosamine-induced carcinogenesis. *Biochem. J.* **110,** 39 (1968).
3. M. Nagao, T. Yokoshima, H. Hosoi and T. Sugimura, Interaction of N-methyl-N'-nitro-N-nitrosoguanidine with Ehrlich ascites cells *in vitro*. *Biochim. biophys. Acta (Amst.)* **192,** 191 (1969).
4. T. J. Anderson and R. H. Burdon, N-methyl-N'-nitro-N-nitrosoguanidine: reactions of significance to biological activity with mammalian cells. *Cancer Res.* **30,** 1773 (1970).
5. N. R. Morris and K. D. Pih, The preparation of soluble DNA methylase from normal and regenerating rat liver. *Cancer Res.* **31,** 433 (1971).
6. D. Drahovsky and R. N. Morris, Mechanism of action of rat liver DNA methylase. II. Interaction with single-stranded methyl-acceptor DNA. *J. molec. Biol.* **61,** 343 (1971).
7. J. Marmur, A Procedure for the isolation of deoxyribonucleic acid from micro-organism. *J. molec. Biol.* **3,** 208 (1961).
8. D. Drahovsky and R. N. Morris, The mechanism of action of rat liver DNA methylase. III. Nucleotide requirements for binding and methylation. *Biochim. Biophys. Acta (Amst.)* **277,** 245 (1972).
9. D. Drahovsky and R. N. Morris, Mechanism of action of rat liver DNA methylase. I. Interaction with double-stranded methyl-acceptor DNA. *J. molec. Biol.* **57,** 475 (1971).

Europ. J. Cancer Vol. 11, pp. 521–522. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Release and Specificity of Inhibitors for Macromolecular Syntheses from Various Suspension Tumor Strains*

D. WERNER and M. SCHULTE
Institute of Cell Research, German Cancer Research Centre, Heidelberg, German Federal Republic

PREVIOUSLY, we have shown that Ehrlich ascites tumor cells incubated for 0–90 min in HBSS release a peptide (500 > mol. wt. < 2000 [1]) which inhibits protein [2] and DNA [3] synthesis. Recently, Ulrich [4] demonstrated that macrophages, lymphocytes and HeLa cells release similar factors when incubated in Krebs–Ringer phosphate buffer. In this paper we present evidence for the release of such factors from cells of various suspension tumor strains. Furthermore, we present data on the specificity of these inhibitors.

Freshly-harvested cells show, at the end of a first 0–90 min incubation period in HBSS, a complete cessation of the incorporation of ^{3}H-thymidine and ^{14}C-amino acids (Fig. 1). We demonstrated earlier that this cessation of the incorporation of isotopically-labeled precursors reflects a delay in protein and DNA synthesis [2, 3] caused by the accumulation of inhibitors during the incubation period [2].

The cells immediately recover their high rates of macromolecular syntheses when the "conditioned" medium is renewed at the beginning of the second 0–90 min incubation period (Fig. 1). This recovery can be explained by the removal of inhibition factor produced during the first 0–90 min incubation period. Other possible explanations for this effect were ruled out earlier [2, 3].

Accepted 1 April 1975.

*This work was supported financially by the "Sonderforschungsbereich 136" of the "Deutsche Forschungsgemeinschaft".

During the second 0–90 min incubation period, more inhibition factor is presumably produced causing a renewed cessation of the macromolecular syntheses (Fig. 1). All cell strains under investigation showed these typical kinetics. Therefore, we conclude that the release of factors causing reversible inhibition of macromolecular syntheses is common to these cells.

This raised the question whether these inhibitors released by the various cell strains are cell-specific. To answer this, cells were incubated for 0–90 min in HBSS. At the end of this incubation period the "conditioned" media of two cell strains were exchanged and the incubation was continued (Fig. 2). As the cells did not recover the high rates of macromolecular syntheses when incubated in the medium "conditioned" by another cell strain, we have to conclude that the inhibitors released by the cells under investigation are not cell-specific.

In previous papers we postulated that the factors released by ascites tumor cell *in vitro* may be involved in the "auto-regulation" of ascites tumors *in vivo*. However, this process is considered to be regulated by substances which are cell-specific [5]. The present results, demonstrating the unspecific nature of the released factors, are not in favor for their role in "autoregulation". The unspecific factors described here could, however, cooperate with unknown cell specific factors to produce "autoregulation" *in vivo*.

It is also possible that the factors detected

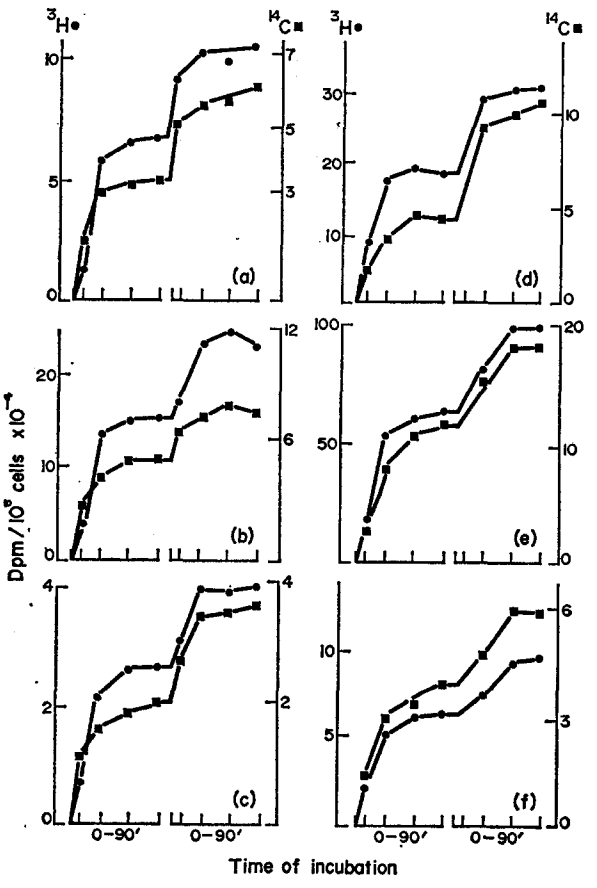


Fig. 1. Incorporation of ^{3}H-thymidine and ^{14}C-amino acids into the cold acid insoluble material of various cell strains during a 0–90 min incubation period and following resuspension of the cells in fresh HBSS during a second 0–90 min incubation period. Incubates contained, per ml of HBSS: 2×10^7 cells, 10 µCi of ^{3}H-thymidine (20 Ci/mmole) and 10 µCi of L-^{14}C-amino acid mixture (57 mCi/matom). The graph represents mean values of five aliquots taken at the times indicated, corrected for dpm $\times 10^{-4}/10^6$ cells. Standard deviations were equal or less than ± 10%.

(*a*) *Ehrlich ascites tumor cells (GØ)*
(*b*) *Ehrlich ascites tumor cells (G+)*
(*c*) *Walker 256 ascites cells*
(*d*) *Ehrlich ascites tumor cells (Karzel)*
(*e*) *L-929 murine fibrocytes*
(*f*) *Zajdela rat hepatoma.*

in our system are directed mainly against immunocompetent cells. Such factors which are released by tumor cells and which allow tumor cells to by-pass the host immunological system of surveillance were described recently by Fauve *et al.* [6].

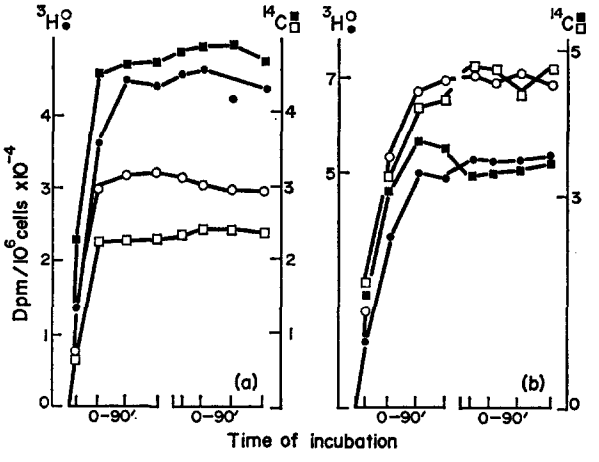


Fig. 2. Incorporation of ^{3}H-thymidine and ^{14}C-amino acids into the cold acid insoluble material of various cell strains during a 0–90 min incubation period and following exchange of the "conditioned" media between two cell strains during a second 0–90 min incubation period.

(*a*) □,○, *Ehrlich ascites tumor GØ*
■,●, *Ehrlich ascites tumor G+*
(*b*) □,○, *Zajdela hepatoma cells*
■,●, *Ehrlich ascites tumor GØ*

Experimental conditions were the same as described under Fig. 1.

REFERENCES

1. G. Maier and D. Werner, A factor reducing protein and DNA synthesis in Ehrlich ascites tumor cells. Purification and characterisation. *Hoppe-Seylers Z. physiol. Chem.* **355,** 59 (1974).
2. D. Werner, G. Maier and R. Lommel, A factor reducing protein synthesis from Ehrlich ascites cells. *Europ. J. Cancer* **9,** 819 (1973).
3. D. Werner and G. Maier, Deficiency of joining of Okazaki type fragments in absence of cellular protein synthesis. *Europ. J. Biochem.* In press (1975).
4. F. Ulrich, A dialyzable protein synthesis inhibitor released by mammalian cells *in vitro*. *Biochem. biophys. Res. Commun.* **60,** 1453a (1974).
5. P. Bichel, Specific growth regulation in three ascitic tumours. *Europ. J. Cancer* **8,** 167 (1972).
6. R. M. Fauve, B. Hevin, H. Jacob, J. Gaillard and F. Jacob, Antiinflammatory effects of murine malignant cells. *Proc. nat. Acad. Sci. (Wash.)* **71,** 4052 (1974).

Europ. J. Cancer Vol. 11, pp. 523–525. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

An Experimental Model for the Treatment of Pulmonary Metastases*

JAMES SHAEFFER, ANAS M. EL-MAHDI and WILLIAM C. CONSTABLE

Section of Experimental Radiation Oncology, University of Virginia Medical Center, Charlottesville, Virginia 22901 *U.S.A.*

ONE OF the major shortcomings in the treatment of malignant disease has been the failure to cope successfully with disseminated disease or metastases. In general, metastatic spread may occur by three routes: by intracavitary seeding, via the lymphatics, or by way of the general circulation.

As a result of the lung receiving the entire systemic venous drainage, pulmonary metastases occur in approximately 30% of all cases exhibiting metastases at the time of necropsy, making the lung the most frequent organ of metastatic disease [1]. In 15% of these cases, the lungs are the only organ involved with metastatic spread [2].

Recently, we have published data relating to the irradiation of pulmonary metastases in an animal model [3, 4]. The purpose of this communication is to provide additional technical details concerning this animal model for pulmonary metastases, with particular emphasis on the size of the metastatic lesion as a function of time.

The maintenance of the C3H/HeJ mice as well as the method of preparation of the C3HBA adenocarcinoma suspension have been previously reported [3, 4].

In order to dilate the tail veins prior to injection, cages of mice were placed approximately 35 cm from a 125 W infrared lamp for approximately 5 min. During injection, tumor cell suspensions were maintained in an ice bath and were aspirated every 15 min to prevent cell aggregation.

About 30 days after i.v. injection, lungs were surgically removed, fixed, and scored as previously described [3, 4]. For colony size determinations, diameters of the lung colonies were measured with the aid of a dissecting microscope. Volumes were then calculated using the formula for sphere: $V = (\P/6) \times \text{dia}^3$.

The distribution of the pulmonary colonies on a lobe by lobe basis in 97 mice is presented in Table 1. The number of colonies per gram of tissue was relatively constant in each of the 5 lobes of the lung, indicating a random distribution of the tumor cells throughout the lungs.

The colony growth curve (Fig. 1) was determined by measuring the colony diameters between 14–42 days post injection. The dotted line portion of the curve represents the back extrapolation to 0 (injection) time. The growth curve follows a Gompertzian relationship with a doubling time of approximately 2 days during the exponential growth phase. Pathological confirmation of tumor growth is depicted in Fig. 2. Note that in 4 and 5 week tumors, areas of central necrosis are visible, presumably containing hypoxic cells.

An experiment was conducted to test the viability of the cells in suspension as a function of the type and temperature of the media in which the cells were suspended. The effect of the time interval between preparation of the cell suspension and animal injection was also tested. Table 2 demonstrates that the tumor cell viability, as measured by the ability to produce lung colonies, is higher when the

Accepted 23 January 1975.

*Supported by USPHS Grant CA 13300–02 from the National Cancer Institute.

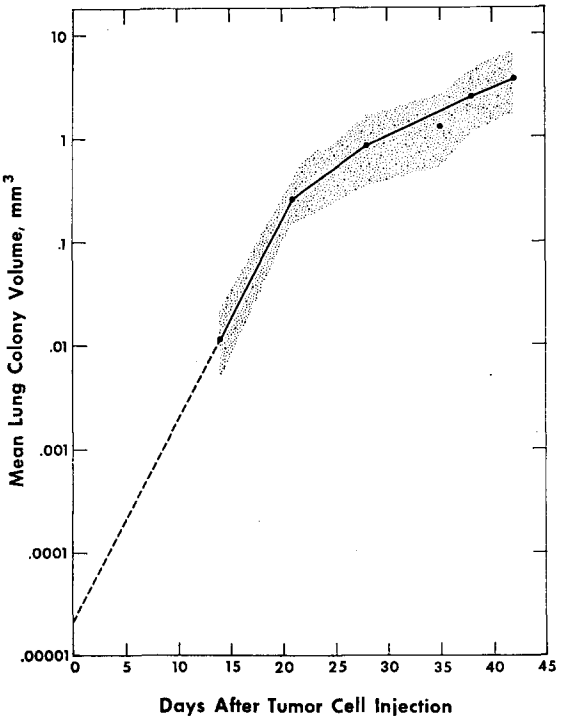


Fig. 1. Lung colony volume as a function of time. Each point was based on an average of 33 tumors. The stippled area represents one standard deviation.

Table 1. Lobular distribution of C3HBA lung colonies in 97 mice

Lung	No. Colonies	Wet Wt., g	Colonies/g	% of Average
Left lung	2178	6.11	356	93
Superior lobe	1147	2.43	472	122
Middle lobe	1002	2.62	382	99
Inferior lobe	1642	4.27	385	100
Post-caval lobe	508	1.36	374	97
Total	6477	16.79	386	100

Table 2. Effect of suspension media and temperature on the viability of C3HBA tumor cell suspension

Age of Suspension, Hrs.	Temperature, °C	Percent of Initial Viability	
		Normal Saline	Eagle's Minimal Essential Medium
0	4	100	100
	37	100	100
1	4	62	95
	37	16	83
2	4	50	89
	37	10	64
4	4	35	60
	37	1	33

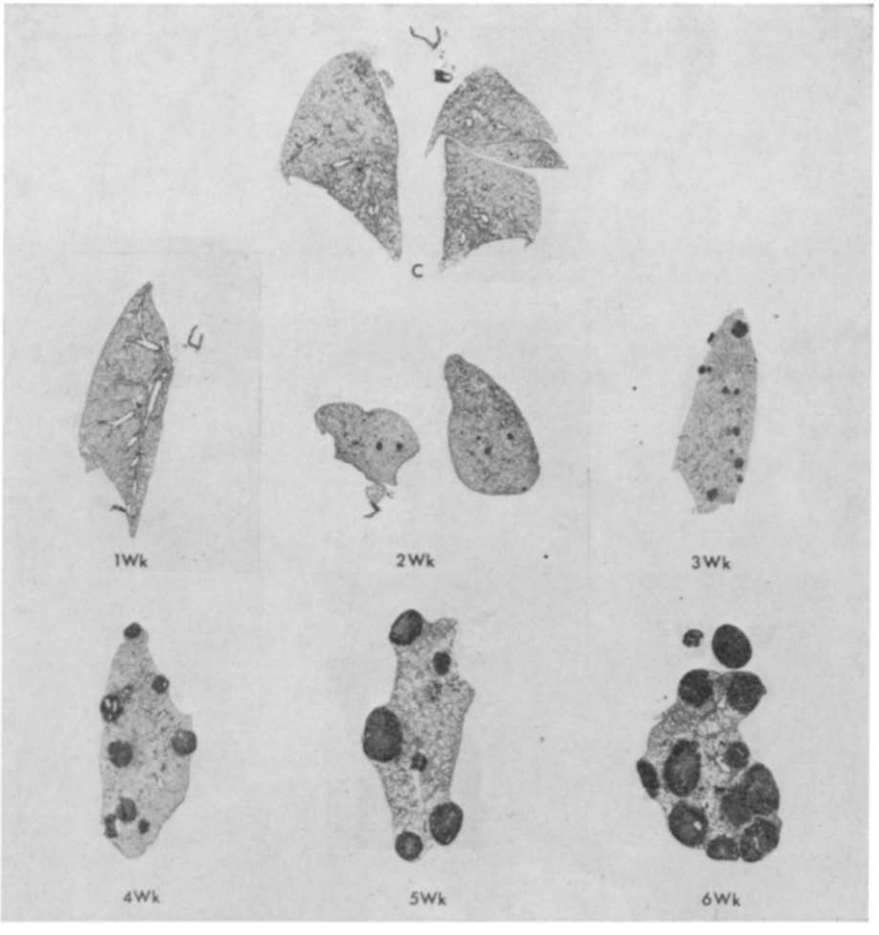


Fig. 2. Histological sections of lungs from uninjected control mouse (C) and from mice sacrificed 1, 2, 3, 4, 5 *and* 6 *weeks after injection of tumor cells.*

(to face page 524)

suspension is kept cold (4°C) as opposed to warm (37°C). In addition, the tumor cell viability is prolonged by storage in nutrient media (Eagle's) compared to storage in normal saline. Thus, storage of the tumor cell suspension in cold media will result in a viability of greater than 90% of the initial viability when the total injection time is kept within 1 hr.

The clonogenic capacity of the cells in the primary tumor does not appear to be affected by whether the cells come from the peripheral area of the tumor as opposed to the central part of the primary tumor. In an experiment in which 1×10^6 total cells from either the central or peripheral area of the 26 mm tumor were injected into two groups of 8 mice each, the average number of lung colonies ± S.E. at 31 days post injection in the peripheral group was $56{\cdot}0 \pm 5{\cdot}2$ compared to $53{\cdot}4 \pm 8{\cdot}9$ for the central group. These values are not significantly different ($P > 0{\cdot}80$) as determined by the student *t*-test.

The average life span of mice bearing different numbers of lung colonies is shown in Fig. 3. Between values of 10–50 colonies per lung, mean survival dropped from approximately 50 to 40 days respectively, but lung colonies in excess of 50 foci per lung did not drastically reduce the life span below 40 days.

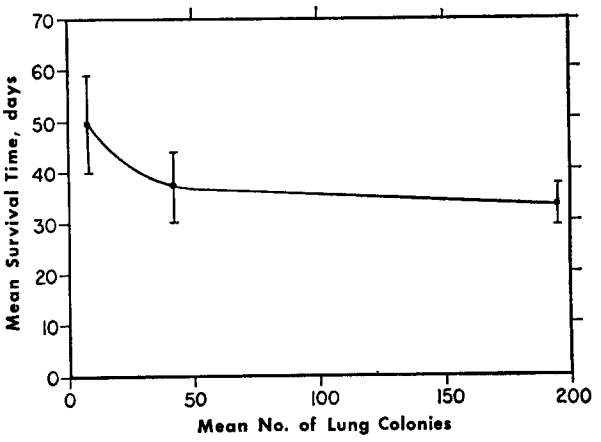


Fig. 3. Mean survival time of C3H/HeJ female mice as a function of number of lung metastases. Each point was based on an average of 70 mice. Bars represent one standard deviation.

In summary, the techniques for the induction of pulmonary metastases in a mouse model have been presented. This *in vivo* model has been used in studies involving the radiation therapy of pulmonary metastases [3, 4], and may prove useful in preclinical studies involving other treatment modalities.

Acknowledgements—The authors wish to thank R. Neal Leake, Kent L. Nelson and Connie M. Allen for their technical assistance.

REFERENCES

1. R. A. Willis, *The Spread of Tumours in the Human Body*. Butterworth, London (1952).
2. J. T. Farrell, Jr., Pulmonary metastases: a pathologic, clinical, roentgenologic study based on 78 cases seen at necropsy. *Radiology* **24,** 444 (1935).
3. J. Shaeffer, A. M. El-Mahdi and W. C. Constable, Radiation control of microscopic pulmonary metastases in C3H mice. *Cancer (Philad.)* **32,** 346 (1973).
4. J. Shaeffer, A. M. El-Mahdi and W. C. Constable, Lung colony assays of murine mammary tumor cells irradiated *in vivo* and *in vitro*. *Radiology* **109,** 703 (1973).

Europ. J. Cancer Vol. 11, pp. 527–528. Pergamon Press 1975. Printed in Great Britain

Announcement

First E.O.R.T.C. *Breast Cancer Working Conference*

Institut Jules Bordet, Brussels, Belgium, September 19–20, 1975.

Secretary: M. Rozencweig

Programme Committee: J. C. Heuson, W. Mattheiem, J. Wybran, A. Billiau, J. L. Pasteels, E. Engelsman, G. Leclerco

---

THIS Working Conference is organized by the E.O.R.T.C. Breast Cancer Cooperative Group. Its purpose is to analyze the present status of the various fields of research on breast cancer, both fundamental and clinical. The main topics will be virology, immunology, experimental models, hormonal receptors and hormone antagonists, treatment of the advanced disease, adjuvant hormonal and cytotoxic therapy of the primary disease, epidemiology and screening.

Each topic will be presented by European and American experts followed by a half-hour round-table discussion. At the end of the meeting, a consultant committee will be created to draft recoms mendations and guidelines to serve the E.O.R.T.C. in the coordination and implementation of it research programmes.

The official language will be English. The registration fee is BF 1000. The number of participants will be limited to the first 200 registrants. Registrations are to be sent to the Secretary of the Conference, Dr. M. Rozencweig, Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Bruxelles, Belgium.

PRELIMINARY PROGRAMME

19 *September* 1975

1. Virology Chairman: A. Billiau (Leuvan, Belgium)

   Viral particles as oncogenic factors in breast cancer

   Participants: J. Hilgers (Amsterdam, Netherlands)
   J. Schlom (Bethesda, USA)
   P. Bentvelzen (Rijswijk, Netherlands)
   A. Burny (Gembloux, Belgium)
   F. J. Cleton (Amsterdam, Netherlands)

2. Immunology Chairman: J. Stjernsward (Lausanne, Switzerland)

   Immunological defense mechanisms

   Participants: Z. L. Lucas (Stanford, USA)
   G. A. Currie (Sutton, Great Britain)
   J. Wybran (Bruxelles, Belgium)
   F. J. Lejeune (Bruxelles, Belgium)
   J. Blonk-van der Wijst (Rotterdam, Netherlands)
   J. Hilderbrand (Bruxelles, Belgium)

3. Experimental Models Chairman: L. M. Van Putten (Rijswijk, Netherlands)

Hormonal aspects and chemotherapy

Participants: J. C. Heuson (Bruxelles, Belgium)
A. E. Bogden (Worcester, USA)
J. L. Pasteels (Bruxelles, Belgium)
Y. Kenis (Bruxelles, Belgium)
H. Maass (Hamburg, Germany)
P. Alberto (Genève, Switzerland)

4. Endocrine Aspects Chairman: R. D. Bulbrook (London, Great Britain)

Hormone receptors and estrogen antagonists

Participants: R. J. B. King (London, Great Britain)
E. Baulieu (Bicêtre, France)
W. L. McGuire (San Antonio, USA)
E. Engelsman (Amsterdam, Netherlands)
S. Saez (Lyon, France)
C. Robyn (Bruxelles, Belgium)

20 *September* 1975

5. Palliative Treatment Chairman: P. Alberto (Genève, Switzerland)

Endocrine, cytotoxic and combined approaches

Participants: H. J. Tagnon (Bruxelles, Belgium)
S. K. Carter (Bethesda, USA)
E. Engelsman (Amsterdam, Netherlands)
H. Hansen (København, Denmark)
E. Baulieu (Bicêtre, France)
G. A. Currie (Sutton, Great Britain)

6. Curative Treatment Chairman: Y. Kenis (Bruxelles, Belgium)

Endocrine and cytotoxic adjuvant therapy of primary disease

Participants: P. P. Carbone (Bethesda, USA)
G. Bonadonna (Milano, Italy)
W. H. Mattheiem (Bruxelles, Belgium)
J. Stjernsward (Lausanne, Switzerland)
M. Tubiana (Villejuif, France)
J. L. Hayward (London, Great Britain)

7. Epidemiology and Screening Chairman: H. J. Tagnon (Bruxelles, Belgium)

Early detection in high-risk population

Participants: R. D. Bulbrook (London, Great Britain)
A. M. Stark (Gateshead, Great Britain)
M. Zelen (Buffalo, USA)
J. Schlom (Bethesda, USA)
A. Clarysse (Brugge, Belgium)
Z. L. Lucas (Stanford, USA)
P. Juret (Caen, France)

8. Conclusion

Conclusive Remarks: P. P. Carbone (Bethesda, USA)

Implementation of an EORTC coordinated
Research Programme: J. C. Heuson (Bruxelles, Belgium)

Europ. J. Cancer Vol. 11, pp. 529–530. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer — *July, 1975*

T. Sairenji and Y. Hinuma: Ultraviolet inactivation of Epstein–Barr virus: Effect on synthesis of virus-associated antigens.

R. M. Schultz, W. A. Woods and M. A. Chirigos: Detection in colorectal carcinoma patients of antibody cytotoxic to established cell strains derived from carcinoma of the human colon and rectum.

C. Desgranges, H. Wolf, G. de-Thé, K. Shanmugaratnam, N. Cammoun, R. Ellouz, G. Klein, K. Lennert and H. zur Hausen: Nasopharyngeal carcinoma. X. Presence of Epstein–Barr genomes in separated epithelial cells of tumours from Singapore, Tunisia and Kenya.

D. M. Murasko and R. N. Lausch: Spleen cell cytotoxicity for cytomegalovirus-transformed cells. II. Inhibition by cytomegalovirus antiserum.

G. Steele, Jr., H. O. Sjögren and M. R. Price: Tumor-associated and embryonic antigens in soluble fractions of a chemically-induced rat colon carcinoma.

T. Nagayo: Microscopical cancer of the stomach—the study on histogenesis of gastric carcinoma.

R. Roubin, J-P. Cesarini, W. H. Fridman, J. Pavie-Fischer and H. H. Peter: Characterization of the mononuclear cell infiltrate in human malignant melanoma.

W. A. Nelson-Rees, R. R. Flandermeyer and P. K. Hawthorne: Distinctive banded marker chromosomes of human tumor cell lines.

R. el Ridi and J. Bubeník: Tumour associated transplantation antigen in sera of rats with large RSV-induced sarcomas.

J. J. Roberts: Inactivation of the DNA template in HeLa cells treated with chlorambucil.

H. Ekert, D. G. Jose, F. C. Wilson, R. N. Matthews and H. Lay: Intermittent chemotherapy and immunotherapy with B.C.G. in remission maintenance of children with acute lymphocytic leukemia: Effects upon immunological function.

F. Vánky, G. Trempe, E. Klein and J. Stjernswärd: Human tumor–lymphocyte interaction *in vitro*: Blastogenesis correlated to detectable immunoglobulin in the biopsy.

G. B. Clements, G. Klein and S. Povey: Production of an EBNA positive subline from an EBNA negative human lymphoma cell line without detectable EBV DNA by EBV infection.

W. Jarrett, O. Jarrett, L. Mackey, H. Laird, C. Hood and D. Hay: Vaccination against feline leukaemia virus using a cell membrane antigen system.

T. Salonen and L. Saxén: Risk indicators in childhood malignancies.

P. Hersey, J. Edwards, A. Edwards, E. Adams, R. Kearney and G. W. Milton: Comparison of ^{51}Cr release and microcytotoxicity assays against human melanoma cells.

P. Hersey, A. Edwards, J. Edwards, E. Adams, G. W. Milton and D. S. Nelson: Specificity of cell mediated cytotoxicity against human melanoma lines—Evidence for "non-specific" killing by activated T cells.

R. L. Tyndall, S. Colyer and N. Clapp: Early alterations in plasma esterases with associated pathology following oral administration of diethylnitrosamine and butylated hydroxytoluene singly or in combination.

British Journal of Cancer *July, 1975*

E. Robinson, E. Bartal, Y. Cohen and R. Haasz: A preliminary report on the effects of methanol extraction residue of BCG (MER) on cancer patients.

M. R. Potter and B. M. Balfour: Stimulation of syngeneic and allogeneic lymphoid cells by tumour cells *in vitro*.

J. J. Miller, P. R. Gaffney, J. A. Rees and M. O. Symes: Lymphocyte reactivity in patients with carcinoma of the breast and large bowel.

R. A. Robins: Serum antibody responses to an ascitic variant of rat hepatoma D23.

D. Sabolovic, N. Sabolovic, A. Moutte, S. Leibovivi, B. Sauvezie, P. Chollet and A. Plagne: Agglutination of peripheral blood lymphocytes from cancer patients and not from healthy controls with F2A1 histone fraction.

M. F. A. Woodruff and Noreen Dunbar: Effect of local injection of C Parvum on the growth of a murine fibrosarcoma.

C. J. Kovacs, H. A. Hopkins, R. M. Simon and W. B. Looney: Effects of 5-fluorouracil on the cell kinetic and growth parameters of hepatoma 3924A.

M. Y. Gordon and N. M. Blackett: Stimulation of granulocytic colony formation in agar diffusion chambers implanted in cyclophosphamide pretreated mice.

M. I. Filipe: Mucous secretion in rat colonic mucosa during carcinogenesis induced by dimethylhydrazine. A morphological and histochemical study.

N. H. Slack and I. P. J. Bross: The influence of site of metastasis on tumour growth and response to chemotherapy.

G. M. Jeffree and C. H. G. Price: The metastatic patterns of osteosarcoma.

N. J. F. Dodd: Electron spin resonance study of changes during the development of a mouse myeloid leukaemia I. Paramagnetic metal ions.

S. M. Macrae and B. V. Cook: A retrospective study of the cancer patterns among hospital in-patients in Botswana.

J. Rumma and D. J. Davies: *Short Communication.* Survival of F1 hybrid rats inoculated with a strain-specific transplantable carcinoma following the induction of a systemic graft-versus-host reaction.

Book Reviews

E. C. Easson: Cancer detection and prevention Vol. 2. Advances in tumour prevention, detection and characterization.

B. W. Fox: Mycotoxins.

Short Communication

R. Favre, Y. Carcassonne and G. Meyer: Loss of anti-tumour immunogenicity of a somatic cell hybrid line with increasing subculture.

Europ. J. Cancer Vol. 11, pp. 531–536. Pergamon Press 1975. Printed in Great Britain

Influence of a Chronic Administration of Diethylnitrosamine on the Relation Between Specific Tissular and Division Functions in the Rat Liver

JACQUES VAN CANTFORT* and HERVÉ BARBASON†
**Service de Chimie médicale and †laboritoire d'Anatomo-Pathologie, Université de Liège, 151, Bd. de la Constitution, 4000 Liège, Belgium*

Abstract—*The evolution of the division and specific tissular functions, in the liver, which are mutually exclusive of young and partially hepatectomised adult rats, was studied during hepatic carcinogenesis induced by the chronic administration of diethylnitrosamine. The results show that one of the phenomena implicated in this chemical carcinogenesis is the deregulation of the normal equilibrium between the specific tissular and the division functions. In effect, during the preneoplasic stages, these two functions are progressively disrupted; they lose their normal exclusive relationship and their specific nycthermeral rhythms. These abnormalities are observed in the unoperated as well as partially hepatectomised rats.*

INTRODUCTION

Previously, [1, 2], we have studied the relationship in the rat liver between the division function and the specific tissular function of the organ. The mitotic index and the rate of DNA and nuclear RNA syntheses were used to measure the division function. The specific tissular function was monitored by measurement of the activity of a specific hepatic enzyme, cholesterol-7α-hydroxylase. As this enzyme presents a short half life (approximately 2½ h) and is directly inhibited by actinomycin D, modifications of its activity might thus reflect the modifications of its neosynthesis at genic level.

On the basis of a number of studies, it has been demonstrated that these two functions follow a circadian rhythm and seem to exclude each other [1, 2]. In the very young animal, the division function is very active, while the specific tissular function is very weak [2]. A circadian rhythm of the two functions does not appear until the 20th post-natal day, i.e., when the activity of the hypothalamo-adrenal axis becomes rhythmic and important. At that time, the mitotic index diminishes rapidly and the cholesterol-7α-hydroxylase activity increases very significantly. In the adult animal, where there are fewer divisions, cholesterol-7α-hydroxylase activity follows a circadian rhythm, rising at night when the animal is awake and eating, and conversely, presenting a minimal level during the day. In contrast, during a regenerative stage, induced by a partial hepatectomy, cholesterol-7α-hydroxylase activity decreases rapidly during the first 24 hr following surgery. Afterwards, successive mitotic cycles with diurnal peaks of decreasing amplitude are recorded, while the activity of cholesterol-7α-hydroxylase again increases progressively, and presents post-mitotic nocturnal peaks of increasing amplitude [1].

These phenomena were interpreted on the basis of a genic-regulating mechanism which would alternately regulate the repression or the derepression of those genes involved in the expression of the two functions. In addition, the

Accepted 25 April 1975.

activity of the hypothalamo-hypophyso-adrenal axis is also directly related to the existence of the circadian rhythm followed by those parameter .

In the present work, we have verified that the nycthemeral rhythm, and the mutually exclusive relationship existing between the division function and the specific tissular function are observed in the course of hepatic carcinogenesis induced by the chronic administration of diethylnitrosamine (DEN), a well known cancerogenous compound [3].

MATERIAL AND METHODS

Male Sprague–Dawley rats (± 200 g) were treated by diethylnitrosamine (DEN), dissolved in the drinking water given *ad libitum*, at a concentration of 40 μl/l (v/v), a dose which corresponds to the absorption of 10 mg/kg per day. Upon observing the volume of water absorbed, it seemed that the animals received an apparently identical dose during the entire experiment. Under these experimental conditions, we have observed that such a treatment, over a period of 100 days, induced hepatomas in all animals, causing their deaths 15–20 days later.

The period which was studied and which lasted from the 25th to 50th days of treatment might be considered as being preneoplasic as during this period the animals did not display observable pathological neoplasic foci [4]. In addition, at this moment, the necroses, due to the toxicity of the drug which is observed during the first 15 days of treatment have practically disappeared [4]. In all cases, this period is characterized by alterations in the structure of the hepatic lobules and by the presence of masses of cells which are observed through the exploitation of their characteristic histochemical properties, such as their positive PAS reaction after 18 hr of fasting, their basophilicity and their loss of different enzymes [3, 5–7]. In a parallel study, it has been shown that the mitotic index of PAS positive areas does not differ from that of the adjacent parenchyma [4].

The animals (150 rats) were divided into three experimental groups: One group serving as a control, the remaining two groups being treated with DEN for 25 and 55 days respectively. At the end of the treatment period, 12 animals were sacrificed in each group, 6 at 10 a.m. and 6 at 10 p.m. respectively. The remaining animals were hepatectomized at 10 a.m. [1] and later sacrificed every 12 hr until the 72nd post-operative hour.

The livers removed at the moment of sacrifice had two purposes: (1) measurement of the mitotic index and (2) measurement of the activity of various microsomal enzymes, specifically, cholesterol-7α-hydroxylase, benzopyrene-hydroxylase and aminopyrine-N-demethylase.

The mitotic index was measured by histological observations after Feulgen reaction [8]. In practice, three samples of $\pm 0{\cdot}1$ cm^3 are taken at random from the different hepatic lobes. Three histological slides are made from different levels of each sample. After fixation in Zenker–Formol and treatment by classical methods, each preparation undergoes the Feulgen reaction. At $1000\times$, 20 fields chosen at random are observed in each slide. Simultaneously, the number of nuclei and mitoses are determined. In total, approximately 9000 cells were observed per animal to determine the mitotic index.

The measurement of microsomal enzymatic activity is effected on the $9{\cdot}000 \times g$ supernatant of a $\frac{1}{5}$ homogenate in isotonic KCl. In all cases, the results are expressed per gram of fresh weight and the protein concentrations of these enzymatic preparations has been verified [9]. The cholesterol-7α-hydroxylase activity is determined using an isotopic method developed in this laboratory and previously described elsewhere [10]. The validity of this method, as well as its qualities of precision, rapidity and reproducibility have also been the object of a detailed study [11]. The measurement of benzopyrene-hydroxylase is effected through the determination of fluorescence of the hydroxylated product using a method previously described [12]. The measurement of aminopyrine-N-demethylase activity is realized by a colorimetric measure of formol released into the incubation medium after the method of Nash [13].

RESULTS AND DISCUSSION

A simultaneous study, in the same animals, of division function and the specific tissular function was made in the liver during chemical carcinogenesis.

The fluctuations of the division rate were followed through the measurement of the mitotic index (Fig. 1). The specific tissular function was studied by measuring the activities of two classes of enzymes: (a) the drug-metabolizing enzymes, which have a rather long half-life and do not present a marked circadian rhythm [14] (Fig. 3,4) and cholesterol-

7α-hydroxylase which controls an important metabolic pathway (bile acids biosynthesis), presents a short half-life (2½ hr) and follows a very important circadian variation in the normal rats [15] (Fig. 2).

In normal and DEN treated rats, we have followed the evolution of the parameters before and after a partial hepatectomy. During the course of DEN treatment and after partial hepatectomy, a slight decrease in the protein concentration of the enzymatic preparation was observed (Table 1). This decrease did not modify the conclusion of our results. Thus, we have expressed these results in units per gram of fresh liver as has usually been done using unpurified enzyme preparations [1, 15].

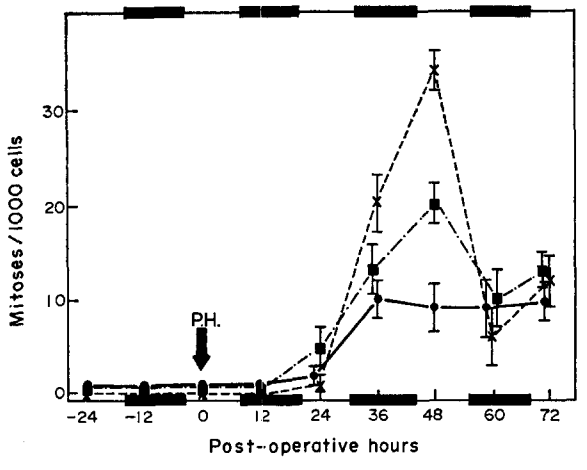


Fig. 1. Mitotic index in control (--x ---x--), in 25 days DEN-treated (—● ■ ● — ● — ■ —) and in 55 days DEN-treated (—●—●—) rats.

The measures were performed every 12 hr before and after a partial hepatectomy realized at 10 a.m. Dark lines on the abscisses represent the night. The vertical bars indicate the standard error of the mean of the measures obtained from at least 5 animals.

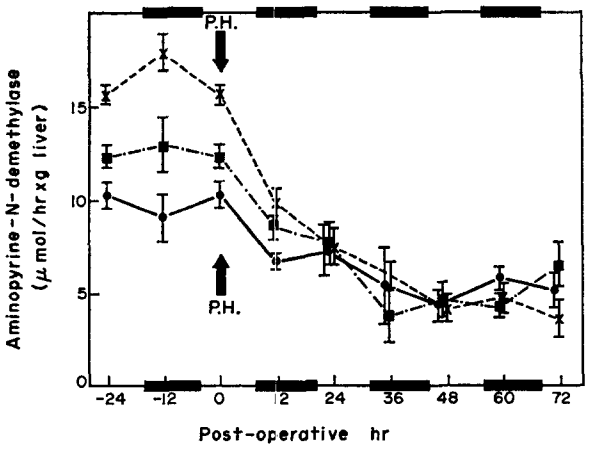


Fig. 3. Benz(a)pyrene hydroxylase activity in control and DEN-treated rats for 25 or 55 days (legend as Fig. 1).

(a) *Relation between the specific tissular and the division functions before a partial hepatectomy*

In DEN-treated animals, the mitotic index is higher (1%) than in normal rats. However, no circadian rhythm of mitoses can be detected (Fig. 1, negative hr).

On the other hand, the tissue-specific function shows an important variation according to the length of the DEN treatment.

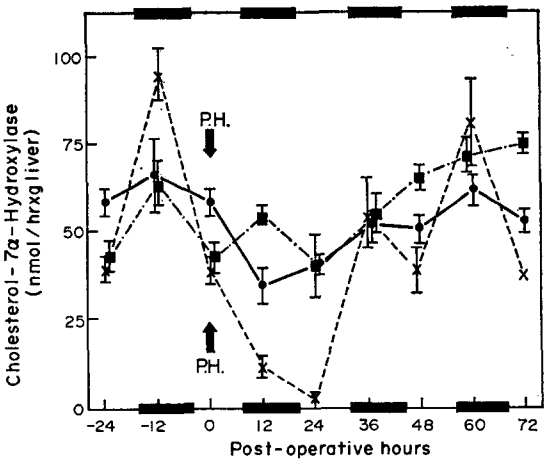


Fig. 2. Cholesterol-7α-hydroxylase activity in control and DEN-treated rats for 25 or 55 days (legend as Fig. 1).

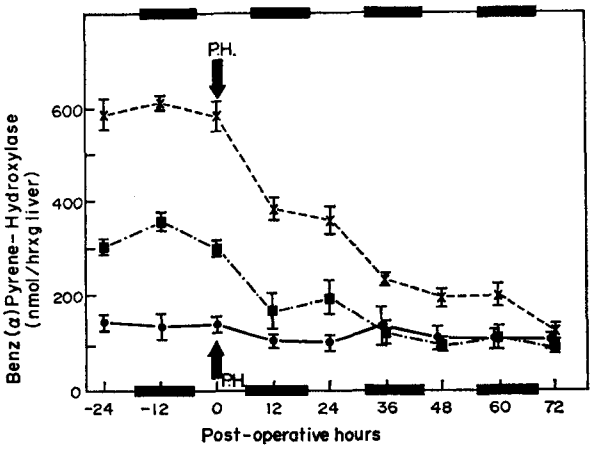


Fig. 4. Aminopyrine-N-demethylase activity in control and DEN-treated rats for 25 or 55 days (legend as Fig. 1).

Cholesterol-7α-hydroxylase circadian rhythm is either partially (25 days) or totally (55 days) abolished by the DEN treatment (Fig. 2, negative hr). Rather than being blocked at its lowest level, the enzymatic activity reaches a higher steady state equilibrium. This new level is nevertheless significantly lower than the 10 p.m. value found in the normal rat.

The drug-metabolizing enzymes are inhibited by the DEN treatment (Fig. 3 and 4).

Table 1

Delay after hepatectomy and time of sacrifice	Non-treated by DEN	Treated by DEN for 25 days	Treated by DEN for 50 days
0 hr at 10 a.m.	124 ± 16	110 ± 10	118 ± 15
0 hr at 10 p.m.	128 ± 16	108 ± 7	108 ± 11
12 hr (10 p.m.)	114 ± 11	111 ± 5	105 ± 10
24 hr (10 a.m.)	109 ± 10	103 ± 14	108 ± 13
36 hr (10 p.m.)	105 ± 8	102 ± 12	106 ± 14
48 hr (10 a.m.)	108 ± 13	107 ± 10	103 ± 11
60 hr (10 p.m.)	104 ± 11	100 ± 8	111 ± 14
72 hr (10 a.m.)	106 ± 10	108 ± 9	103 ± 9

Protein concentration (mg/g liver) in 9000 × ***g*** supernatant of homogenates used as the source of enzymatic activity.

Each series of numbers corresponds to the mean ± S.D. of the results obtained using 6 animals.

After 55 days of the drug administration aminopyrine-N-demethylase activity is decreased by about 50% while benzopyrene hydroxylase activity is almost completely inhibited.

Discontinuation of treatment during the 10 days prior to sacrifice does not change the proliferative properties of the liver, nor the activity of several microsomal enzymes [16]. Therefore the observed effect results not from acute toxicity, but rather from chronic toxicity due to the accumulation of lesions during the treatment period.

The inhibition of microsomal enzymatic activity explains the almost total disappearance of the necrotic toxic action of DEN during the period explored. In effect, the microsomal enzymes metabolize DEN into a toxic product [3].

Therefore, it is not surprising that the toxicity which is observed at the beginning of DEN treatment is extremely reduced during a prolonged treatment.

Thus, in these animals treated by DEN, a complementary relationship between specific tissular and division functions which we have observed previously in the young and in the adult hepatectomised animals [1, 2], still exists; the division function being increased and the specific tissular function being decreased.

(b) *Relation between the specific tissular and the division functions after a partial hepatectomy*

After hepatectomy, the relationship between the two studied functions is different in the treated or in the control rats. The mitotic index is clearly increased, but the cholesterol-7α-hydroxylase activity is not inhibited in the two experimental conditions (DEN treatment during 25 or 55 days). The DEN-treated animals exhibit, after this surgery, a clearly less important increase of the mitotic index than the operated control animals. This relative inhibition affects only the first mitotic wave; afterwards, the mitotic index is comparable in the different groups of animals.

One can also observe important differences in the degree of inhibition of the first mitotic wave and in the kinetics of hepatic regeneration as a function of the length of DEN treatments (25 or 55 days). If the partial hepatectomy is realized after 25 days of treatment, the first mitotic wave is reduced by almost 50% and the mitoses evolve along the normal nycthemeral rhythm. If the operation is performed after 55 days of treatment, the number of mitoses is increased, but is rapidly stabilized at a level corresponding to about 30% of the peak reached in the control rats. In this case, no rhythm is observed.

After partial hepatectomy, the evolution of cholesterol-7α-hydroxylase activity is clearly different in the control and experimental groups. In the former, a partial ablation of the liver produces a total inhibition of this enzyme activity for 24 hr afterwards, followed over the course of successive days, by a progressive and rhythmic restoration of enzymatic activity.

Those animals treated with DEN for 25 days exhibited no reduction in cholesterol-7α-hydroxylase activity after partial hepatectomy (Fig. 2). On the contrary, it was observed that on the first post-operative days, a rhythm of small amplitude still existed. With time, the levels of activity increased slightly, and reached a plateau 2 days later. No nycthemeral variations were observed during the entire experiment.

If partial hepatectomy is realized after 55 days of DEN treatment, i.e., when the circadian rhythm of cholesterol-7α-hydroxylase activity has been abolished in the animal, the reduction

of the observed enzymatic activity is weak and transitory, lasting only the first post-operative day.

The activities of the drug-metabolizing enzymes (aminopyrine-N-demethylase and benzopyrene hydroxylase) which were already decreased by the DEN treatment, are further inhibited following the hepatectomy. This inhibition is recorded in the DEN-treated animals as well as in the normal rats. Finally, the enzyme activities are reduced to the same low level in the different groups of rats. It is worth mentioning that the hepatectomy did not affect the activity of benzopyrene-hydroxylase in the 55-days-treated animals any more. In fact, the enzymatic activity in this group of rats corresponds exactly to the level found in the two other groups 72 hr after surgery.

From all these results, it would appear, therefore, that the antagonism between the division and the specific tissular functions which continues to exist in control animals after hepatectomy, is completely deregulated when the surgery is realized on DEN-treated animals. This would suggest that one of the phenomena implicated in the processes of cellular transformation during carcinogenesis induced by DEN, seems to be, at least in part, a derangement in the simultaneous regulation of the specific tissular and division functions. This lack of regulation, associated with the reduction of enzymatic and mitotic activities over the treatment period should find its origin in the degradation of genetic information induced by the alkylating agent used.

It is also of interest to mention that the drug-metabolizing enzymes are more affected by the treatment than cholesterol-7α-hydroxylase which controls the physiological function of bile acids production.

REFERENCES

1. J. Van Cantfort and H. Barbason, Relation between the circadian rhythms of mitotic rate and cholesterol-7α-hydroxylase activity in the regenerating liver. *Cell Tiss. Kinet.* **5,** 325 (1972).
2. H. Barbason, J. Van Cantfort and N. Houbrechts, Correlation between tissular and division functions in the liver of young rats. *Cell Tiss. Kinet.* **7,** 319 (1974).
3. P. Magee and J. M. Barnes, Cancerogenic nitroso-compounds. *Adv. Cancer Res.* **10,** 163 (1967).
4. H. Barbason, A. Friedman-Manduzio and E. H. Betz, Activité mitotique lors de la période prénéoplasique précédant la cancérisation du foie par la diéthylnitrosamine. Manuscript in preparation.
5. H. Friedrich-Friska, W. Gössner and P. Börner, Histochemische Untersuchungen der Cancerogenese in des Rattenleber nach Dauergaben von Dieithylnitrosamin. *Krebsforsch.* **72,** 226 (1969).
6. W. Gössner and H. Friedrich-Freska, Histochemische Untersuchungen uber die Glucose-6-Phosphatase in der Rattenleber während der Kanzerisierung durch Nitrosamin. *Naturforsch B.*, **19,** 862 (1964).
7. E. Scherrer, M. Hoffmann and P. Emmelot, Quantitative study on foci of altered liver cells induced in the rat by a single dose of diethylnitrosamine and partial hepatectomy. *J. natl Cancer Instl.* **49,** 93 (1972).
8. H. Barbason, Influence du rythme de l'activité circadienne sur l'index mitotique au cours de la régénération hépatique. *C. R. Acad. Sci. (Paris)* **270,** 3295 (1970).
9. O. Lowry, N. Rosenbrough, A. Farr and R. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
10. J. Van Cantfort, Contrôle par les glucocorticoïdes de l'activité circadienne de la cholestérol-7α-hydroxylase. *Biochimie* **55,** 1171 (1973).
11. J. Van Cantfort, J. Renson and J. Gielen, Rat liver cholesterol-7α-hydroxylase I. Development of a new assay based on the enzymatic exchange of the tritium located on the 7α position of the substrate. *Europ. J. Biochem.* In press (1975).
12. J. Van Cantfort and D. Rondia, Mesure et propriétés de la benzo(a)pyrène hydroxylase. *C. R. Acad. Sci. (Paris)* **276,** 3387 (1973).
13. T. Nash, The colorimetric estimation of formaldehyde by means of the Hantz Reaction. *Biochem. J.* **55,** 416 (1953).
14. J. Van Cantfort, P. Kremers and J. Gielen, Genetic and hormonal control of 7α and 16α-steroid hydroxylases. *Fourth European Workshop on Drug Metabolism,* Mainz (Germany) September 74. Manuscript in preparation.

15. J. GIELEN, J. VAN CANTFORT, B. ROBAYE and J. RENSON, Rat liver cholesterol-7α-hydroxylase III New results about its circadian rhythm. *Europ. J. Biochem.* In press (1975).

Europ. J. Cancer Vol. 11, pp. 537–544. Pergamon Press 1975. Printed in Great Britain

Allogeneic Tumor Enhancement by Levamisole, a New Immunostimulatory Compound: Studies on Cell-mediated Immunity and Humoral Antibody Response

A. MANTOVANI and F. SPREAFICO

Instituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea 62—20157 *Milan, Italy*

Abstract—C_3H *mice transplanted with the incompatible L*1210 *leukemia exhibited progressive tumor takes in a high percentage of cases when treated with the same course of the chemical immunostimulant levamisole which could induce a marked rise in the immune response towards allogeneic erythrocytes. Investigations of the specific anti-tumoral immune reactivity revealed in progressor mice a depression of cell-mediated immunity and serum cytotoxic antibodies and an increased serum blocking activity. The relevance of these immunological modifications to enhanced tumor growth and the possible mechanisms of action of the immunostimulant are discussed in this paper.*

INTRODUCTION

STIMULATION of immunity by non-specific immunoadjuvants has been shown to be an effective adjunct in the treatment of experimental and human neoplasms. The search for other agents possessing this stimulatory capacity is therefore particularly active with greater emphasis on compounds with defined chemical structures which may be devoid of some of the side effects displayed by the adjuvants of biological origin currently used such as BCG.

A simple chemical, (-)2,3,5,6-tetrahydro-6-phenylimidazo(2,1-b) thiazole, levamisole, has recently been shown to possess immunostimulatory properties both in animals and in humans [1–7]. Its capacity to effectively synergize with chemotherapy in experimental tumor systems has been described by Chirigos *et al.* [8, 9] and in another paper we have presented our experience in studying the immunoadjuvant capacity of this compound in a series of experimental models [10].

It is known that the effects of adjuvants on immune responsiveness can be difficult to predict since, occasionally, instead of stimulation, a suppression of immune reactivity can be observed [11], hence the need to look at these agents as modulators rather than simply as stimulants of immune responses. In tumorous conditions, enhanced neoplastic growth can also follow immunotherapeutical treatments employing adjuvants [11–15]. Since relatively limited attention has been given so far to studying the modifications in antitumor immune effector mechanisms accompanying enhancing treatments with adjuvants, it was considered of interest to investigate in some detail a condition in which facilitation of allogeneic tumor growth occurred after treatment with levamisole.

Accepted 23 April 1975.

Reprint requests to: Federico Spreafico, Chief, Laboratory of Tumor Immunology and Chemotherapy, Istituto di Ricerche Farmacologioche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy.

MATERIAL AND METHODS

Mice

Female C_3H, 20–22 g body obtained from Charles River, Co., U.S.A. were used for these studies.

Tumor and drug treatment

C_3H ($H\text{-}2^K$) mice were injected i.p. on day 0 with 10^7 cells of the allogeneic L1210 leukemia, which was maintained by weekly i.p. passages in compatible DBA/2 hosts ($H\text{-}2^d$); prior to inoculation, red cells were removed from the ascites by osmotic lysis, followed by washings with phosphate buffered saline (PBS) containing 10% heat-inactivated calf serum (PBS-CS); viability of leukemia cells was checked microscopically by dye exclusion and only préparations with over 95% viability were accepted. When normal C_3H recipients are transplanted with this number of L1210 cells, firstly a phase of leukemia growth can be seen, the total number of neoplastic cells in the peritoneal cavity reaching its peak on days 6–8. Thereafter, tumor rejection ensues as evidenced by the decline in the number of leukemic elements so that by day 10–12 the animals are leukemia-free. Using this high tumor inoculum, only occasionally does a percentage (in our experience never over 20%) of the transplanted animals succumb to progressive leukemia growth, and when lower inocula, such as 5×10^6 cells, are employed, all controls reject the allogeneic tumor.

Levamisole (Leva) was obtained from Janssen Pharmaceutica, Beerse, Belgium. It was freshly dissolved in saline and injected i.p. at a dose of 3 mg/kg from day 1 to day 4; this drug schedule had been found effective in giving a high percentage of cures when administered in conjunction with X-irradiated leukemia cells in L1210-bearing compatible $(Balb/c \times DBA/2)F_1$ hosts [10].

Sera and splenocytes

Blood was collected from the retro-orbital plexus of 4–6 control and treated mice at various intervals after tumor transplantation; the sera were heat-inactivated (56°C for 20 min) and stored at −20°C until tested. Spleens were teased gently in ice-cold Hank's solution containing 5% heat-inactivated calf serum (HBSS-CS), dispersed with a Pasteur pipette and filtered through surgical gauze. The cells were then pelleted by centrifugation and resuspended in Tris buffered ammonium chloride [16] in order to lyse the erythrocytes; this step was repeated twice. The splenocytes were then washed twice with HBSS-CS and finally resuspended in RPMI 1640 medium (Biocult, England), supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI-FCS).

Lymphocytotoxicity assay

A modification of the ^{51}Cr release assay described by Brunner *et al.* [17] was used. Briefly, 5×10^4 labelled leukemia cells in 1 ml RPMI-FCS were incubated in plastic tubes (Nunc, Denmark, Cat. No. 1090) for 4 hr at 37°C in a humidified atmosphere of 95% air–5% CO_2, with normal or immune lymphocytes, final volume being 1·2 ml. The attacker: target cell ratio (A:T) was 100:1 unless otherwise stated. Incubation was stopped by adding to each tube 1 ml of chilled PBS-CS; the cells were then pelleted by centrifugation and the radio-activity present in 0·5 ml of the supernatant determined in a gamma spectrometer (Packard, mod. 578). Per cent ^{51}Cr release was calculated as follows:

$$\frac{^{51}\text{Cr supernatant}}{^{51}\text{Cr release by freezing and thawing} \times \text{total } ^{51}\text{Cr present in single test tubes}} \times 100.$$

The mean total radioactivity released from cells after 4 cycles of freezing and thawing was found to be $75 \pm 1{\cdot}2\%$ of total ^{51}Cr incorporated. Specific lymphocytotoxicity was expressed as the difference between the percent ^{51}Cr released in the presence of test lymphocytes and that released in the presence of the corresponding normal lymphocytes, which was $12{\cdot}5 \pm 2$ in the series of experiments presented here. Results presented are mean of quadruplicate tubes; statistical analysis was performed by Student's *t* test. For specificity testing, control tubes containing labelled EL4 leukemia cells were run in each test; since no cytotoxic activity was detected in these conditions the data for such controls are omitted from the tables.

Serum cytotoxicity and blockade Complement-dependent serum cytotoxicity was evaluated essentially as described by Sanderson [18]: 5×10^4 ^{51}Cr-labelled L 1210 cells in 0·1 ml HBSS-CS were incubated for 30 min at 37°C with 0·1 ml of serial two fold dilutions of test sera; 0·1 ml of 1:2 diluted guinea pig serum, previously absorbed with agarose [19] was then added and the incubation carried on for an additional 30 min. Two ml of ice-cold PBS-CS were then added to each tube, the cells sedi-

mented by centrifugation and the isotope present in 0·5 ml supernatant determined. The mean percentage of isotope release in the presence of normal serum and complement was subtracted. The highest serum dilution giving 20% cytotoxicity was taken as cytotoxic antibody titer.

For the assay of serum blocking activity, 0·2 ml of the test serum were mixed with the leukemia target cells (5×10^4/1 ml) at room temperature and within 5 min spleen lymphoid cells (5×10^6/0·2 ml) were added. The sera remained in the tubes until the termination of the experiment. Percent blocking was calculated as follows:

$$\frac{\text{\% specific lymphocytotoxicity with normal serum} - \text{\% specific lymphocytotoxicity with test serum}}{\text{\%specific lymphocytotoxicity with normal serum}} \times 100$$

In concurrent tests it was found that in the absence of attacker cells, the serum preparations employed for this study did not significantly modify the extent of isotopic release from target elements as compared to normal C_3H serum; therefore, feeder or toxic effects of these sera in our conditions seem excludable.

Response to sheep erythrocytes

C_3H female mice (5 per group) were injected i.p. with 4×10^8 sheep red blood cells (SRBC) on day 0 and treated with levamisole (3 mg/kg i.p.) from day 1 to 4. The number of direct plaque-forming cells in spleens (PFC/spleen) was evaluated on days 4, 5 and 7 after challenge by the technique of Jerne and Nordin [20].

RESULTS

Effect of Leva on tumor allograft rejection

Representative results of the 5 experiments performed are presented in Table 1. It can be seen that when 10^7 incompatible L1210 leukemia cells were injected i.p. into C_3H mice, all controls in some experiments survived this allogeneic tumor challenge, whereas in other experiments a minority of the normal C_3H recipients (never exceeding 20%) died because of progressive tumor growth. On the other hand, if the animals were also treated with Leva from day 1 to 4, the number of mice succumbing to the tumor was in every case significantly higher than in controls, their percentages ranging from 50 to 100% in the various experiments. Survival of these drug-treated progressor mice was not significantly different from that seen in untreated succumbing controls and no Leva-injected allografted animal was ever seen to die later than day 12 in these conditions. When a lower inoculum of 5×10^6 leukemia cells was used, all control C_3H mice were capable of resisting this tumor challenge whereas a substantial proportion, approximately 50%, of drug-treated recipients died and widespread leukemia was present at autopsy.

Effect of Leva on primary response to sheep erythrocytes

Since the immunostimulatory activity of Leva can vary markedly in different mouse strains (Renoux, personal communication), and in order to confirm that the drug treatment employed could indeed result in immunostimulation, the capacity of Leva to influence the primary PFC response of C_3H mice to SRBC was investigated. Table 2 shows that on days 4 and 5 after antigen stimulation, no differences in direct PFC numbers could be found between treated and control mice, whereas a significant increase in PFCs was observed on day 7, the values seen in Leva-treated animals having more than doubled in respect to controls.

It was thus concluded that Leva in the schedule employed was not unspecifically immunodepressive. In an effort, therefore, to elucidate the possible mechanism(s) sustaining

Table 1. *Facilitation of allogeneic tumor growth by levamisole*

	No. L1210 Cells injected	Levamisole	MST*	D/T†
Exp. 1	10^7	–	–	0/10
	10^7	+	8·7 ± 2·1	7/10
	5×10^6	–	–	0/10
	5×10^6	+	9·0 ± 0·8	5/10
Exp. 2	10^7	–	8·5 ± 1·5	3/15
	10^7	+	8·5 ± 2·3	12/15
	5×10^6	–	–	0/15
	5×10^6	+	10·1 ± 3·2	7/15
Exp. 3	10^7	–	–	0/10
	10^7	+	8·3 ± 2·8	5/10
	5×10^6	–	–	0/10
	5×10^6	+	9·5 ± 3·0	6/10

*Mean survival time in days.
†Dead with leukemia over total number of mice injected.

Table 2. Effect of levamisole on primary response to sheep erythrocytes

	PFC/Spleen		
	Day 4	Day 5	Day 7
Control	24,310 (15,640–37,390)	16,680 (19,560–13,940)	338 (263–436)
Levamisole	28,300 (23,580–33,380)	17,070 (14,470–20,000)	935* (1023–851)

*$P < 0.05$.

the observed allogeneic tumor enhancing effect of Leva in these conditions, specific anti-tumoral cell-mediated cytotoxicity (CMC) and the serum cytotoxic and blocking responses were analyzed in mice transplanted with 10^7 incompatible leukemia cells.

Effect of enhancing Leva treatment on CMC

Results obtained are presented in Figure 1a; it will be noticed that although the kinetics of the CMC response were roughly similar in control and drug-injected animals, the cytotoxic activity displayed by splenocytes obtained from Leva-treated mice was always significantly lower than seen with an equal number of control spleen cells. In fact, on day 6, control splenocytes already showed a significant CMC whereas the values of Leva-hosts were still not significantly above background. Cytotoxic activity increased in both groups at later days reaching a peak on day 9 thereafter declining although it was still significant on day 64 after tumor transplantation. Throughout this period, the CMC values observed with Leva splenocytes were 30–50% lower than controls except on day 9 when the difference observed (approximately 10%) was less marked but still statistically significant ($P < 0.05$) and on day 64 when the values in controls and drug-treated regressor animals were similar.

The reduced cytotoxicity displayed by Leva splenocytes was evident also when A:T ratios higher than 100:1, i.e. 500:1, were employed and the difference with controls was evident not only during the phase of active tumor growth but also with splenocytes obtained from Leva-injected mice 11 and 16 days after tumor transplantation (Figure 1b). That these drug-treated animals could be considered regressors was also indicated by the fact that no visible leukemia could be found in the peritoneal

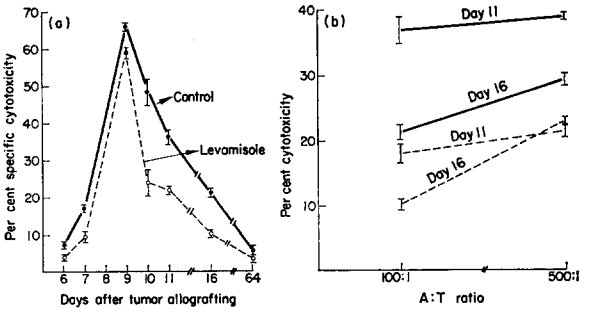


Fig. 1. Cell-mediated immunity of control and levamisole-treated splenocytes (continuous and dotted lines respectively) on different days after tumor allograft (a), and with various attacker: target cell ratios (b).

cavity of these mice when sacrificed for CMC monitoring.

Effect on serum cytotoxic and blocking activities

Complement-dependent serum cytotoxicity was measured in the same animals used for CMC evaluation and representative results are shown in Table 3. Detectable activity was first seen on day 7 in both groups, but whereas in controls a rise in titer could be seen in the following days, in drug-treated animals this increase could not be observed and the similarity in titers found on day 13 between the 2 groups can be ascribed to the fact that at this time the test animals evaluated were regressors. In controls, a drop in cytotoxic antibody concentration was found around day 20 followed by a second rise so that the titers seen 49 days after tumor grafting were higher than observed in the first phase. In drug-treated regressors, this second peak was also present and the titers were in the same range as controls.

Serum blocking activity in control and drug-treated mice was assessed in 2 sets of experiments employing sera obtained from animals sacrificed from 6 to 13 days after allogeneic tumor transplantation. In these tests the

Table 3. Effect of levamisole on serum C′-dependent cytotoxicity

Day of serum collection	Cytotoxic antibody titer	
	Control	Levamisole
6	—	—
7	1:2	1:2
9	1:32	1:4
10	1:32	1:2
13	1:64	1:64
20	1:8	1:8
49	1:248	1:248

attacker cells were splenocytes taken from Leva-treated mice 9 or 13 days (exp. 1 and 2 respectively, Table 4) after leukemia inoculation. For an easier evaluation of this data, it may be worth mentioning that in the first days examined, it was impossible to distinguish between progressor and eventual regressor drug-injected mice.

In controls, a low though significant serum-blocking activity was detected throughout the period investigated and with levels which were practically similar in the various days with the exception of day 6 when in one experiment the value of 18% blocking was significant only at the $P < 0{\cdot}1$ level. In contrast, on the same day, the blocking activity displayed by sera of Leva-treated animals was approximately 3–4 times higher than found in controls, in the two experiments the values being 83 and 84% respectively. On day 7 a significantly higher blocking (86% vs. 22% of controls) was found in drug-treated mice in one experiment, whereas in the second no differences with the appropriate control sera could be detected. In the other days examined, no significant differences in serum blockade could be found between Leva-treated and untreated animals. It should be noted that serum taken from normal mice 48 hr after completion of a 4 day course of Leva did not modify the cytotoxic activity of splenocytes, therefore a direct action of the drug and/or its possible metabolites on cytocydal lymphoid cells seems excludable.

Table 4. Blocking activity in levamisole-treated and control, tumor allografted C_3H mice

Exp.	Day of serum collection	Source of serum	% Cytotoxicity	% Inhibition
1	6	Normal	44·1 ± 0·5	—
		Control	36·1 ± 0·7	18·1
		Levamisole	7·3 ± 3·0	83·5*
2	6	Normal	15·1 ± 1·2	—
		Control	10·9 ± 1·2	27·8†
		Levamisole	3·5 ± 1·6	84†‡
1	7	Normal	44·1 ± 0·5	—
		Control	32·6 ± 1·9	26·1†
		Levamisole	32·0 ± 2·2	27·4†
2	7	Normal	21·3 ± 1·1	—
		Control	17·5 ± 2·0	22·5†
		Levamisole	2·9 ± 1·5	86·5†‡
1	9	Normal	44·1 ± 0·5	—
		Control	34·4 ± 1·4	22+
		Levamisole	30·4 ± 1·9	31+
2	13	Normal	15·1 ± 1·2	—
		Control	7·4 ± 0·4	50·9†
		Levamisole	6·9 ± 0·1	54·3†

* = $P < 0.01$ compared to control serum.
† = $P < 0{\cdot}05$ when compared to normal serum.
‡ = $P < 0.05$ compared to control serum.

DISCUSSION

In conclusion, the results presented have shown that in C_3H mice transplanted with the allogeneic L1210 leukemia, facilitation of tumor takes leading to progressive growths in a high proportion of animals can occur following treatment with Leva, a chemical whose immunostimulatory properties are discussed in an accompanying paper. [10]

In explaining the facilitated allogeneic tumor progression, a direct growth-promoting activity of the drug seems excluded since no accelerated L1210 leukemia growth was observed after administration of Leva to syngeneic DBA/2 or allogeneic C_3H hosts previously immunosuppressed with 400R of X rays. In addition, Leva can be tumor-inhibiting for this and other neoplasms as mentioned earlier.

For other immunostimulants the possibility of obtaining immunodepression has been described [11]. However, the Leva treatment employed in these studies did not appear to induce any generalized immune impairment as evidenced by the stimulation of a primary

response to SRBC. It was thus in the hope of gaining an insight into the mechanisms responsible for this enhanced neoplastic progression that specific antitumoral cell-mediated and humoral responsiveness were analyzed.

CMC has been reported to be the major determinant in the rejection of allogeneic tumors [21] and a depression of cell-mediated reactivity has been shown to be concomitant to progressive tumor growth in syngeneic conditions [22, 23]. It is thus possible that the persistently lower levels of CMC observed in Leva-treated animals in comparison to tumor-rejecting controls, could play an important role in the enhanced tumor growth found in our conditions. The effector cells of CMI responses against allogeneic grafts have been reported to be T lymphocytes [24–26] and in agreement with these findings, in the system employed for this study, the *in vitro* treatment of the immune spleen cells with rabbit anti-theta serum resulted in a complete suppression of cytotoxicity. In mice carrying methylchol–anthrene-induced tumors, a decrease in the percentage of theta-bearing spleen cells was found to be present throughout tumor growth [27]. A similar phenomenon was observed in this laboratory after inoculation of L1210 leukemia into compatible CDF_1 hosts, although only during the first 2–3 days after tumor transplantation (Anaclerio and Spreafico, unpublished observations). The observed impairment of CMC in Leva-treated mice could therefore have resulted from a lower number of effector cells in the splenocyte population employed in our *in vitro* assay. However, this explanation seems untenable since no significant differences from control values were found in the percentage of theta-carrying elements in those spleen cell preparations from Leva-injected mice exhibiting reduced cytotoxic activity. A further mechanism mediating the host's response against syngeneic and allogeneic tumors is represented by antibody-dependent cellular cytotoxicity [28–30]. Modifications in this effector mechanism did not appear to exert a major role in these conditions; in experiments to be reported in detail elsewhere, it was observed in fact that in the presence of arming sera, the capacity to mediate antibody-dependent cellular cytotoxicity of splenocytes from Leva-treated mice was similar to that of controls.

In addition to the depression of lymphocyte-mediated tumor cell killing, another factor which in principle could have contributed to neoplastic progression in drug-treated mice is represented by the depression in serum complement-dependent cytotoxicity as found on days 9 and 10 after tumor transplantation. The importance of this mechanism is unclear since it is possible to consider this finding as secondary to rather than causative of tumor growth.

Serum blockade against allogeneic target cells has previously been described through the use of an isotope-release assay [23–31] but only employing hyperimmune sera, whereas in this study, blocking activity was observed with early sera collected during the period of active tumor growth (days 6 and 7) and displaying very low complement-dependent cytotoxicity. The possible importance of serum blockade in the facilitated tumor growth of Leva-injected mice is difficult to assess since a significant higher blocking activity was consistently demonstrable in drug-treated mice on day 6 whereas at later times the values found were ambiguous or similar to those of controls. However, the coincidence of this observation with the fact that the survival of progressor, Leva-treated mice was in effect 6–7 days in 4 experiments out of 5 is suggestive of the possible biological significance of this finding. Thus, in conclusion, enhanced tumor growth in Leva-injected animals was associated with an impairment of CMC, a depression of cytotoxic antibody titers and a higher serum-blocking activity; whether the first activity of the drug in these conditions consisted in the depression of CMC or in inducing a higher blocking response, is at the present time impossible to state. A mechanism postulating a direct, centrally effective inhibitory activity of Leva as initiating event although not excludable, is difficult to reconcile with the fact that the same treatment enhancing tumor takes could also induce an increase in the PFC response. In addition, the drug has been shown to stimulate other T and T-B cell responses such as the graft-versus-host reaction, allergic encephalomyelitis or syngeneic and allogeneic rejection of normal or neoplastic tissues [1–10]. It could thus be hypothesized that the major initial effect of Leva in these conditions was a stimulation of the production of blocking antibodies or complexes [32]. Since the binding of blocking factors to effector cells can be very strong [33] a possible explanation for the depressed CMC could also be advanced.

Whatever the exact mechanisms, the observed modifications in these immune parameters seem to complement and at the same time be at variance with observations by others on the changes of immunological reactivity following enhancing treatments with immunostimulants.

In fact a reduction of CMC has been found to be concomitant to tumor allograft facilitation after treatment with Freund's adjuvant [13], but no changes in serum blocking activity were detected. On the other hand, enhancement of syngeneic neoplastic growth after treatment with BCG was observed to be accompanied by an increased serum blocking activity whereas no modifications in CMC were seen employing a microcytotoxicity assay [14, 34]. Whether these discrepancies depend on the different experimental conditions and methodologies employed or derive from inherently different biological characteristics of the various immunostimulants investigated, cannot be decided at present. It is of interest to note that the overall pattern of immunological changes detected in these allogeneic conditions appears to be specular to that reported by Bansal and Sjögren [35] in a syngeneic system when successful immunotherapy was performed employing BCG plus unblocking serum.

REFERENCES

1. G. Renoux and M. Renoux, Restauration par le phenyl-imidothiazole de la réponse immunologique des souris âgées. *C. R. Acad. Sci. (Paris)* **274,** 3034 (1972).
2. G. Renoux and M. Renoux, Inhibition par la théophilline de la stimulation immunologique induite par le phenylimidothiazole. *C. R. Acad. Sci. (Paris)* **274,** 3149 (1972).
3. G. Renoux and M. Renoux, Action du phénylimidothiazole (tétramisole) sur la réaction du greffon contre l'hôte. Role des macrophages. *C. R. Acad. Sci. (Paris)* **274,** 3320 (1972).
4. G. Renoux and M. Renoux, Antigenic competition and non-specific immunity after a Rickettsial infection in mice: Restoration of antibacterial immunity by phenyl-imidothiazole treatment. *J. Immunol.* **109,** 761 (1972).
5. J. Brugmans, V. Schuermans, W. De Cock, D. Thienpont, P. Janssen, H. Verhagen, L. Van Nimmen, A. C. Louwagie and E. Stevens, Restoration of host defence mechanisms in man by levamisole. *Life Sci.* **13,** 1499 (1973).
6. D. Tripodi, L. C. Parks and J. Brugmans, Drug-induced restoration of cutaneous delayed hypersensitivity in anergic patients with cancer. *New Engl. J. Med.* **289,** 354 (1973).
7. H. Verhaegen, J. De Cree, W. De Cock and F. Verbruggen, Levamisole and the immune response. *New Engl. J. Med.* **289,** 1148 (1973).
8. M. A. Chirigos, J. W. Pearson and J. Pryor, Augmentation of chemotherapeutically-induced remission of a murine leukemia by a chemical immunoadjuvant. *Cancer Res.* **33,** 2615 (1973).
9. M. A. Chirigos, J. W. Pearson and F. S. Fuhrman, Effect of tumor load reduction on successful immunostimulation. *Proc. Amer. Ass. Cancer Res.* **15,** 116 (1974).
10. F. Spreafico, A. Vecchi, A. Mantovani, A. Poggi, G. Franchi, A. Anaclerio and S. Garattini, Characterization of the immunostimulants levamisole and tetramisole. *Europ. J. Cancer,* In press (1975).
11. G. Mathé, M. Kamel, M. Dezfulian, O. Halle-Pannenko and C. Bourut, An experimental screening for "systemic adjuvants of immunity" applicable in cancer immunotherapy. *Cancer Res.* **33,** 1987 (1973).
12. W. F. Piessen, F. L. Lachapelle, N. Legros and J.-C. Heuson, Facilitation of rat mammary tumour growth by BCG, *Nature (Lond.)* **228,** 1210 (1970).
13. H. Zola, Modulation of the immune response to transplantation antigens. I. The effects of immunization using adjuvants on the immune response of mice to a tumor allograft. *Clin. exp. Immunol.* **11,** 585 (1972).
14. S. C. Bansal and H. O. Sjögren, Effects of BCG on various facets of the immune response against polyoma tumors in rats. *Int. J. Cancer* **11,** 162 (1973).
15. D. O. Chee and A. J. Bodurtha, Facilitation and inhibition of B16 melanoma by BCG *in vivo* and by lymphoid cells from BCG-treated mice *in vitro*. *Int. J. Cancer* **14,** 137 (1974).
16. W. Boyle, An extension of the 51Cr-release assay for the estimation of mouse cytotoxins. *Transplantation* **6,** 761 (1968).
17. K. T. Brunner, J. Mauel, J. C. Cerottini and B. Chapius, Quantitative assay of the lytic action of immune lymphoid cells on 51Cr-labelled allogeneic target cells *in vitro*; inhibition by isoantibody and by drugs. *Immunology* **14,** 181 (1968).
18. A. R. Sanderson, Cytotoxic reactions of mouse isoantisera: preliminary considerations. *Brit. J. exp. Path.* **45,** 398 (1964).

19. A. Cohen and M. Schlesinger, Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. *Transplantation* **10,** 130 (1970).
20. N. K. Jerne and A. A. Nordin, Plaque formation in agar by single antibody-producing cells. *Science* **140,** 405 (1963).
21. G. Berke and D. B. Amos, Cytotoxic lymphocytes in the absence of detectable antibody. *Nature New Biol.* **242,** 237 (1973).
22. M. Hayami, I. Hellström, K. E. Hellström and K. Yamanouchi, Cell-mediated destruction of Rous sarcomas in Japanese quails. *Int. J. Cancer* **10,** 507 (1972).
23. J. C. Leclerc, E. Gomard and J. P. Levy, Cell-mediated reaction against tumors induced by oncornaviruses. I. Kinetics and specificity of the immune response in Murine Sarcoma Virus (MSV)-induced tumors and transplanted lymphomas. *Int. J. Cancer* **10,** 589 (1972).
24. J.-C. Cerottini, A. A. Nordin and K. T. Brunner, *In vitro* cytotoxic activity of thymus cells sensitized to alloantigens. *Nature (Lond.)* **227,** 72 (1970).
25. J.-C. Cerottini, A. A. Nordin and K. T. Brunner, Specific *in vitro* cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. *Nature (Lond.)* **228,** 1308 (1970).
26. P. Goldstein, H. Wigzell, H. Blomgren and E. A. J. Svedmyr, Autonomy of thymus-processed lymphocytes (T cells) for their education into cytotoxic cells. *Europ. J. Immunol.* **2,** 498 (1972).
27. S. Konda, R. T. Smith, The effects of tumor bearing upon changes in cell distribution and membrane antigen characteristics in murine spleen and thymus cell sub-populations. *Cancer Res.* **33,** 1878 (1973).
28. R. Kiessling and E. Klein, Cytotoxic potential of mouse spleen cells on H-2 antibody-treated target cells. *J. exp. Med.* **137,** 527 (1973).
29. P. Perlmann, H. Perlmann and H. Wigzell, Lymphocyte mediated cytotoxicity *in vitro*. Induction and inhibition by humoral antibody and nature of effector cells. *Transplant. Rev.* **13,** 91 (1972).
30. C. Basham and G. A. Currie, Development of specific cell-dependent antibody during growth of a syngeneric rat sarcoma. *Brit. J. Cancer* **29,** 189 (1974).
31. T. G. Canty and J. R. Wunderlich, Quantitative *in vitro* assay of cytotoxic cellular immunity. *J. nat. Cancer Inst.* **45,** 761 (1970).
32. A. Mantovani and F. Spreafico, On the nature of blocking factors and their lymphoid target cells in an allogeneic tumor system. *Europ. J. Cancer.* In press (1975).
33. G. A. Currie and C. Basham, Serum-mediated inhibition of the immunological reactions of the patient to his own tumour: A possible role for circulating antigen. *Brit. J. Cancer* **26,** 427 (1972).
34. J. Ankerst and N. Jonsson, Inhibitory effects of BCG on adenovirus tumorigenesis: Dependence on administration schedule. *Int. J. Cancer* **10,** 351 (1972).
35. S. C. Bansal and H. O. Sjögren, Regression of polyoma tumor metastasis by combined unblocking and BCG treatment-correlation with induced alterations in tumor immunity status. *Int. J. Cancer* **12,** 179 (1973).

Europ. J. Cancer Vol. 11, pp. 545–553. Pergamon Press 1975. Printed in Great Britain

Methotrexate Resistance and Uptake of DDMP by L5178Y Cells

Selective Protection with Folinic Acid*

BRIDGET T. HILL†‡, L. A. PRICE†§ and J. H. GOLDIE
with the technical assistance of S. I. HARRISON
The Department of Medicine, St. Michael's Hospital, and the University of Toronto, Toronto, Canada

Abstract—*The uptake and distribution of 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine (DDMP) by methotrexate (MTX)-sensitive and MTX-resistant L5178Y lymphoblasts has been studied. In both cell lines, DDMP entered by a process which was temperature-dependent and most of the drug remained associated with the trichloracetic acid-soluble fraction. A higher drug concentration was achieved in the MTX-sensitive than in the MTX-resistant cells. Kinetic studies showed that the V_{max} values differed by a factor of approximately 2 (being lower in the resistant cells), although the K_m for DDMP uptake was comparable in both cell lines.*

Pyrimethamine, DDEP, MTX or folic acid did not compete for the uptake of DDMP into either cell type. However, the extent of influx of DDMP into the MTX-sensitive cells was markedly reduced by the simultaneous addition of equimolar folinic acid. In contrast, this effect was not seen with the MTX-resistant cells, where uptake was unaffected by the presence of folinic acid. This finding suggests that a combination of DDMP with folinic acid could result in a differential protection, whereby resistant cells would still be killed. This concept may be exploited clinically.

INTRODUCTION

THE ANTITUMOUR activity of a series of diaminopyrimidines has been tested and demonstrated in a number of experimental animal systems *in vivo* [1–3], and *in vitro* [4, 5], and more recently in man [6–8]. One of these derivatives, DDMP ((2,4-diamino-5-(3',4'-dichlorophenyl) - 6 - methylpyrimidine), BW 50197), has proved superior in inhibiting growth in certain cells resistant to MTX both in animal solid tumours (Walker 256) [9] and in tissue culture (L5178Y lymphoblasts) [5].

Resistance to anticancer agents has frequently been attributed to alterations in membrane permeability, both in animal experimental systems [10–12] and in man [13–15]. The MTX-resistant L5178Y lymphoblasts used in our initial experiments were derived from a cell line in which resistance was associated with impaired MTX influx [10]. Therefore, since human tumours exist which are resistant to MTX by virtue of a transport defect [14, 16], DDMP may be the preferable drug for treatment of these neoplasms. On-going clinical trials show that DDMP has antitumour effects in man [17].

Preliminary experiments [18] have shown that DDMP appears to be transported into

Accepted 15 May 1975.
*J.H.G. wishes to acknowledge the support of the Ontario Cancer and Treatment Research Foundation, Canada.
†Visiting Associates.
‡Present address to which correspondence should be directed: Department of Cellular Pathology, Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.
§Present address: The Division of Medicine, Royal Marsden Hospital and the Institute of Cancer Research, London SW3 6JJ, U.K.

L5178Y mouse lymphoblasts (an MTX-sensitive line) by an active transport process. Therefore, we have extended these studies to determine whether the superiority of DDMP over MTX, in this experimental system, is related to the efficiency with which DDMP is taken up by these MTX-sensitive and MTX-resistant cell lines.

MATERIAL AND METHODS

Chemicals and reagents

Dr. C. A. Nichol, The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, N.C. provided pyrimethamine (PRM), DDMP and DDEP ((2,4-diamino-5-(3′,4′-dichlorophenyl)-6-ethyl pyrimidine), and DDMP-2-^{14}C for these studies. The radiochemical purity of DDMP-2-^{14}C (specific activity 13·8 mCi/m mole) was 98%, as checked by TLC in three systems and by isotopic dilution. Inulin-^{14}C-carboxylic acid (specific activity 8·6 mCi/m mole) was obtained from the Radiochemical Center, Amersham, England, and MTX and folinic acid (CF) as calcium leucovorin from Lederle Products Ltd., Montreal, Canada. Other chemicals were purchased from Fisher Scientific Co. Ltd., Don Mills, Ontario, Canada.

Cell cultures

Two sublines of L5178Y lymphoblasts were grown in suspension culture in Fisher's medium for leukemic cells of mice (Grand Island Biological Supplies Ltd., N.Y.) supplemented with 10% fetal calf serum (Flow Laboratories Ltd., Maryland), as described previously [4–5]. The growth of the MTX-sensitive line was inhibited by continuous exposure to 10^{-8}M MTX, and that of the MTX-resistant line by 5×10^{-6}M MTX. The MTX-resistant line was shown to transport MTX less effectively than the parent sensitive line, with results comparable to those published earlier [19].

Transport studies

Exponentially-growing L5178Y cells, at a density of approximately 2×10^5 cells per ml were removed from suspension by centrifugation at 350 *g* at room temperature for 3 min. The cells were then counted and resuspended to a final concentration of 2×10^6 per ml in Hanks balanced salt solution pH 7·4 (Grand Island Biologicals Ltd., N.Y.) and placed in a water bath at 37°. A drug concentration was selected which was without effect on the colony-forming ability of these cells following a 6 hr exposure in suspension culture. At this drug level, there was a linear relationship between uptake and cell concentration. Therefore, DDMP-2-^{14}C was added to the cell suspensions to a final drug concentration of 5×10^{-5}M (0·625 μCi/ml). At various times after drug addition, aliquots of cell suspension were removed, chilled rapidly by immersion in ice and diluted 10 times with ice-cold Hanks balanced salt solution. The cells were then removed from suspension by centrifugation at 500 *g* for 2 min, washed once with ice-cold Hanks solution, and then twice with ice-cold 0·85% isotonic NaCl. This washing procedure removed not more than 5% of the total radioactivity associated with the cell pellets. The resulting cell pellets were dissolved in 100 μl of N NaOH, and mixed with 10 ml Aquasol (NEN, Dorval, Quebec, Canada). Radioactivty was estimated using a Nuclear-Chicago Scintillation Counter Unilux II, Model No. 6853 at 63% efficiency for ^{14}C.

The distribution of DDMP-2-^{14}C between acid soluble and acid insoluble fractions was estimated under similar experimental conditions, following exposure of both cell lines to 50 μM DDMP. The cell pellets resulting after centrifuging aliquots of cell suspension were extracted in 2×2 ml volumes of 10% trichloracetic acid (TCA) and left to stand at 4° for 20 min before centrifugation to provide TCA-soluble and TCA-insoluble fractions. The pellets representing the TCA-insoluble fractions were finally dissolved in 1N NaOH. The radioactivity associated with aliquots of each cell extract were then measured by scintillation counting.

For measurement of drug uptake at 4°, the cell suspensions were pre-cooled in ice for 15 min before the drug was added.

The rate of efflux of DDMP from the cells at 37° was determined by incubating cells with the drug, using the conditions described above, for 30 min. The cells were then centrifuged at 350 *g* for 2 min at room temperature and resuspended to the same volume in fresh Hanks solution without drug at 37°. Aliquots were removed at subsequent intervals, as described above, in order to determine their radioactivity content.

The intracellular water of both cell types was estimated as the difference between the total water content of the cell pellet and the extracellular water, as determined by incubation with inulin-^{14}C, according to the method of Goldman [20].

RESULTS

Influx of DDMP

The rates of uptake of DDMP-2-^{14}C by MTX-sensitive and MTX-resistant cells are shown in Fig. 1. At 37°, both the early rate (between 1 and 5 min incubation), and the final extent of drug uptake (after 30 min incubation) is greater by the MTX-sensitive than by the MTX-resistant cells. The pattern of drug influx into both cell types is characterised by an early rapid association of the drug with the cells, followed by a period during which the concentration of drug within the cell increases linearly as a function of time, until a steady-state concentration is achieved after 30 min incubation. The intracellular drug

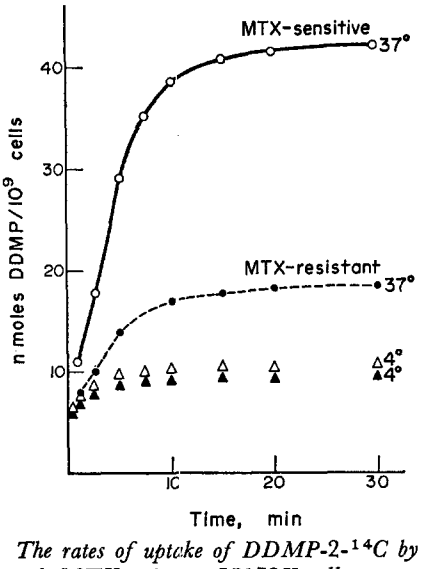


Fig. 1. The rates of uptake of DDMP-2-^{14}C by MTX-sensitive and MTX-resistant L5178Y cells expressed in n moles of drug per 10^9 cells as a function of time (min) of incubation at 37° or 4° following exposure to 5×10^{-5}M drug. Each figure represents the mean of 8 determinations. Overall scatter not in excess of 10%.

concentrations achieved at these plateau levels of the graphs after 30 min incubation at 37° in the presence of 5×10^{-5}M drug are $68 \pm 9\%$ μM and $44 \pm 7\%$ μM for MTX-sensitive and MTX-resistant cells respectively. These values were calculated using measurements of the intracellular free space determined as described above. The intracellular water content was $6{\cdot}1 \pm 3\%$ and $5{\cdot}0 \pm 8\% \times 10^{-7}$ ml/cell for the sensitive and resistant cells respectively. MTX-resistant L5178Y cells have been shown previously to have a smaller cellular volume than MTX-sensitive cells, however, the intracellular concentration of DDMP is still greater in the sensitive line (approximately 150% of the value for resistant cells). Under these experimental conditions, there was no evidence that the drug was accumulated against a concentration gradient by these cells.

The effect of temperature on drug uptake is also shown in Fig. 1. Uptake of DDMP is dependent on temperature, being depressed at the lower temperature, after a 30 min incubation, to approximately 25% and 50% of those values achieved at 37°C in MTX-sensitive and MTX-resistant cells respectively. However, even at this low temperature (4°) there is still a considerable quantity of DDMP, which associated with the cells and which does not increase markedly with time. This rapidly associating fraction of drug, which is seen at both temperatures, may represent non-specific binding to the cell surface of within the cell membrane, which has been shown in other studies using folate analogues [21, 22]. However, to confirm this would require more precise evaluation of the association of the drug with the cells a few seconds after exposure to DDMP. The fact that this fraction represents a substantial amount of DDMP uptake is probably related to the highly lipophylic nature of the drug.

Table 1 shows that at both temperatures the majority of the intracellular drug remains associated with the TCA-soluble fraction. Approximately 25% of the total drug taken up by the two cell lines is associated with the TCA-insoluble fraction at both temperatures, indicating that this proportion may be bound to cell macromolecules.

Efflux of DDMP

L5178Y lymphoblasts were preloaded with DDMP by incubation for 30 min at 37° with drug at 5×10^{-5}M. The cells were then centrifuged, washed and resuspended in drug-free medium at 37°. Aliquots of cell suspension were removed at the times shown in Fig. 2, which illustrates the rate and extent of efflux of DDMP from these two cell types.

There is a rapid efflux of DDMP from both MTX-sensitive and MTX-resistant cells. The final amount of drug associated with the cells is slightly higher (20%) in the sensitive than the resistant cells, but when corrections are made for their differing cellular volumes, similar intracellular drug concentrations result after efflux. This level of drug remaining associated with the cells after efflux is comparable to that amount which is associated with the cells within 1 min of drug addition at 37° and after a 10 min incubation at 4° (see Fig. 1).

Table 1. *Distribution of DDMP-2-^{14}C following a* 30 *min exposure of L*5178*Y cells to a* 5×10^{-5}*M drug concentration*[a]

Temperature of Incubation	n moles per 10^9 cells					
	Sensitive L5178Y cells			Resistant L5178Y cells		
	Total	TCA - insoluble	TCA - soluble	Total	TCA - insoluble	TCA - soluble
4°	10.7±6%	3.1±4%	7.6±6%	9.3±7%	2.6±11%	6.7±10%
37°	42.1±4%	10.9±5%	31.1±5%	18.2±7%	5.3±8%	12.9±8%

[a]Distribution of radioactivity between TCA-soluble and TCA-insoluble cell extracts following drug exposure was determined as described in Materials and Methods section, using 2×10^6 cells per assay.
Each value represents the mean of at least 6 determinations.

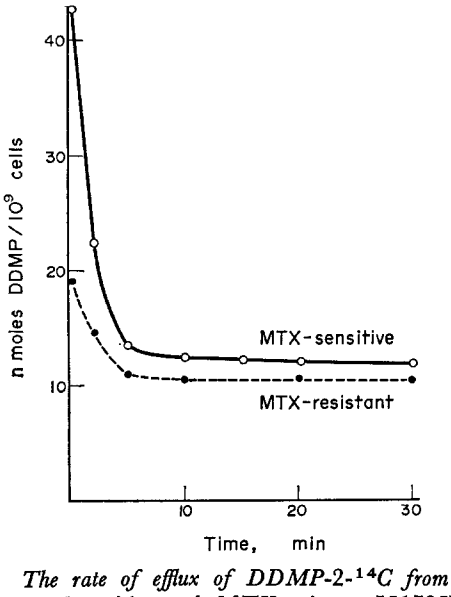


Fig. 2. *The rate of efflux of DDMP-2-^{14}C from 'pre-loaded' MTX-sensitive and MTX-resistant L*5178*Y cells expressed in n moles of drug per* 10^9 *cells as a function of time of re-incubation in drug-free medium at* 37°. *Each figure represents the mean of* 6 *determinations. Overall scatter not in excess of* 15%.

Kinetics of drug uptake

For these kinetic studies involving determinations of the initial velocity of drug uptake, the uptake velocity was determined from the difference between drug associated with the cells after 2 min vs 5 min incubation at 37°, when the rate of influx approximated to linearity (see Fig. 1). Uptake of DDMP by MTX-sensitive and MTX-resistant L5178Y lymphoblasts approximated to Michaelis–Menten kinetics for drug concentrations from 10^{-5}to10^{-4}M. However, deviation from simple Michaelis–Menten kinetics was observed on exposure of the cells to extracellular drug concentrations in excess of 10^{-4}M. The results are shown in Fig. 3.

The data from the curves in Fig. 3(*b*), which are clearly biphasic, would be consistent with at least two uptake mechanisms for DDMP. One, a facilitated (saturable) transport process and the second a "first-order" component. The latter is most probably passive diffusion,

Table 2. *Kinetic data for the uptake of DDMP by L*5178*Y lymphoblasts*

Cell line	Values for low drug concentrations[a] (less than 10^{-4}M)		Recalculated values for low drug concentrations[b]		
	K_m[c]	V_{max}[d]	K_m[c]	V_{max}[d]	First order rate constant[e]
MTX-resistant	0.13±5%	11.6±4%	0.087±16%	3.8±7%	38.7±6%
MTX-sensitive	0.13±8%	18.4±6%	0.084±18%	8.9±8%	51.2±5%

[a]The data was derived by calculation of enzyme Michaelis–Menten parameters with standard errors by the statistical method of Wilkinson [23].
[b]The data was derived using the method of Hoare [24], taking into account that at high drug concentrations (in excess of 10^{-4}M) there is a Michaelis-type saturable transport system *plus* a first order mechanism.
[c]K_m is expressed as mM concentration.
[d]V_{max} is expressed in n moles per min per 10^9 cells.
[e]First order rate constant is expressed in n moles per min per 10^9 cells per mM concentration.

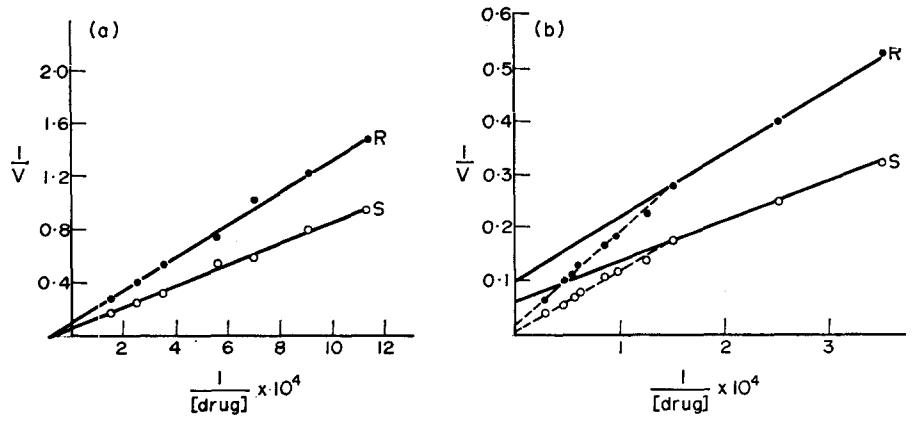


Fig. 3. Uptake of DDMP-2-^{14}C by MTX-sensitive and MTX-resistant L5178Y cells. The uptake velocity was determined from the difference between the amount of drug associated with the cells after 2 min vs 5 min incubation at 37° for both cell types. Lineweaver–Burk plots of the data with ordinate expressing the reciprocal uptake velocity in 1/(n moles/min/10^9 cells), and abscissa expressing the reciprocal substrate concentration as 1/(DDMP-2-^{14}C × 10^{-4}M).

(a) Represents values obtained over the DDMP concentration range of $10^{-5} - 10^{-4}$M.

(b) Represents values obtained over the DDMP concentration range of $10^{-3} - 10^{-4}$M.

or it could be another facilitated pathway with a very high K_m. Using the data from Fig. 3(*a*), K_m and V_{max} values were calculated by the statistical method of Wilkinson [23] for low drug concentrations only (less than 10^{-4}M). These kinetic parameters were then recalculated, using the method of Hoare [24] which was specifically designed for transport studies, to take account of the two mechanisms involved at the higher drug concentrations (in excess of 10^{-4}M). The K_m's calculated in this manner (see Table 2) are comparable for both cell lines, whilst the V_{max} for sensitive cells is 2·3 times the value for resistant cells.

Table 3. The effect of various folate derivatives on the intracellular level of DDMP by MTX-sensitive and MTX-resistant L5178Y cells[a]

Added folate compound	Concentration (10^{-5}M)	Extent of uptake (%)[b] MTX-sensitive cells	MTX-resistant cells
PRM	5	98±3%	100±2%
DDEP	5	99±6%	98±3%
MTX	5	89±6%	91±6%
Folic Acid	5	98±2%	98±4%
	10	97±1%	95±5%
CF	0.5	78±5%	100±4%
	1	60±1%	100±2%
	2.5	48±2%	97±1%
	5	26±3%	87±5%
	10	-[c]	72±5%

[a]Incubations were carried out in Hanks balanced salt solution, using 2×10^6 cells/ml and 5×10^{-5}M DDMP as described in Materials and Methods section, for 30 min at 37°.

[b]The extent of uptake in the absence of added folate compound = 100%.

[c]Not estimated.

Each value represents the mean of at least 6 estimations.

Effect of adding various folate derivatives and other diaminopyrimidines on the uptake of DDMP

The effect of equimolar unlabelled compounds on the level of DDMP-2-^{14}C associated with the cells following incubation at 37° for 30 min (by which time the "steady-state" level has been reached, see Fig. 1) was determined. The results in Table 3 show that the presence of equimolar PRM or DDEP do not influence the level of DDMP achieved in either MTX-sensitive or -resistant cells under these conditions. MTX (5×10^{-5}M) causes a very slight reduction in drug influx into both cell types, but competition between the two drugs at these drug concentrations is not significant. Similarly equimolar CF and folic acid do not markedly reduce the uptake of DDMP into MTX-resistant cells, neither does folic acid have any effect on uptake by the sensitive cells. However, the addition of folinic acid does markedly reduce the uptake of DDMP into MTX-sensitive cells and confirms our earlier preliminary data [18]. This significant difference in the influence of CF on DDMP influx into these two cell types was studied in more detail (see Fig. 4). The addition of CF ($2{\cdot}5 \times 10^{-5}$M) has little effect on the amount of drug associated with the cells within 1 min of

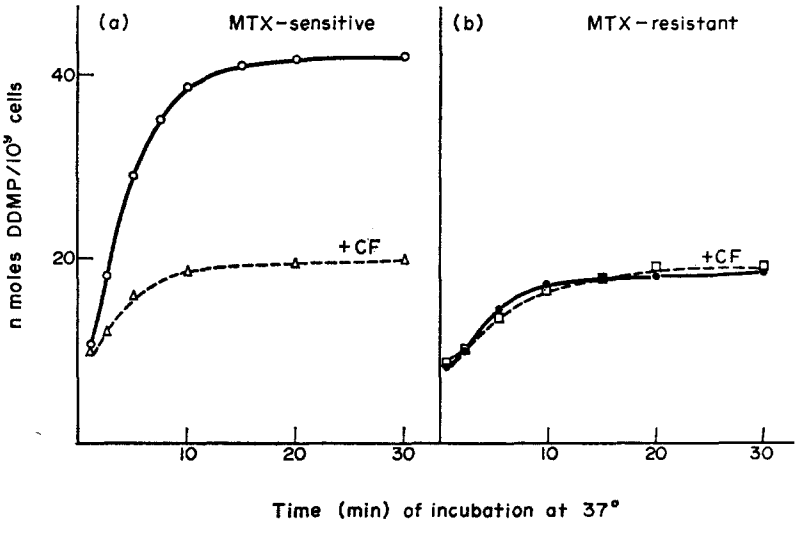


Fig. 4. The influence of simultaneous addition of CF ($2{\cdot}5 \times 10^{-5}M$) with DDMP ($5 \times 10^{-5}M$) on the rate and extent of uptake of DDMP-2-^{14}C into MTX-resistant and MTX-sensitive cells expressed in n moles of drug per 10^9 cells as a function of time of incubation at 37°. Each figure represents the mean of 6 determinations. Overall scatter not in excess of 10%.

drug addition to the cell suspension. However, the rate of uptake (between 1 and 5 min incubation) and the final extent of DDMP uptake by the sensitive cells in the presence of CF is reduced to approximately 48% of the value achieved in the absence of added CF. This is in contrast to the negligible effect that added CF has on the rate or extent of DDMP uptake by MTX-resistant cells.

DISCUSSION

These results show that DDMP enters MTX-sensitive and MTX-resistant L5178Y cells readily. Drug uptake, in both cell lines, is temperature dependent (being reduced at 4°). Since passive diffusion ordinarily exhibits little temperature dependence, this could be indicative of uptake which is at least partially by a carrier-mediated process. Most of the DDMP is associated with the TCA-soluble fraction (after a 30 min incubation at 37°) with only approximately 30% being associated with the TCA-insoluble fraction. Uptake against a concentration gradient was not demonstrated. However, the rate of uptake during 1–5 min of incubation and the final extent of influx of the drug is greater in MTX-sensitive cells compared with the resistant line. The MTX-resistant cells used in the study are resistant by virtue of impaired transport of MTX [10, 19]. These results show, however, that they would also have a reduced capacity to take up DDMP. Even so, they are not resistant to DDMP, since their viability can be reduced markedly by exposure to the drug [5]. Therefore, even when uptake is reduced, sufficient drug must enter the resistant cells to produce cytotoxic concentrations.

These findings show that the extent of reduced uptake of DDMP reported here does not appear sufficient to impair the cytoxicity of the drug. Therefore, under these conditions the ability of a drug to kill cells cannot necessarily be equated with the initial ability of the target cell to take up the compound. This observation would be in agreement with previous studies which have shown that the rate of influx alone is probably an inadequate parameter for evaluating possible therapeutic efficacy [11, 21, 22, 25].

Efflux of DDMP from cells "pre-loaded" to a "steady-state" drug concentration was rapid and comparable in both cell lines. Under these conditions the addition of equimolar CF to the extracellular compartment had no effect on the flow of DDMP out of the cell, (unpublished observations, B. T. Hill, L. A. Price and J. H. Goldie).

This rapid influx and efflux of DDMP using these L5178Y cells differed from results obtained studying the transport of some benzylaminobenzoyl-aspartate diaminopyrimidine analogues into L1210 cells, where influx was slow and efflux rapid so that only low intracellular drug levels were achieved [22], therefore perhaps serving to illustrate the wide stereochemical specificity of these different pyrimidines.

The K_m for uptake of DDMP was comparable in both cell lines, however the V_{max} in the resistant cells was approximately half that for

the sensitive cells. The kinetic studies over two dose ranges showed that Michaelis–Menten kinetics are obeyed at lower drug concentrations, but deviations from this relationship are observed at drug concentrations in excess of 10^{-4}M. It is possible that membrane-permeability becomes rate-limiting at higher DDMP concentrations. However, this data is also consistent with the suggestion that at least two mechanisms are involved in the uptake of DDMP by L5178Y lymphoblasts, i.e. facilitated diffusion and a nonsaturable component which may or may not reflect passive diffusion. At low drug concentrations facilitated diffusion apparently predominates, whereas at high drug concentrations the facilitated pathway appears saturated and the nonsaturable component increases. These theoretical mechanisms of membrane transport have been recently reviewed by Goldman [11]. This finding may be relevant to the previous report that DDMP was most effective in reducing the viability of MTX-resistant L5178Y cells growing in culture when high drug concentrations were maintained over an extended period of time [5].

The uptake of DDMP by both sensitive and resistant cell lines is not affected by the presence of equimolar concentrations of other diaminopyrimidines (e.g. PRM or DDEP), or MTX or folic acid. This implies that DDMP enters the cells by different routes from these compounds. This is in agreement with the previous observations that cells which are relatively insensitive to MTX are sensitive to DDMP [5, 9]. In this study, however, we have mainly considered equimolar concentrations of DDMP and inhibitors at levels about half the K_m when the transport system would be at a low level of saturation and inhibition might not be measurable. Therefore, this aspect clearly requires further study so as to determine the effects of higher concentrations of inhibitors on DDMP transport. However, the fact that the addition of equimolar folinic acid considerably reduces the uptake of DDMP in the sensitive cells, suggests that these agents may either share a common transport site or bind to the same intracellular sites. In an attempt to distinguish between these two possibilities, further information is needed about the transport of folinic acid itself. The possible existence of different transport systems for folate and its derivatives into cells has been proposed by other workers. They have demonstrated the relative ineffectiveness of 5-formyl-tetrahydrofolate in displacing folate from tissues [26], and have shown that folic acid did not inhibit uptake of 5-methyltetrahydrofolate [27], although at high levels folic acid had an effect on 5-formyltetrahydrofolate transport [28]. However, other evidence has been presented for the involvement of a single carrier for influx and efflux [21, 22, 25, 29, 30] for all the folate analogues. But, as Goldman [21] pointed out, a single carrier system may in reality be a composite of more than one mechanism, which are indistinguishable by the sensitivity of the currently available techniques. Our data would be consistent with the latter suggestions since in the sensitive cells the presence of folinic acid reduces DDMP uptake, but MTX has no effect. It is possible therefore, that the nature of the composite parts of the transport mechanism may differ in sensitive and resistant cells.

This finding, that the presence of equimolar amounts of folinic acid reduces the uptake of DDMP into sensitive cells but not into resistant cells may be of considerable clinical significance. It has been shown that the simultaneous addition of equimolar folinic acid with DDMP to cells growing in suspension culture prevented the reduction in cell viability in the MTX-sensitive cells seen with DDMP alone. This "protection" by folinic acid was not seen with the MTX-resistant cells (unpublished observations, B. T. Hill, L. A. Price and J. H. Goldie). Similar results have been obtained earlier using PRM in this same experimental system [31]. This suggests that while MTX-sensitive cells could be protected from DDMP by using folinic acid simultaneously, the entry of DDMP into MTX-resistant cells would be unaffected. Thus, there is a definite possibility, at least in theory, that in a mixed population of cells a "selective protection or rescue" might be achieved whereby MTX-sensitive cells are protected from DDMP by folinic acid, whereas its tumouricidal effect on resistant cells would continue. Further experimental work with this model system is underway and the clinical application of this concept is being studied. Preliminary clinical studies suggest that this concept of differential rescue can be applied successfully to human cancer chemotherapy using DDMP [17].

Acknowledgements—The authors wish to thank Dr. R. C. Jackson for his extremely valuable help in advising on and carrying out the statistical evaluation of their kinetic data. The helpful comments and criticisms of Dr. I. D. Goldman, during the preparation of the manuscript, are gratefully acknowledged.

REFERENCES

1. J. H. Burchenal, S. K. Goetchius, C. C. Stock and G. H. Hitchings, Diaminodichlorophenylpyrimidines in mouse leukemia. *Cancer Res.* **12,** 251 (1952).
2. D. A. Clarke, S. M. Buckley, S. S. Sternberg, C. C. Stock, C. P. Rhoades and G. H. Hitchings, Effects of 2,4-diaminopyrimidines on mouse sarcoma 180. *Cancer Res.* **12,** 255 (1952).
3. K. Sugiura, Effects of various compounds on the Ehrlich ascites carcinoma. *Cancer Res.* **13,** 431 (1953).
4. J. H. Goldie, M. E. Furness and L. A. Price, Comparison of the effects of methotrexate and pyrimethamine on L5178Y lymphoblasts in culture. *Europ. J. Cancer* **9,** 709 (1973).
5. B. T. Hill, J. H. Goldie and L. A. Price, Studies concerned with overcoming resistance to methotrexate: A compatison of the effects of methotrexate and 2,4-diamino-5-(3′4′-dichlorophenyl)-6-methylpryrimidine (BW 50197) on the colony forming ability of L5178Y cells. *Brit. J. Cancer* **28,** 263 (1973).
6. G. F. Geils, C. W. Scott, Jr., C. M. Baugh and C. E. Butterworth, Treatment of meningeal leukemia with pyrimethamine. *Blood* **38,** 131 (1971).
7. L. M. Murphy, R. R. Ellison, D. A. Karnofsky and J. H. Burchenal, Clinical effects of dichloro- and monochlorophenyl analogues of diaminopyrimidines: Antagonists of folic acid. *J. clin. Invest.* **33,** 1388 (1954).
8. D. R. Stickney, W. S. Simmons, R. L. Deangelis, R. W. Rundles and C. A. Nichol, Pharmacokinetics of pyrimethamine (PRM) and 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DMP) relevant to meningeal leukemia. *Proc. Amer. Ass. Cancer Res.* **14,** 52 (1973).
9. C. A. Nichol, Studies on dihydrofolate reductase related to the drug sensitivity of microbial and neoplastic cells. *Advanc. Enzyme Reg.* **6,** 305 (1968).
10. G. A. Fischer, Defective transport of amethopterin (methotrexate) as a mechanism of resistance of the anti-metabolite in L5178Y leukemia cells. *Biochem. Pharmacol.* **11,** 1233 (1960).
11. I. D. Goldman, Uptake of drugs and resistance. In *Drug Resistance and Selectivity*. Edited by E. Mihich, p. 299. Academic Press, New York and London (1973).
12. G. P. Wheeler, Studies related to mechanisms of resistance to biological alkylating agents. *Cancer Res.* **23,** 1334 (1963).
13. T. C. Hall, *Predictions of Response in Cancer Therapy*. Edited by T. C. Hall, p. 140. U.S. Government Printing Office Washington, D.C. (1971).
14. D. Kessel, T. C. Hall and D. Roberts, Modes of uptake of methotrexate by normal and leukemic human leucocytes *in vitro* and their relation to drug response. *Cancer Res.* **28,** 564 (1968).
15. M. Lane, Clinical resistance to cancer chemotherapy. In *Drug Resistance and Selectivity*. Edited by E. Mihich, p. 453. Academic Press, New York and London (1973).
16. J. R. Bertino, Current studies of the folate antagonists in patients with acute leukemia, *Cancer Res.* **25,** 1614 (1965).
17. L. A. Price, J. H. Goldie and Bridget T. Hill, Methodichlorophen as an antitumour drug: Preliminary report, *Brit. Med. J.* **2,** 20 (1975).
18. Bridget T. Hill, L. A. Price, S. Harrison and J. H. Goldie, Studies on the transport and distribution of diaminopyrimidines in L5178Y lymphoblasts in cell culture. *Biochem. Pharmacol.* **24,** 535 (1975).
19. K. R. Harrap, Bridget T. Hill, M. E. Furness and L. I. Hart, Sites of action of amethopterin: Intrisic and acquired drug resistance. *Ann. N.Y. Acad. Sci.* **186,** 312 (1971).
20. I. D. Goldman, Transport energetics of the folic acid analogue, methotrexate, in L1210 leukemia cells. *J. biol. Chem.* **244,** 3779 (1969).
21. I. D. Goldman, The characteristics of the membrane transport of amethopterin and the naturally occurring folates. *Ann. N.Y. Acad. Sci.* **188,** 400 (1971).
22. F. M. Sirotnak and R. C. Donsbach, Stereochemical characteristics of the folate-antifolate transport mechanism in L1210 leukemia cells. *Cancer Res.* **34,** 371 (1974).
23. G. N. Wilkinson, Statistical estimations in enzyme kinetics. *Biochem. J.* **80,** 324 (1961).

24. D. G. Hoare, A curve-fitting procedure utilizing a small computer for equations containing three or four unknown constants. *Ann. Biochem.* **46,** 604 (1972).
25. F. M. Sirotnak and R. C. Donsbach, Comparative studies on the transport of aminopterin, methotrexate and methasquin by the L1210 leukemia cell. *Cancer Res.* **32,** 2120 (1972).
26. D. G. Johns and I. H. Plenderleith, Folic acid displacement in man. *Biochem. Pharmacol.* **12,** 1071 (1963).
27. A. Nahas, P. F. Nixon and J. R. Bertino, Transport of 5-methyltetrahydrofolate by L1210 mouse leukemia cells. *Fed. Proc.* **28,** 389 (1969).
28. A. Nahas, P. F. Nixon and J. R. Bertino, Uptake and metabolism of N^5-Formyl-tetrahydrofolate by L1210 leukemia cells. *Cancer Res.* **32,** 1416 (1972).
29. I. D. Goldman, N. S. Lichtenstein and V. T. Oliverio, Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. *J. biol. Chem.* **243,** 5004 (1968).
30. D. Kessel and T. C. Hall, Amethopterin transport in Ehrlich ascites carcinoma and L1210 cells, *Cancer Res.* **27,** 1539 (1967).
31. J. H. Goldie, S. I. Harrison, L. A. Price and Bridget T. Hill, Impaired responsiveness to folinic acid protection in methotrexate-resistant L5178Y cells. *Europ. J. Cancer.* In press (1975).

Europ. J. Cancer Vol. 11, pp. 555–563. Pergamon Press 1975. Printed in Great Britain

Characterization of the Immunostimulants Levamisole and Tetramisole

FEDERICO SPREAFICO*, ANNUNCIATA VECCHI, ALBERTO MANTOVANI, ANDREINA POGGI, GIUSEPPE FRANCHI, ALESSANDRO ANACLERIO and SILVIO GARATTINI

Istituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea 62, 20157 *Milan, Italy.*

Abstract—*The immunostimulatory activity of tetramisole and its levo isomer levamisole, has been investigated in tumorous and non-tumorous experimental animals. These compounds were shown to raise the anti-SRBC response in normal and aged mice and to increase the incidence, severity and duration of allergic encephalomyelitis in rats.*

Treatments with levamisole were capable of increasing survival and inducing cures in L1210 leukemia-bearing mice when administered in conjunction with irradiated tumor cells. Increased anti-leukemia resistance to subsequent tumor challenges were also observed in animals primed with inactivated tumor cells and injected with levamisole. Single doses of these chemicals could also reduce the number of lung metastases in Lewis lung carcinoma bearing C57B1/6 mice whereas significant life-span prolongation and cures were seen in this system when these agents were employed in a combined chemo-immunotherapeutic approach.

INTRODUCTION

AT THE present time, clinical immunotherapeutical approaches to cancer rely heavily on the use of non-specific immunostimulants such as Bacillus Calmette–Guerin (BCG) and *Corynebacterium parvum*. Although proven of value in a variety of experimental and human neoplastic conditions [1–3], these agents have also been shown to possess a number of disadvantages, some of which are directly related to their bacterial nature, such as the difficulties in standardization of the material and the possibility of infectious complications [4, 5]. It is also obvious that for a more effective treatment, a better knowledge of the mechanisms of action of immunomodulators would be desirable, a goal which is not facilitated by the complexity of available materials. The need, therefore, exists for a larger number of compounds with defined chemical structures displaying this capacity. The aim of the present report is to present our experience in the characterization of the immunostimulants tetramisole and levamisole, the racemic and levo isomer of 2,3,5,6-tetra-hydro-6-phenylimidazo (2,1-b)thiazole. These compounds, in use for a number of years for their broad and potent anti-helmintic activity [6–8], have been shown recently to stimulate immunological responsiveness to a variety of experimental [9–14] and clinical stimuli [15–18]. Considering also that these compounds appear to have a very low direct toxicity, it was of interest to further investigate their immunostimulatory activity in experimental tumoral and non-tumoral conditions.

Accepted 25 April 1975.

*Reprint requests to: Federico Spreafico, Chief, Laboratory of Tumor Immunology and Chemotherapy, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy.

MATERIAL AND METHODS

Animals

Mice of both sexes of the following strains: CD_1, C57Bl/6, DBA/2, (DBA/2 × Balb/c)F_1 (hereafter referred to as CDF_1) were obtained from Charles River Co., U.S.A. At the time of use these animals were 2–3 months old. When the term "old mice' is used it refers to C57Bl/6 mice more than 1 year of age when used. Female Wistar–Lewis rats, 200 ± 25 g body weight (Charles River, France, S.A., Elbeuf, France) were employed for the allergic encephalomyelitis studies.

Drugs

Tetramisole (Tetra) and levamisole (Leva) were kindly supplied by Dr. J. Brugmans, Janssen Pharmaceutica, Beerse, Belgium. The drugs were dissolved in saline immediately prior to use. Methyl-CCNU (Me-CCNU; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso, NSC-95441), obtained through the courtesy of Dr. H. B. Wood, Jr., NCI, NIH, Bethesda, Maryland, USA, was prepared as described by Chirigos, *et al.* [14]. Cyclophosphamide (Cyclo-P, 2H-1,3,2-oxaza-phosphorine, 2-[bis(2-chloroethyl)-amino] tetrahydro-2-oxide, monohydrate; NSC-26271) was dissolved fresh in buffered saline. Pertussis vaccine was purchased from the Istituto Sieroterapico Milanese, Milan.

Tumors

The L1210 leukemia was maintained by weekly i.p. passages in DBA/2 mice, whereas for experimental tests, the tumor was inoculated in CDF_1 hosts. Viability of ascites cells was assessed microscopically by Trypan blue dye exclusion and only preparations showing over 95% viability were accepted. Tumor vaccination was performed by injecting i.p. 2×10^7 X-irradiated (5000 R) L1210 leukemia cells into CDF_1 mice either 14 days before or 24 hr after inoculation of viable leukemia cells.

The Lewis lung carcinoma (3LL) was maintained by transplantation of 2×10^5 cells in isogeneic C57Bl/6 mice; test animals were transplanted i.m. with the same number of tumor cells. At death, tumor weight was measured after excision, the number of lung nodules evaluated as described by Wexler [19] and their value estimated by the formula $4/3\ \pi r^3$ where r is the semiaxis of each nodule measured on a millimetric scale; the volume was then converted to weight assuming unit density. Observation time for all tumor transplanted mice was 90 days and gross autopsy analysis was carried out on all dead or sacrificed animals.

Response to sheep erythrocytes

CD_1 and old C57Bl/6 mice were injected i.p. with a standard dose of 10^8 and 4×10^8 sheep red blood cells (SRBC) respectively and the response monitored by the technique of Jerne and Nordin [20]. Results presented are geometric means after logarithmic transformation of the data [21].

Experimental allergic encephalomyelitis (EAE)

For the induction of EAE, the procedure described by Levine and Wenk [22] was followed. Rats were injected into the right hind footpad with 0·2 ml of an emulsion of equal volumes of 20% rat spinal cord homogenate in saline and Freund's complete adjuvant (Difco). At the same time, pertussis vaccine (20 billion killed microorganisms in 0·1 ml) was given in the dorsal surface. Assessment of the incidence and clinical severity of the disease was initiated on day 10 and signs were scored blindly by two independent observers and graded: 0 = normal mobility; 1+ = muscular weakness; 2+ = paralysis.

Statistical analysis

The animal data were statistically analyzed by analysis of variance.

RESULTS

Effect of Leva on L1210 leukemia

Two types of experimental conditions were used for these studies; in the first CDF_1 mice were injected i.p. with 2×10^7 irradiated L1210 cells, treatment with Leva was initiated 24 hr later (3 mg/kg i.p. per 4 consecutive days) and different inocula of viable leukemia transplanted i.p. 14 days after tumor vaccination. As shown in Table 1, which gives the mean of 2 representative experiments, when the challenge consisted of 10^7 leukemia cells, no differences in survival were noted between the control and Leva-treated groups, although the previous tumor vaccination significantly prolonged mean survival time (MST) in these animals over unprimed controls. After an inoculum of 10^6 cells, a significantly higher number of Leva-treated than control mice (12/20 v 4/20) was able to resist this tumor challenge and were long-term survivors, although no differences in MSTs were present as regards those mice eventually succumbing to the leukemia. Similar results were seen when an inoculum of 10^5 viable L1210 cells was employed; in these conditions, 40% in fact of

Table 1. *Survival in CDF_1 mice given different inocula of L1210 leukemia after vaccination with irradiated tumor cells and treatment with levamisole*

2×10^7 X-rayed L1210 cells i.p. Day 0	Levamisole (3 mg/kp i.p.) Day 1–4	No. L1210 cells i.p. Day 14	MST*	D/T†
–	–	10^7	6·3 ± 0·8	20/20
–	–	10^6	8·2 ± 1·1	20/20
–	–	10^5	10·4 ± 0·7	20/20
+	–	10^7	20·5 ± 2·9	20/20
+	+	10^7	21·2 ± 3·3	19/20
+	–	10^6	24·5 ± 2·7	16/20
+	+	10^6	26·8 ± 3·2	8/20
+	–	10^5	32·3 ± 4·0	12/20
+	+	10^5	—	2/20

*Mean survival time in days.
†Dead with leukemia over total number of mice injected.

the controls survived whereas the percentage reached 90% in the group treated with the immunostimulant. If mice primed with the inactivated L1210 leukemia and treated with Leva, were then injected with 10^5 cells of the L5178Y leukemia, no increase in survival over unprimed controls could be observed.

In another protocol, CDF_1 mice were transplanted s.c. on day 0 with 10^4 or 10^5 L1210 cells and given 2×10^7 irradiated leukemia cells i.p. 24 hr later; treatment with Leva was from day 1 to 4 at 3 mg/kg i.p. Additional groups were also given an immunosuppressive treatment with 200 mg/kg i.p. of cyclo-P on day −1. The injection of inactivated tumor cells after transplantation of 10^4 leukemia cells resulted in a significantly prolonged mean survival time (Table 2) and this increased survival was not present in hosts previously immunosuppressed with cyclo-P. In the group also treated with Leva, the majority of the animals (from 60 to 80% in various experiments) were able to completely resist this tumor inoculum. This resistance appeared to require a functional immune apparatus since in previously immunosuppressed mice, the MSTs observed were undistinguishable from those recorded for controls injected only with leukemia cells. Long term survivors obtained in these conditions after Leva treatment were capable of resisting a second challenge of 10^4 L1210 cells given 30 days after the first tumor transplantation, whereas their MSTs after inoculation of 10^4 L5178Y cells were not significantly prolonged over normal CDF_1 hosts. It may be worth noting that of the 5 such experiments performed, 4 gave the described evidence of Leva's immunostimulatory activity, whereas in 1, the same drug treatment was not followed by any significant life-prolonging activity over appropriate controls.

The injection of only 10^7 irradiated L1210 cells in mice transplanted s.c. 24 hr before with 10^4 leukemia cells also resulted (Table 3) in a significant increase in MST over controls, although lifespan prolongation was shorter

Table 2. *Survival in leukemia-bearing CDF_1 mice treated with irradiated tumor cells and levamisole*

Cyclo-P 200 mg/kg i.p. Day −1	10^4 L1210 s.c. Day 0	2×10^7 X-rayed L1210 i.p. Day 1	Leva 3 mg/kg i.p. Day 1–4	MST*	D/T†
–	+	–	–	14·1 ± 0·8	10/10
+	+	–	–	14·0 ± 1·1	10/10
–	+	+	–	24·8 ± 1·7‡	10/10
+	+	+	–	13·5 ± 1·2	10/10
–	+	+	+	27·0 ± 0·4‡	2/10
+	+	+	+	13·6 ± 1·5	10/10

*Mean survival time in days.
†Dead with leukemia over total number of mice injected.
‡$P < 0·05$.

Table 3. Survival in L1210 bearing CDF_1 mice treated with 10^7 irradiated leukemia cells and levamisole

Treatment				
Day 0	Day 1	Day 1–4	MST*	D/T†
10^4 L1210 cells s.c.	10^7 X-rayed L1210 cells i.p.	Leva 3 mg/kg i.p.	29·5 ± 2·3‡	13/16
+	+	−	18·2 ± 0·3‡	16/16
+	−	−	13·2 ± 0·6	16/16
10^5 L1210 cells s.c.	+	+	11·5 ± 0·8	16/16
+	+	−	13·0 ± 1·3	16/16
+	−	−	13·2 ± 0·7	16/16

*Mean survival time in days.
†Dead with leukemia over total number of mice injected.
‡$P < 0.05$.

than seen after treatment with 2×10^7 inactivated tumor cells. Administration of Leva appeared to be beneficial also in these conditions since a further significant lifespan prolongation was observed together with a small number of long-term survivors. On the contrary, this number of irradiated cells could not increase MST when 10^5 live leukemia cells had been transplanted and a treatment with Leva was ineffective in these conditions.

Effects of Tetra and Leva on the 3 LL tumor

It may be noted, that single or repeated injections of Tetra at 10 mg/kg i.p. given on various days after the i.m. transplantation of 2×10^5 tumor cells, did not significantly modify from control values the MST nor the primary tumor weight at death; however, in 2 experiments, significant though not very marked reductions in the number and weight of metastatic nodules in the lung were observed. As shown by experiment 2, Table 4, differences in this activity on lung metastasis could be recognized between the various treatments with Tetra employed. Similar findings in terms of decreases in lung metastasis involvement in one half of the experiments, without modifications in survival or primary tumor weight were observed when Leva was administered at 3 mg/kg i.p. in the same experimental conditions.

On the other hand, significant prolongations in survival could be obtained when Tetra was given after a treatment with MeCCNU (16 mg/kg i.p. on day 7), but variations in the degree of this synergism among the various schedules were observed in different experiments. In the first experiment in fact, a synergistic effect with significantly longer MSTs over appropriate controls and 3/10 long-term survivors was seen only when Tetra was injected on day 19. Treatments with single doses of the drug on day 15 or 21 did not induce increases in MST over the MeCCNU alone group, but in both cases the number of lung metastases was decreased significantly. In the second experi-

Table 4. Effect of combinations of methyl-CCNU with tetra or leva in Lewis lung carcinoma-bearing $C_{57}B1/6$ mice

		Experiment 1			Experiment 2		
Exp. group	Days of treatment	MST (Day ± s.e.)	Primary tumor WT. (g ± s.e.)	No. lung nodules (± s.e.)	MST (Day ± s.e.)	Primary tumor WT. (g ± s.e.)	No. lung nodules (± s.e.)
Control	—	31·1 ± 1·1	8·6 ± 0·4	26·9 ± 2·7	28·1 ± 0·7	6·6 ± 0·5	32·2 ± 2·5
Tetramisole	15	31·2 ± 1·1	7·4 ± 0·6	27·3 ± 2·5	28·8 ± 1·5	6·6 ± 0·3	19·3 ± 2·8*
Tetramisole	19	30·2 ± 0·7	7·4 ± 0·2	26·8 ± 4·4	30·4 ± 1·4	6·3 ± 0·6	15·3 ± 2·1*
Tetramisole	21	30·4 ± 1·2	8·7 ± 0·6	24·2 ± 2·4	28·3 ± 1·9	6·8 ± 0·5	16·5 ± 3·1*
Tetramisole	15, 19, 21	29·7 ± 1·2	6·6 ± 0·5	22·5 ± 1·9	27·6 ± 1·2	5·8 ± 0·6	15·5 ± 2·9*
MeCCNU	7	47·9 ± 2·9*	6·5 ± 0·8	19·9 ± 2·2	35·5 ± 2·2*	6·6 ± 0·8	12·7 ± 2·7*
MeCCNU + Tetramisole	7 15	45·9 ± 3·9	7·0 ± 0·6	24·1 ± 2·9	35.8 ± 2.7*	3·8 ± 0·9*	9·0 ± 4·7*
MeCCNU + Tetramisole	7 19	57·3 ± 2·3†‡	3·4 ± 0·2†	15·7 ± 2·3*	43·1 ± 2·8*	3·8 ± 0·6*	18·3 ± 3·6*
MeCCNU + Tetramisole	7 21	49·2 ± 2·8	6·4 ± 0·8	15·1 ± 2·5*†	42·5 ± 2·5*†	4·8 ± 1·1	13·3 ± 3·8*
MeCCNU + Tetramisole	7 15, 19, 21	34·5 ± 0·5	7·7 ± 0·6	16·7 ± 13·1*	47·1 ± 2·3†§	3·3 ± 0·8*	8·5 ± 3·6*

Table 4 Continued

Exp. group	Days of Treatment	Experiment 3 MST (Days ± s.e.)	Primary tumor WT. (± s.e.)	No. lung nodules (± s.e.)
Control	—	28·3 ± 0·9	6·6 ± 0·3	20·3 ± 1·2
Levamisole	15	27·0 ± 1·4	5·9 ± 0·6	18·7 ± 1·9
Levamisole	19	28·5 ± 1·0	6·9 ± 0·5	14·7 ± 2·0*
Levamisole	21	26·4 ± 1·6	5·8 ± 0·4	13·8 ± 2·3*
Levamisole	15, 19, 21	29·4 ± 1·4	5·9 ± 0·5	15·8 ± 2·6*
MeCCNU	7	42·5 ± 1·5*	5·4 ± 0·6	14·2 ± 3·8*
MeCCNU + Levamisole	7 15	44·4 ± 1·3*	6·5 ± 0·7	18·4 ± 2·7
MeCCNU + Levamisole	7 19	49·4 ± 4·6*	5·0 ± 0·2	11·4 ± 2·2*
MeCCNU + Levamisole	7 21	48·3 ± 2·2*	6·0 ± 0·3	13·0 ± 0·7*
MeCCNU + Levamisole	7 15, 19, 21	55·5 ± 1·5*† §	5·6 ± 0·3	9·0 ± 1·3*†

*$P < 0·05$ in respect to controls.
†$P < 0·05$ in respect to MeCCNU controls
‡3/10 long-term survivors
§2/10 long-term survivors.

ment presented, treatments with Tetra on day 19 or 21 gave both significant lifespan prolongation of similar degrees; however the greatest increase in MST with 2/10 long-term survivors was seen when MeCCNU was followed by 3 injections of Tetra on days 15, 19 and 21. The apparent superior activity of repeated over single injections of Tetra was seen in 2 of the 4 experiments, in the third no differences were present, whereas in one (experiment 1, Table 4), a 3 injection course was actually followed by shorter survivals than seen in mice given MeCCNU alone. In 2 further experiments, Leva (3 or 10 mg/kg i.p.) was injected instead of Tetra; using the same schedules, results obtained with Leva were analogous both in quantitative terms as well as regarding the variability in efficacy between the various experiments and differences also could not be observed between the 2 drug doses investigated. In the experiment presented (experiment 3, Table 4) a significant synergism in terms of MST increase over treatments with MeCCNU alone, was seen with repeated injections of Leva, a schedule which also gave the greatest reduction in lung metastases. However, in the second experiment of this type performed, a significant increase in survival was seen only with injections of Leva on day 21, whereas the more prolonged survivals seen with the other schedules tested were not statistically significant as compared to the MeCCNU controls.

Effects of Leva on anti-SRBC immune responsiveness

When CD_1 mice were inoculated i.p. with 10^8 SRBC on day 0 and given a single Leva injection of 3 mg/kg i.p. on day 1, no increases

Table 5. Effect of levamisole treatment on spleen PFC numbers in mice

Exp. group	PFC/Spleen Day 2	Day 4	Day 7
Control	395 (190–565)	112,570 (105,500–121,000)	2240 (1690–3125)
Levamisole	230 (120–370)	266,540* (255,600–278,300)	7335* (6510–8960)

*$P < 0·05$.
CD_1 mice were injected i.p. with 10^8 SRBC on day 0; Leva (3 mg/kg i.p.) was given on day 1. Results presented are geometric means (in parentheses ± one s.e.) after logarithmic transformation of the data. Values shown are means of 3 experiments, with at least 5 mice per group.

in the number of spleen PFCs were seen when the organs were examined 48 hr after antigenic stimulation, whereas at peak response (day 4 in these conditions) the values of PFC were more than doubled in respect to controls. Table 5 also shows that the levels of PFC found on day 7 were significantly higher than in controls; the latter finding, however, was not observed consistently in every experiment. No increase in background spleen PFC values could be observed when this dose of Leva was injected in unstimulated animals examined from day 2 to day 6 after drug treatment. Very marked increases in PFC production were induced by Leva in old C57Bl/6 mice (Table 6); the highest PFC numbers, with values approximately 10 times higher than in controls, were seen when the drug dose employed (3 mg/kg i.p.) was administered on the same day or 24 hr after SRBC, but a significant increase was found also when the drug treatment preceded antigen injection by 24 hr.

Table 6. Effect of levamisole on anti-SRBC immune responsiveness in old C_{57}Bl/6 mice

Exp. group	Day of Treatment	PFC/Spleen
Control	—	1400 (1120–1650)
Levamisole	−1	2,200* (2040–2580)
Levamisole	0	8600† (7535–9320)
Levamisole	+1	10300† (9620–11540)

*$P < 0.05$ †$P < 0.01$.

C_{57}Bl/6 mice over 1 year were injected i.p. with 4×10^8 SRBC on day 0; assessment of response was performed on day 4. Levamisole was injected i.p. in a 3 mg/kg dose.

Effects of Leva on EAE

The effects of treatments with Leva (3 mg/kg) when administered to rats for 4 consecutive days beginning 24 hr after injection of allogeneic cord tissue are presented in Table 7; data shown are means of 2 experiments. It may be seen that the drug treatment employed was able to increase both the incidence and the severity of disease. In fact, on day 12 after immunization, all Leva-treated rats showed evidence of EAE whereas a number of controls still exhibited normal mobility. Similarly, on day 14, 4/20 control rats were clinically normal and only 4 animals showed a clear bilateral hind limb paralysis, whereas there were 10/20 paralyzed rats in the Leva group. Duration of EAE signs also appeared to be prolonged by Leva, and on day 18 all treated rats still showed signs of reduced mobility or paralysis, whereas 20% of controls had already returned to normal.

DISCUSSION

These results, showing that Leva could induce an increased primary response to SRBC in normal and aged mice, and enhance the severity and incidence of EAE in rats, clearly indicate that this compound possesses immunostimulatory activity, a conclusion in agreement with recent observations by others, in animals [9–14] and in man [15–18]. The findings described with a SRBC challenge are generally similar to those reported by Renoux and Renoux [10] employing Tetra; the type and dose of drug employed and/or variations in experimental conditions could account for the discrepancies observed with this study such as the fact that immunostimulation at peak response was higher in our conditions, the absence of an increase in PFCs on day 2 and the effectiveness of Leva also when administered before antigen. The results obtained in these tumor systems would also seem to confirm the immunostimulatory activity of these compounds.

Table 7. Incidence and severity of allergic encephalomyelitis signs in control and levamisole-treated rats

	Number of rats showing EAE signs on days											
	12			13			14			18		
Exp. group	−	+	++	−	+	++	−	+	++	−	+	++
Control	4	8	8	—	12	8	4	12	4	4	16	—
Levamisole	—	8	12	—	8	12	—	10	10	—	16	4

Both agents are not cytotoxic *in vitro* for normal or neoplastic cells of various origin even at concentrations of 100 μg/ml [23] Morasca, unpublished observations); at variance with other immunostimulants [24–36], these phenylimidothiazoles have not been found to induce interferon production [14]. A direct antineoplastic activity of Leva can also be excluded since no prolongation of survival could be seen in previously immunosuppressed, L1210 transplanted mice, whereas a state of specific tumor resistance was present in Leva-treated, long-term survivors. However, whether in addition to specific immunostimulation, other mechanism(s) also play a role in the tumor-inhibitory activity of these agents cannot be definitely ruled out at the present time.

As observed for other adjuvants, for these compounds a careful choice of conditions appears essential as well for obtaining significant anti-tumoral effects. Although cures were occasionally seen in the L1210 system after treatments with Leva alone, positive results were consistently observed only when the agent was associated with additional immunostimulation as given by concomitant treatment with inactivated leukemia cells. Moreover, also in the latter conditions, increases in survival were observed only when the tumor inoculum was within defined size limits. Similarly, in the 3LL tumor model, prolonged survivals with Tetra or Leva were obtained only when the tumor mass had presumably been reduced by previous chemotherapy with MeCCNU. In this respect our findings are analogous to those of Chirigos and his group [14, 27] with virally-induced leukemias or solid tumors in mice, since long-term survivors were seen only after induction of remission with cytotoxic treatments. In view of the fact that with i.m. 3LL, death is not dependent on the extent of lung metastatic involvement [28], it was not surprising that no increase in MST was seen in those mice with reduced lung nodules after Leva or Tetra alone. On the other hand, since the reduction in metastasis was obtained with single or limited treatments administered late in the course of the disease, this finding seems worthy of special note. Renoux and Renoux [29] reported that single injections of Leva on the first day after 3LL transplantation markedly retarded tumor growth and induced cures in a high proportion of animals. In experiments not reported here, repetition of the conditions employed by these authors failed to evidentiate any antineoplastic effect of the drug.

As regards the cellular target(s) for the immunostimulatory activity of these agents, no definite conclusions can be drawn yet. T lymphocytes would appear to be probably implicated in view of the finding that resistance towards syngeneic tumors can be increased and development of EAE facilitated by Leva and Tetra; in both reactivities T cells are thought to play a major role [12, 14, 27, 30]. Consistent with this view is also the increase in GVHR observed in animals [12], the restoration of cutaneous hypersensitivity reactions in patients treated with Leva [15–18] and the success obtained with this agent in Job's syndrome, a condition in which cellular immune deficiency exists [31]. Whether the increase in the PFC response and the stimulation of humoral antibody production observed in animals and man to a variety of antigenic stimuli [10, 11, 15] is the consequence of a direct action of these agents on B cells or is mediated through cooperating T elements is still to be determined. Preliminary results indicate that the response to a T-independent antigen such as the pneumococcal polysaccharide SS-III (Tagliabue, Mantovani and Spreafico, unpublished observations) is not influenced by treatment with Leva capable of stimulating an increased anti-SRBC PFC response. On the other hand, rises in reticuloendothelial activity have been observed in rodents and humans treated with these compounds [18, 32]; the possibility that an activation of macrophages plays a role in immunostimulation therefore remains open.

In conclusion, these drugs appear to represent a useful addition to the list of available immunomodifiers; much still remains to be elucidated however, before their real potential in clinical immunotherapy can be assessed precisely since examples of inactivity in experimental systems have been reported [33] and, as for other immunostimulants [34–36], treatments with Leva can be followed by enhanced tumor progression [37].

REFERENCES

1. G. Mathé, Experimental basis and first clinical controlled trials of leukemia active immunotherapy. In *Progress in Immunology*. (Edited by B. Amos) p. 959. Academic Press, New York (1971).
2. R. L. Powles, D. Crowther, C. J. T. Bateman, M. E. J. Beard, T. J. McElwain, J. Russell, T. A. Lister, J. M. A. Whitehouse, P. F. M. Wrigley, M. Pike, P. Alexander and G. Hamilton-Fairley, Immunotherapy for acute myelogenous leukaemia. *Brit. J. Cancer* **28,** 365 (1973).
3. J. U. Gutterman, C. McBride, E. J. Freireich, G. Mavligit, E. Frei III and E. M. Hersh, Active immunotherapy with BCG for recurrent malignant melanoma. *Lancet* **1,** 1208 (1973).
4. F. C. Sparks, M. J. Silverstein, J. S. Hunt, C. M. Hasken, Y. H. Pilch and D. L. Morton, Complications of BCG immunotherapy in patients with cancer. *New Engl. J. Med.* **289,** 827 (1973).
5. C. W. Aungst, J. E. Sokal and B. V. Jager, Complications of BCG vaccination. *Proc. Amer. Ass. Cancer Res.* **14,** 108 (1973).
6. D. Thienpont, O. F. J. Vanparijs, A. H. M. Raeymaevers, J. Vanderberk, P. J. A. Demoen, F. T. N. Allewijn, R. P. H. Marsboom, C. J. E. Niemegeers, K. H. L. Schellenkens and P. A. J. Janssen, (R 8299) a new, potent broad spectrum anthelmintic. *Nature (Lond.)* **209,** 1084 (1966).
7. D. Thienpont, J. Brugmans, K. Abadi and S. Tanamal, Tetramisole in the treatment of nematode infections in man. *Amer. J. trop. Med. Hyg.* **18,** 520 (1969).
8. F. Gatti, F. Krubwa, J. Vandepitte and D. Thienpont, Control of intestinal nematodes in African schoolchildren by the trimestrial administration of levamisole. *Ann. Soc. belge Méd. Trop.* **52,** 19 (1972).
9. G. Renoux and M. Renoux, Effet immunostimulant d'un Imidothiazole dans l'immunization des souris contre l'infection par Brucella abortus. *C.R. Acad. Sci (Paris)/D/* **272,** 349 (1971).
10. G. Renoux and M. Renoux, Action immunostimulant de dérivés du phénylimidothiazole sur les cellules spléniques formatri ces d'anticorps. *C.R. Acad. Sci (Paris)/D/* **274,** 756 (1972).
11. G. Renoux and M. Renoux, Restauration par le phenylimidothiazole de la réponse immunologique des souri âgées. *C.R. Acad. Sci.* (Paris)/*D/* **274,** 3034 (1972).
12. G. Renoux and M. Renoux, Action de phénylimidothiazole (tétramisole) sur la réaction du greffon contre l'hôte. Role des macrophages. *C.R. Acad. Sci (Paris)/D/* **274,** 3320 (1972).
13. G. Renoux and M. Renoux, Antigenic competition and non-specific immunity after a Rickettsial infection in mice: Restoration of antibacterial immunity by phenyl-imidothiazole treatment. *J. Immunol.* **109,** 761 (1972).
14. M. A. Chirigos, J. W. Pearson and J. Pryor, Augmentation of chemiotherapeutically induced remission of a murine leukemia by a chemical immunoadjuvant. *Cancer Res.* **33,** 2615 (1973).
15. J. Brugmans, V. Scheurmans, W. De Cock, D. Thienpont, P. Janssen, H. Verhaegen, L. Van Nimmen, A. C. Louwagie and E. Stevens, Restoration of host defense mechanisms in man by levamisole. *Life Sci.* **13,** 1499 (1973).
16. D. Tripodi, L. C. Parks and J. Brugmans, Drug-induced restoration of cutaneous delayed hypersentivity in anergic patients with cancer. *New Engl. J. Med.* **289,** 354 (1973).
17. Y. Hirshaut, C. Pinsky, H. Marquardt and H. F. Oettgen, Effects of levamisole on delayed hypersensitivity reactions in cancer patients. *Proc. Amer. Ass. Cancer Res.* **14,** 109 (1973).
18. H. Verhaegen, J. De Cree, W. De Cock and F. Verbruggen, Levamisole and the immune response. *New Engl. J. Med.* **289,** 1148 (1973).
19. H. Wexler, Accurate identification of experimental pulmonary metastases. *J. nat. Cancer Inst.* **36,** 641 (1966).
20. N. K. Jerne and A. A. Nordin, Plaque formation in agar by single antibody-producing cells. *Science* **140,** 405 (1963).
21. D. W. Dresser and H. H. Wortis, Localized haemolysis in gel. In *Handbook of Experimental Immunology*. Edited by D. M. Weir, p. 1054. Blackwell Scientific Publ., Oxford (1967).

22. S. LEVINE and E. J. WENK, Hyperacute allergic encephalomyelitis. *Amer. J. Path.* **50,** 465 (1967).
23. D. COPELAND, T. STEWART and J. HARRIS, Effect of levamisole (NSC 177023) on *in vitro* human lymphocyte transformation. *Cancer Chemother. Rep.* **58,** 167 (1974).
24. W. BRAUN, W. REGELSON, Y. YAJIMA and M. ISHIZUKA, Stimulation of antibody formation by pyran copolymer. *Proc. Soc. exp. Biol. (N.Y.)* **133,** 171 (1970).
25. A. E. MUNSON, J. A. MUNSON, W. REGELSON and G. L. WAMPLER, Effect of tilorone hydrochloride and congeners on reticuloendothelial system, tumors and the immune response. *Cancer Res.* **32,** 1397 (1972).
26. P. S. MORAHAN, J. A. MUNSON, L. G. BAIRD, A. M. KAPLAN and W. REGELSON, Antitumor action of pyran copolymer and tilorone against Lewis lung carcinoma and B-16 melanoma. *Cancer Res.* **34,** 506 (1974).
27. M. A. CHIRIGOS, J. W. PEARSON and F. S. FUHRMAN, Effect of tumor load reduction on successful immunostimulation. *Proc. Amer. Ass. Cancer Res.* **15,** 116 (1974).
28. F. SPREAFICO and S. GARATTINI, Selective antimetastatic treatment—Current status and future prospects. *Cancer Treat. Reviews* **1,** 239 (1974).
29. G. RENOUX and M. RENOUX, Levamisole inhibits and cures a solid malignant tumour and its pulmonary metastases in mice. *Nature New Biol.* **240,** 217 (1972).
30. V. A. LENNON and W. J. BYRD, Role of T lymphocytes in the pathogenesis of experimental auto-immune encephalomyelitis. *Eur. J. Immunol.* **3,** 243 (1973).
31. R. VANHEULE and J. DE CREE, Treatment of a child suffering from Job's syndrome using levamisole. Personal communication.
32. J. HOEBEKE and G. FRANCHI, Influence of tetramisole and its optical isomers on the mononuclear phagocytic system. I. Effect on carbon clearance in mice. *J. reticuloendoth. Soc.* **14,** 317 (1973).
33. C. W. POTTER, I. CARR, R. JENNINGS, R. C. REES, F. MCGINTY and V. M. RICHARDSON, Levamisole inactive in treatment of four animal tumours. *Nature (Lond.)* **249,** 567 (1974).
34. W. F. PIESSENS, F. L. LACHAPELLE, N. LEGROS and J. C. HEUSON, Facilitation of rat mammary tumour growth by BCG. *Nature (Lond.)* **228,** 1210 (1970).
35. J. ANKERST and N. JONSSON, Inhibitory effects of BCG on adenovirus tumorigenesis: dependence on administration schedule. *Int. J. Cancer* **10,** 351 (1972).
36. D. O. CHEE and A. J. BODURTHA, Facilitation and inhibition of B16 melanoma by BCG *in vivo* and by lymphoid cells from BCG treated mice *in vitro*. *Int. J. Cancer* **14,** 137 (1974).
37. A. MANTOVANI and F. SPREAFICO, Allogeneic tumor enhancement by levamisole, a new immunostimulatory compound: studies on cell-mediated immunity and humoral antibody response. *Eur. J. Cancer* **11,** 537 (1975).

22. S. LEVINE and E. J. WENK, Hyperacute allergic encephalomyelitis. *Amer. J. Path.* **50,** 465 (1967).
23. D. COPELAND, T. STEWART and J. HARRIS, Effect of levamisole (NSC 177023) on *in vitro* human lymphocyte transformation. *Cancer Chemother. Rep.* **58,** 167 (1974).
24. W. BRAUN, W. REGELSON, Y. YAJIMA and M. ISHIZUKA, Stimulation of antibody formation by pyran copolymer. *Proc. Soc. exp. Biol. (N.Y.)* **133,** 171 (1970).
25. A. E. MUNSON, J. A. MUNSON, W. REGELSON and G. L. WAMPLER, Effect of tilorone hydrochloride and congeners on reticuloendothelial system, tumors and the immune response. *Cancer Res.* **32,** 1397 (1972).
26. P. S. MORAHAN, J. A. MUNSON, L. G. BAIRD, A. M. KAPLAN and W. REGELSON, Antitumor action of pyran copolymer and tilorone against Lewis lung carcinoma and B-16 melanoma. *Cancer Res.* **34,** 506 (1974).
27. M. A. CHIRIGOS, J. W. PEARSON and F. S. FUHRMAN, Effect of tumor load reduction on successful immunostimulation. *Proc. Amer. Ass. Cancer Res.* **15,** 116 (1974).
28. F. SPREAFICO and S. GARATTINI, Selective antimetastatic treatment—Current status and future prospects. *Cancer Treat. Reviews* **1,** 239 (1974).
29. G. RENOUX and M. RENOUX, Levamisole inhibits and cures a solid malignant tumour and its pulmonary metastases in mice. *Nature New Biol.* **240,** 217 (1972).
30. V. A. LENNON and W. J. BYRD, Role of T lymphocytes in the pathogenesis of experimental auto-immune encephalomyelitis. *Eur. J. Immunol.* **3,** 243 (1973).
31. R. VANHEULE and J. DE CREE, Treatment of a child suffering from Job's syndrome using levamisole. Personal communication.
32. J. HOEBEKE and G. FRANCHI, Influence of tetramisole and its optical isomers on the mononuclear phagocytic system. I. Effect on carbon clearance in mice. *J. reticuloendoth. Soc.* **14,** 317 (1973).
33. C. W. POTTER, I. CARR, R. JENNINGS, R. C. REES, F. MCGINTY and V. M. RICHARDSON, Levamisole inactive in treatment of four animal tumours. *Nature (Lond.)* **249,** 567 (1974).
34. W. F. PIESSENS, F. L. LACHAPELLE, N. LEGROS and J. C. HEUSON, Facilitation of rat mammary tumour growth by BCG. *Nature (Lond.)* **228,** 1210 (1970).
35. J. ANKERST and N. JONSSON, Inhibitory effects of BCG on adenovirus tumorigenesis: dependence on administration schedule. *Int. J. Cancer* **10,** 351 (1972).
36. D. O. CHEE and A. J. BODURTHA, Facilitation and inhibition of B16 melanoma by BCG *in vivo* and by lymphoid cells from BCG treated mice *in vitro*. *Int. J. Cancer* **14,** 137 (1974).
37. A. MANTOVANI and F. SPREAFICO, Allogeneic tumor enhancement by levamisole, a new immunostimulatory compound: studies on cell-mediated immunity and humoral antibody response. *Eur. J. Cancer* **11,** 537 (1975).

Europ. J. Cancer Vol. 11, pp. 565–569. Pergamon Press 1975. Printed in Great Britain

Study of Growth Hormone Secretion in Lung Carcinoma

P. CLAEYS-DE CLERCQ, S. LEVIN and A. BORKOWSKI
Service de Médecine et d'Investigation Clinique de l'Institut J. Bordet, Bruxelles, Belgium

Abstract—*We investigated the possibility of ectopic growth hormone (HGH) secretion in 26 patients with lung carcinoma by measuring plasma HGH during an oral glucose tolerance test (GTT). Only 4 patients had raised basal HGH concentrations; however, these concentrations were only slightly above normal, remained well below the range of our acromegalics and did not result from an autonomous secretion since they were modified by the GTT. Five patients had a paradoxical response to glucose. In the other 18 patients, basal HGH was normal and remained within normal limits after glucose administration. Consequently, we did not find any evidence for a significant ectopic HGH secretion. Nevertheless, a slight ectopic secretion without increased plasma HGH concentrations cannot be excluded since during the GTT some plasma HGH remained constantly measurable in most of our 26 patients. There was no correlation between plasma HGH and any other clinical or biological parameter, particularly clubbing.*

INTRODUCTION

LUNG carcinoma is particularly prone to secrete ectopic polypeptides. This might be due to its embryological origin [1]. The ectopic polypeptides can be hormones, pro-hormones or hormone subunits [2, 3]; their biological activity is very variable [3]; some, like the placental hormones, are not normally present in man and can thus be viewed as specific markers of the neoplasm or of particular clones within the neoplasm [4]; whereas the ectopic production of big ACTH [2] seems very frequent, other ectopic productions in lung carcinoma might be rare events. As concerns human growth hormone (HGH), it has been shown to be produced *in vitro* by a culture of lung carcinoma [5]; this HGH had the same immunologic and chromatographic properties as normal HGH. Therefore, ectopic secretion of HGH by bronchogenic tumors presumably occurs *in vivo*. However, its frequency and its biological and clinical consequences are unknown. Some authors have reported that significant amounts of HGH are commonly found within bronchogenic tumors [6]. Others have suggested that excessive HGH production might be responsible for the hypertrophic pulmonary osteoarthropathy secondary to lung carcinoma [7, 8]. Since ectopic HGH secretion, like most endocrine secretions from malignant neoplasms, might be expected to be autonomous, we investigated the possibility of ectopic HGH secretion in 26 patients with lung carcinoma by measuring plasma HGH during an oral glucose tolerance test (GTT). Our observations extend those by G. Ennis, D. Cameron and H. Burger [9] obtained in a similar fashion. In addition, we compared our measurements with those obtained in acromegalics who had a proven pituitary tumor and we tried to correlate our findings with various clinical and biological parameters. The results of this investigation are reported in the present paper.

Accepted 21 April 1975.

*This work was supported by the Fondation Cancérologique de la Caisse Générale d'Epargne et de Retraite de Belgique, by contract Euratom-ULB-Pisa and by the Fonds National de la Recherche Scientifique, Bruxelles, Belgium.

MATERIAL AND METHODS

The magnitude and autonomy of HGH secretion was evaluated in 26 male patients with lung carcinoma admitted successively to the Service of Medicine of the Institut Jules

Bordet for chemotherapy or palliative radiotherapy; 15 had an epidermoid carcinoma, 7 had an oat-cell carcinoma and 4 had an undifferentiated large cell carcinoma. These patients were compared with 5 acromegalics who had a proven pituitary tumor and were being investigated during the same period.

A standard oral glucose tolerance test with 100 g of glucose [10] was performed in each patient, in the morning, after an overnight rest and fasting. Blood samples were obtained at hourly intervals and collected into heparinized tubes on ice. The following substances were measured in plasma = D glucose [11], human growth hormone [12], basal insulin [12], basal cortisol (i.e. around 8 a.m.) and cortisol in the evening of the same day (around 10 p.m.) [13].

Plasma immunoreactive growth hormone was measured using a modification of the method of Hales and Randle [14]; basal plasma HGH concentrations determined in 19 normal male controls ranged from 0·0 to 5·4 ng/ml (mean: 1·96 ng/ml—s.d.: 1·44 ng/ml); consequently, we took as upper normal limit: 4·8 ng/ml (i.e. the mean +2 s.d.). Basal plasma immunoreactive insulin was measured in a similar fashion; normal range: 6–25 μU/ml. Normal range for plasma cortisol in the morning: 5–25 μg/ml.

In addition to a clinical evaluation, the following parameters among others were followed in each patient: serum ionogram, calcium, non-protein nitrogen, proteins and electrophoresis, standard liver tests; if liver metastases were suspected, a liver scan was performed; if there was any suspicion of cerebral metastases, and EEG, a cerebral scan and eventually a cerebral arteriography were also performed.

RESULTS

Basal plasma HGH concentrations were found to be increased (higher than 4·8 ng/ml) in only 4 of our 26 patients (Table 1); these raised basal concentrations ranged from 5·7 to 8·7 ng/ml (mean: 7·0 ng/ml) and thus remained well below the basal concentrations measured in the five acromegalics with a pituitary tumor (range: 11·3 to 48·5 ng/ml—mean: 27·0 ng/ml) (Table 2).

None of the 4 patients with lung carcinoma and raised basal HGH concentrations was unresponsive to the oral GTT: one had a somewhat paradoxical response (Vers); in the other three patients plasma HGH fell, at least momentarily, to normal values with a nadir of 1·8 to 4·5 ng/ml (mean: 2·9 ng/ml), whereas in our acromegalics the mean nadir was almost ten times higher: 27·7 ng/ml and ranged from 5·6 to 54·4 ng/ml.

Five patients with lung carcinoma had a paradoxical HGH response during the oral GTT (Table 3); plasma HGH was highest 1 hr after the glucose administration and increased HGH levels persisted throughout the whole test.

In the other 18 patients with lung carcinoma (Table 4) basal plasma HGH was normal and remained within normal limits after glucose administration except for occasional and transitory increases; however, even at the nadir

Table 1. Patients with a high basal concentration of plasma growth hormone

Patients	Plasma growth hormone (ng/ml) during the glucose tolerance test				Fasting plasma insulin (μU/ml)	Fasting glycaemia (mg/100 ml)	Plasma cortisol (μg/100 ml)	
	0 hr	1 hr	2 hr	3 hr			8 a.m.	10 p.m.
Tas.	8·7	4·5	13·5	10·6	14	80	20	10
Vdm.	6·0	6·3	2·7	1·8	20	85	13	10
Vin.	5·7	—	3·5	2·4	11	80	47	20
Vers.	7·4	30·0	7·8	5·8	54	65	15	8
Means	7·0				25	78	24	12

Table 2. Acromegalic patients

Patients	Plasma growth hormone (ng/ml) during the glucose tolerance test			
	0 hr	1 hr	2 hr	3 hr
Jan.	48·5	37·8	42·6	72·6
Cro.	30·0	68·5	54·4	56·1
Lad.	30·0	30·0	29·0	47·5
Sad.	15·3	13·2	5·6	14·7
Clo.	11·3	23·6	11·6	15·0
Means	27·0	34·6	28·6	41·2

Table 3. *Patients with a paradoxical growth hormone response*

Patients	Plasma growth hormone (ng/ml) during the glucose tolerance test				Fasting plasma insulin (μU/ml)	Fasting glycaemia (mg/100 ml)	Plasma cortisol (μg/100 ml)	
	0 hr	1 hr	2 hr	3 hr			8 a.m.	10 p.m.
Ros.	3·9	40·0	23·2	18·2	16	90	51	49
Ded.	2·6	27·0	18·8	6·8	24	75	12	5
Col.	4·4	13·6	6·9	6·3	35	130	19	10
Gil.	3·6	17·8	8·1	9·6	20	80	19	7
Vers.	7·4	30·0	7·8	5·8	54	65	15	8
Means	4·4	25·7	13·0	9·3	30	88	23	16

Table 4. *Patients with normal concentrations of plasma growth hormone* ($N = 18$)*

	Plasma growth hormone (ng/ml) during the glucose tolerance test				Fasting plasma insulin (μU/ml)	Fasting glycaemia (mg/100 ml)	Plasma cortisol (μg/100 ml)	
	0 hr	1 hr	2 hr	3 hr			8 a.m.	10 p.m.
Means	2·2	3·7	3·5	2·1	23	97	18	12
Ranges	(0·0–4·5)	(0·0–12·6)	(0·0–14·1)	(0·0–6·9)	(6–49)	(75–165)	(8–35)	(0–25)

*Normal concentrations, except for occasional and transitory increases; N = Number of patients.

(mean: 1·5 ng/ml—range: 0·0–4·7 ng/ml) some HGH (more than 0·5 ng/ml) was still measurable in the plasma of most of these patients.

No correlation was found between the plasma HGH concentrations and any of the histological, clinical or biological parameters. Seven of the eight patients with high basal levels or paradoxical HGH responses (Tables 1, 3) had an epidermoid carcinoma and 1 (Ros., Table 3) had an oat-cell carcinoma. Only 1 of these 8 patients (Gil.) had a moderate digital hippocratism whereas hippocratism was found to be moderate to marked in 5 of the other 18 patients. Five of the eight patients with HGH abnormalities were cachectic or had lost much weight (2 had evidences of liver metastases), 9 of the other 18 patients were in a similar poor nutritional state (3 with evidences of liver metastases); only one (Ros.) in the former group had cerebral metastases against 3 in the latter. Because of inadequate previous food intake, a diabetic glucose tolerance curve was common (in 18 of the 26 patients) but only 4 patients had a fasting hyperglycaemia. Fasting insulin was increased in 2 of the 8 patients with HGH abnormalities and in 5 of the 18 other patients; it did not correlate with plasma HGH nor with plasma cortisol concentrations. With regard to the latter, it should be noted that we did not find any parallelism between basal morning cortisol concentrations and HGH basal concentrations.

DISCUSSION

According to some authors, concentrations of HGH which are significantly higher than in the adjacent uninvolved lung are found within almost 40% of all bronchogenic tumors [6]. Although this finding might be much more exceptional [15], one does not know what an increased concentration within a tumor means in terms of *in vivo* secretion. Some patients with lung carcinoma have also been reported to have high plasma HGH concentrations with return to normal after lung resection [8]. However, unless one measures an arterio-venous gradient of plasma concentration across the tumor, the *in vivo* demonstration of ectopic HGH secretion is difficult. The difficulty arises, of course, from the interference of pituitary HGH secretion; the latter is highly variable depending on exercise, stress, time and quality of food intake, nutritional state, and presence of central nervous system lesions [12, 16]. These parameters should particularly be taken into account in patients with lung carcinoma where loss of weight and cerebral metastases are frequent. In opposition to pituitary HGH secretion, the

ectopic HGH secretion might be expected to be autonomous, i.e. should not be modified by acute variations of the glycaemia [10]. It is remarkable therefore, that the 4 patients of our series who had raised basal levels of plasma HGH responded in some fashion to the oral GTT (one responded paradoxically, the other 3 responded by a clearcut decrease of plasma HGH). In addition, the basal level of HGH in these 4 patients was only slightly above the normal range and remained well below the range of our "control" acromegalics. Consequently, we did not find any evidence for a significant ectopic HGH secretion. Nevertheless, a slight ectopic secretion without increased plasma HGH concentrations cannot be excluded since during the oral GTT some plasma HGH remained constantly measurable in most of our 26 patients. Five of our patients had a paradoxical response to the glucose administration. Paradoxical responses have already been reported in lung carcinoma [9, 16] and in other neoplasms [17]. This anomaly cannot be specifically attributed to the tumor; it can have nutritional causes, and in some patients at least, it might be due to cerebral metastases [12, 17]. The significance of a paradoxical response in malnutrition has not been established but might have an adaptative value since HGH is most efficient as an anabolic agent when plasma insulin is increased, the high levels of insulin during hyperglycaemia inhibiting the fat mobilizing action of HGH and catalizing its protein-anabolic effects [18]. With regard to the presence of cerebral metastases, it should be noted that all patients with cerebral metastases did not have a paradoxical response; the latter cannot be viewed therefore as an early or constant manifestation of the former.

The clinical evaluation of the consequences of ectopic HGH secretion appears difficult because of the lack of specific metabolic or clinical manifestations when an HGH hypersecretion has not been of very long duration. The clinical similarity between patients with acromegaly and chronic pulmonary osteoarthropathy secondary to lung carcinoma suggested excess HGH as a possible cause of the latter [7, 8]; in this regard, as Ennis, Cameron and Burger [9], we did not find any correlation between clubbing and plasma HGH concentration. Neither did we find any correlation between increased basal levels of HGH and increased basal levels of insulin, paradoxical HGH responses and diabetic GTT, abnormalities of HGH secretion and abnormalities of cortisol secretion. Since the stress increases both HGH and cortisol secretions it is interesting that we did not find any parallelism between the basal plasma levels of these two hormones in the morning; this indicates that factors other than stress must be considered [16].

REFERENCES

1. R. Weichert III, The neural ectodermal origin of peptide secreting endocrine glands. *Amer. J. Med.* **49,** 232 (1970).
2. G. Gewirtz and R. S. Yalow, Ectopic ACTH production in carcinoma of the lung. *J. clin. Invest.* **53,** 1022 (1974).
3. B. Weintraub and S. Rosen, Ectopic production of the isolated beta subunit of human chorionic gonadotropin. *J. clin. Invest.* **52,** 3135 (1973).
4. A. Tashjian, B. Weintraub, N. Barowsky, A. Rabson and S. Rosen, Subunits of human chorionic gonadotropin: unbalanced synthesis and secretion by clonal cell strains derived from a bronchogenic carcinoma. *Proc. natl. Acad. Sci. (Wash.)* **70,** 1419 (1973).
5. P. Greenberg, C. Beck, T. Martin and H. Burger, Synthesis and release of human growth hormone from lung carcinoma in cell culture. *Lancet* **1,** 350 (1972).
6. C. Beck and H. Burger, Evidence for the presence of immunoreactive growth hormone in cancers of the lung and stomach. *Cancer* **30,** 75 (1972).
7. B. Dupont, I. Hoyer, S. Borgeskov and J. Nerup, Plasma growth hormone and hypertrophic osteoarthropathy in carcinoma of the bronchus. *Acta Med. Scand.* **188,** 25 (1970).
8. H. Steiner, O. Dahlbach and J. Waldenstrom, Ectopic growth hormone production and osteoarthropathy in carcinoma of the bronchus. *Lancet* **1,** 783 (1968).
9. G. Ennis, D. Cameron and H. Burger, On the aetiology of hypertrophic pulmonary osteoarthropathy in bronchogenic carcinoma: lack of relationship to elevated growth hormone levels. *Aust. N.Z. med. J.* **3,** 157 (1973).
10. S. Glick, J. Roth, R. S. Yalow and S. A. Berson, The regulation of growth hormone secretion. *Recent Progr. Hormone, Res.* **21,** 241 (1965).

11. W. WERNER, H. REY and H. WIELINGER, Unber die Eigenschaften eines neuen Chromogens für die Blutzuckerbestimmung nach der GOD/POD Methode. *Z. analyt. Chem.* **252,** 224 (1970).
12. V. KEYMOLEN-JARDINI, E. MOUAWAD, P. CLAEYS-DE CLERQ, E. VAN LAER, S. LEVIN and A. BORKOWSKI, Glucose metabolism in traumatic brain injury. *Acta endocr. (Kbh)* **77,** 103 (1974).
13. B. MURPHY, Some studies of the protein-binding of steroids and their application to the routine micro- and ultramicro measurement of various steroids in body fluids by competitive protein-binding radioassay. *J. clin. Endocr.* **27,** 973 (1967).
14. C. N. HALES and P. RANDLE, Immunoassay of insulin with insulin-antibody precipitate. *Biochem. J.* **88,** 137 (1963).
15. M. SPARAGANA, G. PHILLIPS, C. HOFFMAN and L. KUCERA, Ectopic growth hormone syndrome associated with lung cancer. *Metabolism* **20,** 730 (1971).
17. J. B. MARTIN, Neural regulation of growth hormone secretion. *New Engl. J. Med.* **288,** 1384 (1973).
18. D. RABINOWITZ and K. ZIERLER, A metabolic device based on the actions of human growth hormone and of insulin, singly and together, on the human forearm. *Nature (Lond.)* **199,** 913 (1963).
16. D. CZARNY, V. JAMES, J. LANDON and F. C. GREENWOOD, Corticosteroid and growth hormone response to synthetic lysine-vasopressin, natural vasopressin, saline solution and venepuncture. *Lancet* **2,** 126 (1968).

Europ. J. Cancer Vol. 11, pp. 571–579. Pergamon Press 1975. Printed in Great Britain

The Effect of Synthetic Anti-oestrogens on the Growth and Biochemistry of Rat Mammary Tumours

ROBERT I. NICHOLSON AND MERIEL P. GOLDER
Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, CF4 4XX, U.K.

Abstract—*The effect of a series of potential oestrogen antagonists on the growth and biochemical properties of DMBA-induced mammary tumours in rats was studied. Plasma prolactin and oestradiol-17β levels were also measured and no difference was observed between animals with tumours that regressed and those in which tumours continued to grow. ICI* 46474 (*Tamoxifen*) *and CI* 628 (*Parke-Davis*) *were more effective than ICI* 79792, meso-*dihydrodibutylstilboestrol and diethylstilboestrol in causing tumour regression. The results suggest that the initial action of these oestrogen antagonists is at the tissue level rather than through an effect on plasma hormone concentrations.*

INTRODUCTION

HUGGINS, Grand and Brillantes [1] demonstrated that multiple mammary adenocarcinomas can be induced in female Sprague–Dawley rats by the single administration of the polycyclic hydrocarbon 7,12-dimethylbenz(a)-anthracene (DMBA). Many of these mammary tumours are hormone-dependent and can be observed to regress after ovariectomy [1], hypophysectomy [2] or after the administration of various steroids or antihormones [3–6].

The hormone-dependent nature of this experimental tumour system therefore provides a useful model for the evaluation of the endocrine factors which are concerned in the growth and maintenance of the mammary tumour and also for the assessment of various drugs considered to have some potential in the clinical management of human breast cancer.

This paper describes the effects of ICI 46474 (Tamoxifen) and its *nitrogen mustard* derivative ICI 79792 on the DMBA-induced mammary tumour and their influence on the plasma levels of prolactin and oestradiol-17β. The endocrine effects produced by these two compounds have been compared to those produced by diethylstilboestrol, *meso*-dihydrodibutylstilboestrol, and CI 628 (Parke-Davis).

Accepted 19 March, 1975.

MATERIAL AND METHODS

Animals

Mammary tumours were induced in virgin female Sprague–Dawley rats (50 ± 2 days old) by intubation with a single dose of 20 mg DMBA in 1 ml sesame oil. Animals were housed in groups of 4 and fed diet and water *ad libitum*. After 5 weeks, the rats were palpated for tumours at weekly intervals and the size recorded as the mean of two perpendicular diameters, one measured across the greatest width. No tumour was used in experimentation which did not attain an approximate size of 20 mm mean diameter in the 23-week period following carcinogen administration.

Chemicals

Oestradiol-17β was purchased from Sigma Chemical Company Ltd., London. Diethyl-

stilboestrol (DES) was purchased from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire. All other compounds were gifts from the manufacturers. CI 628, 1-, 2-[p-α-(p-methoxyphenyl)-β-nitrostyryl phenoxy] ethyl-pyrrolidine mono-citrate was provided by Parke-Davis and Co; ICI 46474, the trans-isomer of 1-(p-β-dimethylamino-ethoxy-phenyl)-1,2-diphenyl-but-1-ene, ICI 79792, the trans-isomer of 1-{4-β-[bis(2-chloroethyl)-amino] ethoxyphenyl}diphenyl-but-1-ene hydrochloride and *meso*-dihydrodibutylstilbo-estrol(*meso*-DHBS) 5,6-bis(p-hydroxyphenyl)-decane were supplied by Dr. A. L. Walpole and Dr. D. N. Richardson, ICI Ltd., Alderley Edge, Cheshire.

Experimental

Three experiments were undertaken:

(i) Nine animals bearing 17 tumours (approximate size 20 mm mean dia.) were ovariectomised. Surgery was undertaken under ether anaesthesia and the ovaries removed through two lateral incisions. Seven days post-operatively, a small portion (approximately 100 mg) of each tumour was removed, under ether anaesthesia, for histological and biochemical studies. The remainder of the tissue was left in the animals for a further period of 6 weeks. Tumour size was measured weekly.

(ii) Forty animals bearing tumours (approximate size 20 mm mean dia) were divided randomly into five groups of eight animals. Each group received daily i.m. injections of various compounds in 0·1 ml sesame oil for 14 days and then further injections every 2 days for 5 weeks. Tumour size was measured weekly and a biopsy taken on day 7. The compound and the amount administered at each injection are given below:
(a) sesame oil, (b) ICI 46474 (100 μg), (c) ICI 79792 (100 μg), and (d) ovariectomised and sham injected with sesame oil.

(iii) One hundred and twenty-two tumour-bearing animals (approximate tumour size 2 mm mean dia) were divided randomly into nine groups. Each group of animals received daily i.m. injections of various compounds in 0.1 ml sesame oil for six days. The groups were treated as follows:
(a) sesame oil, (b) ICI 46474 (100 μg), (c) ICI 79792 (100μg) (d) CI 628 (100μg), (e) DES (100 μg), (f) *meso*-DHBS (100 μg), (g) oestradiol-17β (5 μg), (h) ovariectomised and sham injected with sesame oil and (i) ovariectomised and injected with oestradiol-17β (5 μg). Tumour size was determined at the initiation and termination of the experiment.

On day seven, all animals were anaesthetised with ether and exsanguinated through the dorsal aorta. Plasma samples were stored at −20°. The tumours, uterus, pituitary gland, adrenal glands and ovaries were removed, dissected free of fat and weighed. A small portion from each tumour was immediately immersed in Bouins liquid for histology. Fixed tissue was stained with haemotoxylin and eosin. The remainder of the tissue was used for biochemical studies.

Based on the histology, animals were classified as either possessing "growing" or "regressing" tumours.

Enzyme studies

A portion of the tissue was washed in saline (0·15 M), cut into small pieces and a homogenate (20% w/v) prepared in ice-cold 50 mM Tris–HCl buffer pH 7·4, containing 1 mM EDTA, using a Potter and Elvehjem glass on Teflon homogeniser of clearance 14–16 nm (10 strokes at 5000 rev/min).

The procedures for the estimation of p-nitrophenylphosphatase activity and protein concentration have been previously described [7]. The combined activity of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6 PGDH) was assayed at 25° by the method of Kornberg *et al.* [8]. The method utilises absorption changes at 340 nm due to the reduction of NADP. The results are referred to as dehydrogenase activity; one unit of dehydrogenase activity being defined as the amount of enzyme which will convert 1 μmole of substrate per min at its optimal conditions.

Assessment of oestradiol-17β binding capacity of tumour tissue

Tissue from a selected number of tumours was homogenised to determine the oestradiol-17β binding capacity. This was carried out at 4° in 10 mM Tris–HCl buffer, pH 7·6 containing 1 mM EDTA and using a Silverson homogeniser. The homogenate (20% w/v) was centrifuged at 800 ***g*** for 10 min in a refrigerated Mistral 4L centrifuge. Duplicate samples of 0·2 ml of the supernatant were incubated for 30 min at 30° with a saturating concentration of ^{3}H oestradiol-17β, (5×10^{-9}M[2,4,6,7,$^3H_4$]-oestradiol-17β, specific activity 85 Ci/mole,

Radiochemical Centre, Amersham). Non-specific oestradiol-17β binding was determined in control incubations which had been pre-heated for 60 min at 45° [9]. After the incubation period the samples were cooled in ice, 0·5 ml of dextran-coated charcoal added (0·5% Norit A plus 0·05% dextran) mixed, and incubated for 10 min at 30° with shaking, followed by centrifugation at 800 *g* for 10 min. A sample of the supernatant (0·5 ml) was added to scintillant (5 ml) (Aquasol, New England Nuclear) and radioactivity measured in a Nuclear Chicago (Mark I) liquid scintillation spectrophotometer. Oestradiol-17β binding capacity was expressed as counts/min per mg protein after subtracting the non-specific binding value.

Hormone assays were performed on 8 randomly-selected plasmas from each group.

Assay for prolactin

Plasma prolactin levels were measured in a double antibody radioimmunoassay system using the kit distributed by the National Institute for Arthritic, Metabolic and Digestive Diseases, (NIAMDD), Bethesda, MD U.S.A., and described previously [10].

Assay for oestradiol-17β

Plasma oestradiol-17β levels were measured by radioimmunoassay using the method established in these laboratories by Cameron and Jones [11].

RESULTS

The histology of the tissue was used as an assessment of tumour growth or regression. "Growing tumours" were composed of glandular acini, which were arranged in various conformations, within a fibrous stroma (Fig. 1). The lumina, where present, were small and sometimes contained eosin-positive material. A major proportion of the cell mass was taken up by the presence of large, lightly stained nuclei. Mitotic figures were a common feature, especially in poorly differentiated tumours. The most common feature associated with tumour regression was cellular atrophy (Fig. 2), resulting from a considerable loss of cytoplasm. Nuclei were small and intensely stained. The net result of cell shrinkage was to increase the size of the lumina which were often surrounded only by a single layer of low cuboidal cells. Tumours displaying these characteristics were referred to in the text as 'regressing".

The correlation which exists between the histological and biochemical properties of tumour tissue removed seven days post-ovariectomy and tumour size at seven weeks post-ovariectomy is shown in Table 1 (Experiment 1). The p-nitrophenylphosphatase activity of "regressing" tumours was higher than that of "growing" tumours, whereas dehydrogenase activity was lower in the "regressing" tissue. At the end of the experimental period (7weeks) the mean diameter of "regressing" tumours was significantly smaller than that of "growing" tumours.

A large proportion (13/16) of mammary tumours regressed after ovariectomy (Fig. 3(a)) (Experiment 2). The remainder (3/16) continued to grow in a manner similar to the majority (7/8) of tumours in the control group. Of the tumours in animals receiving ICI 46474 treatment, approximately half regressed, (9/16) (Fig. 3(b)). Only 2/8 of tumours treated with ICI 79792 responded in this manner. The mean diameters of the tumours which regressed following treatment with ICI 46474 were not

Table 1. *Effect of ovariectomy on DMBA-induced mammary tumours. Seven days post-ovariectomy tumours were classified histologically as either "growing" or "regressing". Enzyme activities from samples of tumours were also determined at this time. The tumours were then left intact in the animals and tumour size measured* 40 *days post ovariectomy. Number of tumours assayed given in parentheses*

	Growing Tumours (4)	Regressing Tumours (13)
p-Nitrophenylphosphatase activity after 7 days ($^{u}/_{g}$ protein)	33.1 ± 9.24	58.4 ± 12.26*
Dehydrogenase Activity after 7 days ($^{u}/_{g}$ protein)	169 ± 35.2	96 ± 25.4*
Average Tumour size after 40 days (mean diameter mm).	24 ± 5.0	6 ± 6.0*

*$P < 0·05$ (v "growing" tumours) determined by Student's *t*-test. Results are expressed as mean ± S.D.

significantly different from those regressing following ovariectomy. It was interesting that a number of "regressing" tumours showed a re-stimulation of tumour growth after approximately 6 weeks of treatment. No changes in tumour size could be detected within the first week of treatment.

Table 2 lists the numbers of growing and regressing tumours found in animals used in Experiment 3. Ovariectomy caused a histologically recognised regression in approximately 80% of tumours. The regression of tumour growth could be partially countered by the administration of 5 μg oestradiol-17β per day, a dose shown to be sufficient to maintain growth of all tumours in the intact animals studied. Approximately 10% of the tumours of intact, non-treated animals were found to show histological evidence of regression. ICI 46474 and CI 628 were the most effective agents in promoting tumour regression (60–65% of treated tumours), whereas only 20% of the tumours responded to either ICI 79792, DES or *meso*-DHBS. No changes in tumour size could be detected following any of the treatments.

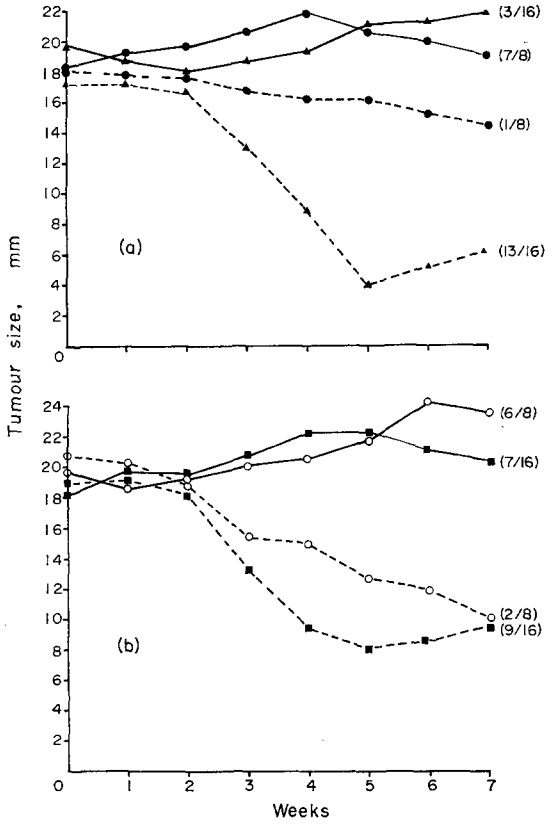


Fig. 3(*a*). *Effect of ovariectomy on DMBA-induced mammary tumour growth.*

Tumours were classified histologically after 7 *days as either "growing" or "regressing". Tumour size was measured weekly. Figures given in parentheses show the fraction of tumours, within a group, that were classified as either "growing"* (——) *or "regressing"* (– – –), ●, *intact animals (sham injected).* ▲. *ovariectomised animals (sham injected).*

Fig. 3(*b*). *Effect of ICI* 46474 *and ICI* 79792 *on mammary tumour growth.*

Animals were injected daily for 14 *days and then every* 2 *days for* 5 *weeks with* 100 μg *(per injection) of either ICI* 46474 *or ICI* 79792. *Tumour size (mean diameter) was measured weekly. Classification of tumours as either "growing" or "regressing" was determined on biopsy samples at day* 7. *Figures given in parentheses show the fraction of tumours, within a group, that were classified as either "growing"* (——) or *"regressing"* (– – –). ■. *ICI* 46474 *injected,* ○, *ICI* 79792 *injected.*

Table 3 shows clearly that the activity of the p-nitrophenylphosphatase enzyme system is higher in "regressing" tumour tissue than in the "growing" tumours of control animals. The dehydrogenase activity however was again lower in these "regressing" tissues particularly in tumour tissue from animals treated with either ICI 46474 or CI 628.

Prolactin concentrations were decreased significantly in the plasma of ovariectomised animals (Table 4), an effect which could be abolished by the daily administration of 5 μg oestradiol-17β. Administration of either 5 μg oestradiol-17β or 100 μg DES each day to intact animals resulted in a significant elevation of the plasma prolactin concentration. Furthermore, administration of ICI 46474, ICI 79792, CI 628 or *meso*-DHBS failed to result in any significant change in the plasma prolactin concentration. No apparent differences were detectable between plasma hormone levels of animals bearing "growing" or "regressing" mammary tumours.

Oestradiol-17β concentrations were significantly reduced in ovariectomised animals and also in animals given injections of DES, *meso*-DHBS, and CI 628 (Table 4). Administration of oestradiol-17β to ovariectomised animals resulted in an elevation of the plasma oestradiol-17β concentration to values which were not statistically different from control levels. Oestradiol-17β levels, however, were unaffected, by the administration of ICI 46474 and ICI 79792. Again, no apparent differences were detectable in hormone levels of animals bearing "growing" or "regressing" mammary tumours.

Low speed supernatants from both "growing" and "regressing" tumours had the capacity to bind oestradiol-17β (Table 5), ovariectomy did not affect the ability of the regressing tumour to bind oestradiol-17β. However, a tumour that continued to grow after ovariectomy did not contain any specific binding capacity.

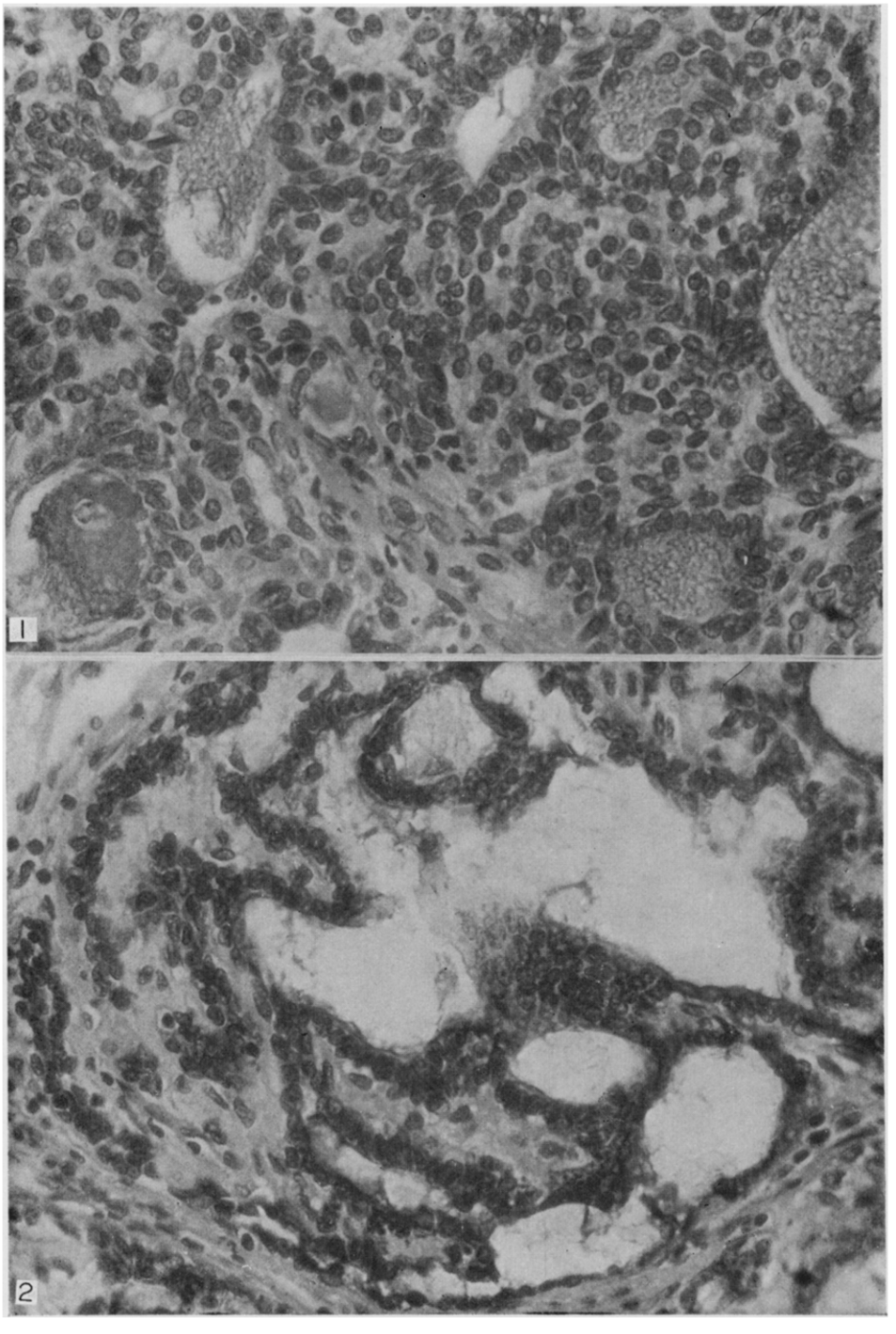


Fig. 1. "*Growing*" *tumour. H* and *E* × 340.

Fig. 2. "*Regressing*" *tumour. H and E* × 340.

(to face p. 574)

Table 2. Histological classification of 186 tumours after treatment with oestradiol-17β or oestrogen antagonists. Groups of animals were given daily injections (i.m.) of 5 μg oestradiol-17β or 100 μg oestrogen antagonist in 0·1 ml sesame oil. Control and ovariectomised animals received 0·1 ml of vehicle alone. Animals were killed 7 days after ovariectomy and their tumours classified histologically as "growing" or "regressing"

		Growing	Regressing	% Regressing
INTACT ANIMALS	(Dose/day)			
Control	-	20	2	9
ICI 46474	(100μg)	7	13	65
ICI 79792	(100μg)	18	4	18
CI 628	(100μg)	7	10	59
meso-DHBS	(100μg)	15	4	21
DES	(100μg)	16	4	20
Oestradiol-17β	(5μg)	14	0	0
OVARIECTOMISED ANIMALS				
Control	-	6	28	82
Oestradiol-17β	(5μg)	11	8	42

Table 3. Enzyme activities in DMBA-induced mammary tumours from intact and ovariectomised Sprague–Dawley rats. Groups of animals were given daily injections (i.m.) of 5 μg oestradiol-17β or 100 μg oestrogen antagonist in 0·1 ml sesame oil. Control and ovariectomised animals received 0·1 ml of vehicle alone. Animals were killed 7 days after ovariectomy and their tumours classified histologically as "growing" or "regressing". Enzyme activity was measured as Units per g protein. Results are expressed as mean ±S.D. Groups containing less than 4 samples were not analysed statistically

		p-Nitrophenylphosphatase Activity		Dehydrogenase Activity	
INTACT ANIMALS	(dose/day)	Growing	Regressing	Growing	Regressing
Control	-	35.3 ± 6.14 (20)	45.4 ± 6.44 (2)	175 ± 24.8 (20)	125 ± 14.8 (2)
ICI 46474	(100μg)	33.3 ± 5.40 (7)	50.3 ± 12.32 (13)*	164 ± 20.6 (7)	74 ± 26.2 (13)*
ICI 79792	(100μg)	38.2 ± 8.43 (18)	42.6 ± 11.20 (4)*	159 ± 21.2 (18)*	152 ± 29.0 (4)*
CI 628	(100μg)	40.3 ± 8.06 (7)	51.5 ± 14.80 (10)*	163 ± 14.3 (7)	70 ± 23.0 (10)*
meso-DHBS	(100μg)	35.7 ± 6.49 (15)	44.8 ± 6.50 (4)*	170 ± 15.3 (15)	133 ± 44.8 (4)*
DES	(100μg)	40.3 ± 7.44 (16)*	36.7 ± 7.84 (4)	201 ± 16.0 (16)*	134 ± 45.6 (4)*
Oestradiol-17β	(5μg)	40.6 ± 6.38 (14)*	-	205 ± 13.3 (14)*	-
OVARIECTOMISED ANIMALS					
Control	-	42.7 ± 9.52 (6)*	59.0 ± 9.75 (28)*	192 ± 12.2 (6)*	88 ± 26.6 (28)*
Oestradiol-17β	(5μg)	45.2 ±10.21 (11)*	50.5 ± 10.61 (8)*	169 ± 14.2 (11)*	110 ± 9.7 (8)*

*$P < 0.05$ (v. intact "growing" controls). P estimated by Student's t-test.

Table 4. Effect of ovariectomy, oestradiol-17β and oestrogen antagonists on plasma prolactin and oestradiol-17β levels in mature female Sprague–Dawley rats. Groups of animals were given daily injections (i.m.) of 5 μg oestradiol-17β or 100 μg oestrogen antagonist in 0·1 ml sesame oil. Control and ovariectomised animals received 0·1 ml of the vehicle alone. Animals were killed seven days after ovariectomy and their tumours classified histologically as "growing" or "regressing". Results are expressed as mean ±S.D. Groups containing less than 4 samples were not analysed statistically

		PROLACTIN (ng/ml)		OESTRADIOL-17β (pg/ml)	
INTACT ANIMALS	(Dose/day)	Growing	Regressing	Growing	Regressing
Control	-	37 ± 23.2 (7)	31 (1)	37 ± 15.6 (7)	32 (1)
ICI 46474	(100μg)	28 ± 17.5 (3)	33 ± 14.4 (5)	28 ± 7.0 (3)	33 ± 13.4 (5)
ICI 79792	(100μg)	23 ± 9.2 (6)	22 ± 11.3 (2)	33 ± 15.6 (6)	44 ± 15.0 (2)
CI 628	(100μg)	35 ± 15.6 (3)	39 ± 21.0 (5)	18 ± 3.5 (3)	13 ± 4.0 (5)*
meso-DHBS	(100μg)	22 ± 10.1 (7)	20 (1)	18 ± 1.9 (7)*	22 (1)
DES	(100μg)	82 ± 20.8 (6)*	63 ± 1.4 (2)	8 ± 3.2 (6)*	18 ± 4.2 (2)
Oestradiol-17β	(5μg)	69 ± 20.4 (8)*	--	62 ± 14.6 (8)*	--
OVARIECTOMISED ANIMALS					
Control	-	12 (1)	10 ± 5.2 (7)*	9 (1)	9 ± 5.4 (7)*
Oestradiol-17β	(5μg)	59 ± 15.3 (4)	56 ± 12.9 (4)	39 ± 10.6 (4)	37 ± 6.6 (4)

*$P < 0.05$ (v. intact "growing" controls). P estimated by Student's t-test.

Table 5. Effect of ovariectomy, oestradiol-17β and oestrogen antagonists on the oestradiol-17β binding capacity in DMBA-induced rat mammary tumours. Group of animals were given daily injections (i.m.) of 5 μg oestradiol-17β or 100 μg oestrogen antagonist in 0·1 ml sesame oil. Control and ovariectomised animals received 0·1 ml vehicle alone. Animals were killed 7 days after start of treatment and tumours classified histologically as "growing" or "regressing" Oestradiol-17β binding capacity was measured as cpm/mg protein. Results are expressed as median with range in parenthesis. NCB = No counts bound. Groups containing less than 4 samples were not analysed statistically

		Oestradiol-17β binding capacity (cpm/mg protein)					
		Growing		P	Regressing		P
INTACT ANIMALS (Dose/day)							
Control	-	815 (264 - 1675)	(n=8)		604	(n=1)	
ICI 46474	(100μg)	NCB	(n=3)		NCB	(n=4)	<0.01
ICI 79792	(100μg)	386 (NCB - 859)	(n=5)	NS	235 (194 - 275)	(n=2)	
CI 628	(100μg)	NCB	(n=3)		NCB	(n=5)	<0.01
meso-DHBS	(100μg)	796 (114 - 1833)	(n=7)	NS	651	(n=1)	
DES	(100μg)	50 (NCB - 794)	(n=6)	<0.01	112 (NCB - 221)	(n=2)	
Oestradiol-17β	(5μg)	92 (14 - 241)	(n=8)	<0.01	-		
OVARIECTOMIZED ANIMALS							
Control	-	NCB	(n=1)		482 (257 - 1698)	(n=6)	NS
Oestradiol-17β	(5μg)	36 (NCB - 134)		<0.01	29 (NCB - 220	(n=4)	<0.01

P (v. intact growing controls) was determined by Mann Whitney U test.

Table 6. Effect of ovariectomy, oestradiol-17β and oestrogen antagonists on uterine and pituitary weights in mature Sprague–Dawley rats. Groups of animals were given daily injections (i.m.) of 5 μg oestradiol-17β or 100 μg oestrogen antagonist in 0·1 ml sesame oil. Control and ovariectomised animals received 0·1 ml of the vehicle alone. Animals were killed 7 days after ovariectomy and their tumours classified histologically as "growing" or "regressing". Results are expressed as mean ±S.D. Groups containing less than 4 samples were not analysed statistically

		ORGAN WEIGHT (mg/g initial body weight.)			
		UTERUS		PITUITARY	
		Growing	Regressing	Growing	Regressing
INTACT ANIMALS (Dose/day)					
Control	-	2.06 ± 0.435(10)	1.96 ± 0.532(2)	0.057 ± 0.021(10)	0.059 ± 0.016(2)
ICI 46474	(100μg)	1.23 ± 0.160(5)*	1.19 ± 0.03 (8)*	0.053 ± 0.006(5)	0.052 ± 0.010(8)
ICI 79792	(100μg)	1.72 ± 0.491(10)	1.52 ± 0.410(3)	0.059 ± 0.013(10)	0.058 ± 0.016(3)
CI 628	(100μg)	1.34 ± 0.300(5)*	1.41 ± 0.320(9)*	0.054 ± 0.010(5)	0.051 ± 0.010(9)
meso-DHBS	(100μg)	1.69 ± 0.210(10)	1.62 ± 0.13 (2)	0.053 ± 0.011(10)	0.054 ± 0.012(2)
DES	(100μg)	2.14 ± 0.361(3)	2.04 ± 0.335(9)	0.058 ± 0.012(3)	0.056 ± 0.006(9)
Oestradiol-17β	(5μg)	2.38 ± 0.329(10)	-	0.055 ± 0.007(10)	-
OVARIECTOMISED ANIMALS					
Control	-	1.06 ± 0.293(3)	1.10 ± 0.332(11)*	0.053 ± 0.016(3)	0.053 ± 0.022(11)
Oestradiol-17β.	(5μg)	1.91 ± 0.466(7)	1.93 ± 0.339(6)	0.067 ± 0.019(7)	0.068 ± 0.016(6)

*$P < 0·05$ (v. intact "growing" controls). *P* estimated by Student's *t*-test.

Administration of oestradiol-17β to both intact and ovariectomised animals resulted in a significant drop in binding capacity of both "growing" and "regressing" tissue (Table 5). Tumours from animals treated with DES, CI 628 and ICI 46474 exhibited significantly reduced capacities to bind [^{3}H]oestradiol-17β, whereas animals treated with ICI 79792 and *meso*-DHBS exhibited binding similar to that seen in control animals.

The weight of the uterus was significantly reduced by ovariectomy and the administration of either ICI 46474 or CI 628 (Table 6). All the other compounds had no effect on uterine wt. Administration of oestradiol-17β to ovariectomised animals maintained a uterine weight similar to that observed in intact animals. The wt of the pituitary gland was unaffected by all forms of treatment. No differences were detectable between animals bearing "growing" or "regressing" tumours.

DISCUSSION

It is well established that the growth of hormone-dependent mammary tumours is altered significantly by the removal of the ovaries from the host [1]. In earlier papers we reported a correlation between tumour regression after ovariectomy, and the levels and

properties of certain acid hydrolases [7, 12, 13]. The present experimental data show the validity of using histological or biochemical parameters as indicators of hormonal effects on short term growth patterns of such tumours. After ovariectomy or administration of anti-oestrogens, hormone dependent tumours show early alterations of enzyme activity, which precede changes in tumour volume. The levels of activity relate to well-defined histological characteristics and were found to be different from values determined in "growing" or autonomous tumours. Such enzyme assays can therefore provide an accurate means of monitoring tumour regression and reflect the hormone dependency of the tissue.

There is still a great deal of conjecture surrounding the identity of the hormone or hormones which control the growth of the DMBA-induced mammary tumour. Prolactin and oestradiol-17β, both individually and also synergistically, have been implicated in this process [14–16]. There can be little doubt that tumours which are responsive to ovariectomy contain oestradiol-17β receptors and that autonomous tumours have reduced amounts of this protein [17–21]. An early study by Terenius [6] established that the oestrogen antagonist, U11, 100A, was capable of inhibiting DMBA-induced mammary tumour growth and that the drug inhibited the *in vitro* binding of oestradiol-17β to the specific tumour receptors. The possibility of a second oncolytic mechanism resulting from a decrease in circulating prolactin levels following the administration of the anti-oestrogen cannot be excluded. In fact, the increase of prolactin secretion caused by oestradiol-17β administration to ovariectomised animals can be partially blocked by U11, 100A, treatment [22].

The studies described in this paper were performed on a population of tumours attaining an approximate size of 20 mm mean diameter within 23 wk of DMBA administration and showing an 80% response to ovariectomy. Other studies in these laboratories have led us to believe that a second population of tumours exist in older animals which have only a 50% response to ovariectomy (unpublished data). The results are consistent with the data of Griswold and Green [23], who reported a gradual increase in autonomy in DMBA induced tumours with increasing time between tumour induction and treatment. In view of the experimental procedure, however, it is unlikely that the results reported here are due to an increased population of autonomous tumours within the test groupings.

The present report clearly demonstrates that the plasma prolactin and oestradiol-17β levels together with uterine weight were decreased within seven days of ovariectomy. Administration of oestradiol-17β to ovariectomised animals, whilst restoring prolactin and oestradiol-17β levels to those of control animals and preventing a fall in uterine wt, was unable to prevent the post-operative regression in all tumours studied. The plasma concentration of hormones and uterine wt were similar in animals bearing responsive and non-responsive tumours. These results suggested that there may be factors inherent within the tumour which are responsible for control of tumour growth rather than purely host factors. "Growing" tumours in ovariectomised animals given oestradiol-17β supplements, contained elevated acid hydrolase levels and reduced dehydrogenase activities. Such levels are normally associated with tumour "regression" and may reflect an imminent regression state within these tumours.

Administration of DES, like oestradiol-17β, caused an elevation of the plasma prolactin concentration in intact animals and may well account for the lack of an oncolytic effect at this dose level. As expected, however, administration of oestradiol-17β decreased the binding capacity of the tumour tissue. Similar effects were produced by DES, probably reflecting the compound's known oestrogenic properties [24].

Meso-DHBS, a structural analogue of DES, was ineffective in causing tumour regression despite reducing the plasma oestradiol-17β concentration. This lack of oncolytic activity may relate to its ineffectiveness in reducing the oestradiol-17β binding capacity of the tumour. This compound does, however, produce prostatic atrophy in the male rat [10].

Treatment of intact animals with the anti-oestrogens ICI 46474 and CI 628 had little effect on the circulating levels of prolactin and oestradiol-17β. Clearly, the effect of these compounds, the administration of which resulted in uterine atrophy and extensive inhibition of the growth of certain tumours, was not mediated through changes in the plasma hormone levels. After treatment, responsive and non-responsive tumour tissue had a reduced capacity to bind oestradiol-17β, as determined by the procedure used. It was not possible to determine whether this was due to a loss of available receptor binding protein or to a saturation of the available binding sites by the anti-oestrogen. Callintine and his colleagues [25] have demonstrated that CI 628 was weakly oestrogenic when administered to

ovariectomised or immature rats and this may account for its failure to produce an 80% regression response. There is no evidence for oestrogenic properties of ICI 46474 under the experimental conditions used (Nicholson—unpublished data).

ICI 79792, a *nitrogen mustard* derivative of ICI 46474, was ineffective in causing tumour regression and its administration did not result in changes in the circulating levels of oestradiol-17β or prolactin. Treatment of animals with this compound did not significantly reduce the oestradiol-17β binding capacity of tumour tissue. This observation relates to evidence from other workers in this Institute [26] who have shown that ICI 79792 is less effective than ICI 46474 in reducing the oestradiol-17β binding to specific receptor protein isolated by sucrose density gradient centrifugation.

The observation that in a number of "regressing" tumours, restimulation of growth occurred after approximately 6 wks was of particular interest. Studies to determine the reasons for the resultant growth could provide valuable information relevant to the treatment of recurrent breast cancer in the human.

Acknowledgements—We are very grateful to Dr. A. L. Walpole and Dr. D. N. Richardson (ICI Ltd) for gifts of ICI 46474, ICI 79792 and *meso*-dihydrodibutylstilboestrol.

We are indebted to Dr. D. Fahmy and Miss K. Baker for the measurement of plasma oestradiol-17β concentrations and to Mrs. D. Jenner for expert technical assistance. We thank Professor K. Griffiths for his advice and encouragement and the Tenovus Organization for generous financial support.

REFERENCES

1. C. Huggins, L. G. Grand and F. P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature (Lond.)* **189,** 204 (1961).
2. P. N. Daniel and M. M. L. Pritchard, The response of experimentally induced mammary tumours in rats to hypophysectomy and to pituitary stalk section. *Brit. J. Cancer* **17,** 446 (1963).
3. M. N. Teller, C. C. Stock, G. Stohr, P. C. Merker, R. J. Kaufman, G. C. Escher and M. Bowie, Biological characteristics and Chemotherapy of 7,12-dimethylbenz(a)anthracene-induced tumours in rats. *Cancer Res.* **26,** 245 (1966).
4. D. P. Griswold, H. E. Skipper, W. R. Laster, Jr., W. S. Wilcox and F. M. Schabel, Jr., Induced mammary carcinoma in the female rat as a drug evaluation system. *Cancer Res.* **26,** 2169 (1966).
5. K. D. Schulz, B. Haselmeier and F. Holzel, The influence of clomid and its isomers upon dimethylbenzanthracene-induced rat mammary tumours. *Acta endocr. (Kbh).* Suppl. 138, 236 (1969).
6. L. Terenius. Anti-oestrogens and breast cancer. *Europ. J. Cancer* **7,** 57 (1971).
7. R. I. Nicholson, I. Bagnall and M. Davies, Lysosomal enzymes in 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma after ovariectomy. *Europ. J. Cancer* **9,** 313 (1973).
8. A. Kornberg and B. L. Horecker, *Methods in Enzymology*, 1, Academic Press, New York (1955).
9. C. B. Korsten and J.-P. Persijn, A simple assay for specific oestrogen binding capacity in human mammary tumours. *Z. Klin. Chem. Klin. Biochem.* **10,** 502 (1972).
10. V. Danutra, M. E. Harper, A. R. Boyns, E. N. Cole, B. G. Brownsey and K. Griffiths, The effect of certain stilboestrol analogues on plasma prolactin and testosterone in the rat. *J. Endocr.* **57,** 207 (1973).
11. E. H. D. Cameron and D. A. Jones, Some observations on the measurement of oestradiol-17β in human plasma by radioimmunoassay. *Steroids* **20,** 737 (1972).
12. R. I. Nicholson and M. Davies, Distribution and some properties of acid phosphatase in the 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinoma. *Europ. J. Biochem.* **44,** 25 (1974).
13. R. I. Nicholson and M. Davies, *Europ. J. Biochem.* Submitted for publication.
14. O. H. Pearson, A. Molina, T. P. Butler, L. Llerena and H. Nasr, Estrogens and prolactin in mammary cancer. In *Estrogen Target Tissues and Neoplasia* (Edited by T. L. Dao), p. 287. The University of Chicago Press, Chicago (1972).

15. A. Sterental, J. M. Dominguez, C. Weismann and O. H. Pearson, Pituitary role in oestrogen dependency of experimental mammary cancer. *Cancer Res.* **23,** 481 (1963).
16. D. Sinha, D. Cooper and T. L. Dao, The nature of oestrogen and prolactin effect on mammary tumourgenesis. *Cancer Res.* **33,** 411 (1973).
17. S. Sanders, *In vitro* uptake of oestradiol in DMBA-induced breast tumours of the rat. *Acta. path. microbiol. scand.* **75,** 520 (1969).
18. P. W. Jungblut, E. R. DeSombre and E. V. Jenson, Estrogen receptors in induced rat mammary tumours. In *Hormone in Genese und Therapie des Mammary Carcinomas. Abhandlungen der deutschen Akademie der Wissenschaften,* p. 109. Academie Verlag, Berlin (1967).
19. R. J. B. King, D. M. Cowan and D. R. Inman, The uptake of 6,7-^{3}H oestradiol by DMBA-induced rat mammary tumours. *J. Endocr.* **32,** 83 (1965).
20. B. G. Mobbs, The uptake of tritiated oestradiol by dimethylbenzanthracene induced rat mammary tumours. *J. Endocr.* **36,** 409 (1966).
21. S. Sanders and A. Attramadal, The *in vitro* uptake of oestradiol-17β by hormone responsive and unresponsive breast tumours in the rat. *Acta path. microbiol. scand.* **74,** 169 (1968).
22. J. C. Heuson, C. Waelbroeck, N. Legros, G. Gallez, C. Robyn and N. L'Hermite, Inhibition of DMBA-induced mammary carcinogenesis in the rat by 2-Br-α-Ergocryptine (CB154), an inhibitor of prolactin secretion, and by Nafoxidine (U 11,100A), an oestrogen antagonist. *Gynecol. Invest.* **2,** 130 (1972).
23. D. P. Griswold, Jr. and C. H. Green, Observations on the hormone sensitivity of 7,12-dimethylbenz(a)anthracene-induced mammary tumours in the Sprague–Dawley rat. *Cancer Res.* **30,** 819 (1970).
24. J. Grundy, Artificial estrogens. *Chem. Reviews* **57,** 281, (1957).
25. M. R. Callantine, R. R. Humphrey, L. L. Lee, B. L. Windsor, N. H. Schottin and O. P. O'Brien, Action of an oestrogen antagonist on reproductive mechanisms in the rat. *Endocrinology* **79,** 153 (1966).
26. W. Powell-Jones, D. A. Jenner, R. W. Blamey, P. Davies and K. Griffiths, *Biochem. J.* **150,** 71 (1975).

Europ. J. Cancer Vol. 11, pp. 581–583. Pergamon Press 1975. Printed in Great Britain

Lethal and Sublethal Cellular Injury in Multifraction Irradiation

H. RODNEY WITHERS

Section of Experimental Radiotherapy, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, 6723 Bertner Avenue, Houston, Texas, U.S.A.

The dose D_r measured by Dutreix, Wambersie and Bounik, and reported in their recent paper in this journal [1] is not the same as $(D_N - D_1)/(N-1)$ which is the usual measure of recovery per fractionation interval and for which D_r would be a more apt symbol. The value measured by Dutreix *et al* would be better described as ΔD_r since they measured the *increase in the dose, expressed as the incremental dose per fraction*, required to achieve an iso-effect when the fraction number was doubled.

The difference between D_r and ΔD_r can be best illustrated by an example. Assume a certain effect could be achieved with a single dose of 1350 rads, 2 doses of 800 rads, 4 doses of 450 rads or 8 doses of 240 rads. If there had been no additional sparing from dividing the dose into 4 instead of 2 fractions, the dose schedule would have been 4×400 rads. Since the dose needed per fraction was 450 rads, $\Delta D_r = 50$ rads. Similarly, had there been no additional recovery from dividing the dose into 8 fractions instead of 4, the dose per fraction would have been 1800/8 = 225 rads: since a dose of 8×240 was needed, $\Delta D_r = 15$ rads for an increase from 4 to 8 fractions.

It is not necessary that the number of dose fractions be doubled to measure ΔD_r. Since it is the increase in dose per fraction needed for an iso-effect when the number of dose fractions is increased (from N to N'), a more general expression is:

$$\Delta D_r = \frac{\textit{Total dose in } N' \textit{ fractions}}{N'} - \frac{\textit{Total dose in } N \textit{ fractions}}{N'}$$

$$= \frac{D_{N'} - D_N}{N'}$$

Since the value for ΔD_r is not only dependent upon doses per fraction, but also upon the magnitude of change in fraction number, the expression will be written as:

$$\Delta D_{r(N'/N)} = \frac{D_{N'} - D_N}{N'}$$

For comparison,

$$D_r = \frac{D_{N'} - D_1}{N'-1} \quad \text{or} \quad \frac{D_N - D_1}{N-1}$$

The differences between D_r and ΔD_r for the hypothetical example used above are shown in Table 1.

Table 1

$2N$	D_r (rads)	$\Delta D_{r(2N/N)}$ (rads)
2	$\frac{1600-1350}{1} = 250$	$\frac{1600-1350}{2} = 125$*
4	$\frac{1800-1350}{3} = 150$	$\frac{1800-1600}{4} = 50$
8	$\frac{1920-1350}{7} = 93$	$\frac{1920-1800}{8} = 15$

*This value is only conceptually valid if the single dose, 1350 rads, is considered to be two equal doses of 675 rads.

Although it is not shown in Table 1, the increase in dose-per-fraction if the fraction number were increased from 2 to 8 would be

$$\frac{D_8 - D_2}{8} = \frac{1920-1600}{8} = 40 \text{ rads.}$$

Thus, instead of replacing 2×800 rads by 8×200 rads, the new dose schedule for the same effect would be 8×240 rads. Since possible changes in fraction number are limitless, further discussion in this letter will be restricted, for convenience,

Accepted 10 October 1973.
*This work was supported in part by Grants CA 11138 and CA 06294 from the National Institutes of Health.

to examples in which the number of fractions is doubled.

Values for ΔD_r and D_r diverge rapidly at low doses-per-fraction (Table 1). A simple explanation of this is as follows: The amount of sublethal injury deposited is proportional to dose and hence, as dose is increased, so is the amount of sublethal injury deposited and repaired. Thus, D_r increases with dose, even at very low doses. However, at low doses, when all sublethal damage is completely repaired and cell killing results only from non-repairable injury, decreasing the dose-per-fraction cannot increase the amount of sublethal injury repaired, and hence, cannot increase further the sparing effect of dose-fractionation. Therefore, at low doses-per-fraction, ΔD_r approaches zero.

As Dutreix *et al.* have emphasized, the absolute amount of sublethal injury repaired per fractionation interval (D_r) is not as important to radiotherapists as the change in the amount repaired (ΔD_r) when the dose-per-fraction is altered. ΔD_r is a practical value and is the type of information radiotherapists seek from iso-effect formulae [2, 3]. In their Fig. 6, Dutreix *et al.* plotted values for ΔD_r on the same graph as values of D_r for skin and other tissues. Other data for ΔD_r are presented here on the same graph (Fig. 1) as the data of Dutreix *et al.*, for human skin. The value for ΔD_r for human skin at a dose of 200 rads is shown as 25 rads or less, but it may be closer to 0 than 25 rads (1, 13). Thus the human and murine data may be more separated at low doses than suggested by the symbols in Fig. 1.

Except for gastric mucosa [5] and bone marrow (measured by $LD_{50/30}$ [6]), *most* values for ΔD_r are not greatly different from those for human skin [1]. The overall treatment durations in experiments on gastric mucosa and hemoleucopoietic tissue were 9 and 11 days respectively and rapid repopulation probably increased ΔD_r above that expected to result from repair of sublethal injury alone. The data for colon [7] and jejunum [8] came from experiments using 3-hr fractionation intervals which minimized repopulation. Callus-forming tissue [9], lung [10] and human skin [1] would be expected to undergo minimal repopulation in the experiments from which the data in Fig. 1 were drawn. Repopulation was also excluded as a significant factor in the 5 and 10 dose fraction data obtained by Howes *et al.* [11] for mouse mammary carcinoma. Although the differences between data in Fig. 1 are small they could be significant, especially since they would be multiplied by fraction number in clinical practice.

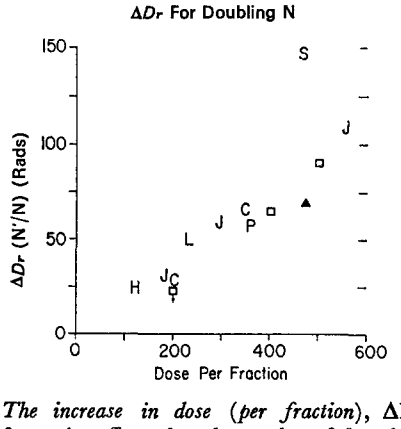


Fig. 1. The increase in dose (per fraction), $\Delta D_{r(2N/N)}$ necessary for an iso-effect when the number of dose fractions is increased from N to N′ plotted as a function of the new (lower) dose-per-fraction. The data were taken from experiments in which 3, 4, 5, 10 or 20 dose fractions were given and all ΔD_r values relate to a doubling in the number of fractions. The tissues irradiated were human skin (□), hemoleucopoietic tissue (H), jejunum (J), descending colon (C), lung (L), callus-forming periosteal tissue (P), stomach (S) and a mouse mammary carcinoma (▲).

The data in Fig. 1, derived from a variety of tissues, support the conclusion of Dutreix *et al.*, that when a dose is divided into a large number of fractions, further increase of fraction number is of less importance to tissue sparing than is suggested by the iso-effect formulae of Ellis [2] or Cohen [3]. This reflects the importance of non-repairable injury in determining the response of tissues to low doses.

The other implication from the data in Fig. 1 is that the survival curve shape at low doses must be similar for the cells of the eight various tissues—that is, their sensitivity to non-repairable damage must be similar as must their capacity for sublethal damage and its repair. Furthermore, there is no appreciable difference in ΔD_r between normal tissues and the one malignant tissue for which data are available. This is similar to the data for D_r [4, 12]. The data for stomach probably reflect the large effect of repopulation on the incremental dose necessary for an iso-effect in a rapidly proliferating tissue when the overall time is extended. Similar conclusions have been reached by Wambersie *et al.* [13] based on experiments in which mouse intestine and skin were exposed to multiple doses.

In summary, although there is a critical divergence at low doses, the data for mouse tissues are similar to those given for human skin by Dutreix *et al.* [1] and support the conclusions:

i. that the capacity of many normal cells for accumulating and repairing sublethal radiation injury is probably not greatly different [4, 12].

ii. that fixed exponents used for fraction number and time in iso-effect formulae [2, 3] are inappropriate. At low doses-per-fraction, repair of sublethal injury is complete, or nearly so, and hence, additional fractionation of dose does not give appreciable additional sparing, whereas rapidly-regenerating tissues, due to the lengthening of overall time, would continue being spared by repopulation.

Acknowledgements—I am indebted to Dr. A. E. Howes for permitting me to use unpublished data.

REFERENCES

1. J. Dutreix, A. Wambersie and C. Bounik, Cellular recovery in human skin reactions: Application to dose fraction number overall time relationship in radiotherapy. *Europ. J. Cancer* **9,** 159 (1973).
2. F. Ellis, Nominal standard dose and the ret. *Br. J. Radiol.* **44,** 101 (1971).
3. L. Cohen, The statistical prognosis in radiation therapy. *Amer. J. Roentgenol.* **84,** 741 (1960).
4. H. R. Withers, Recovery in normal and malignant tissues. IVth International Congress of Radiation Research, Evian, France (1970).
5. K. Y. Chen and H. R. Withers, Survival characteristics of stem-cells of gastric mucosa exposed to localized gamma irradiation in C_3H mice. *Int. J. Radiat. Biol.* **21,** 521 (1972).
6. T. L. Phillips and E. J. Ainsworth, Altered split-dose recovery in mice irradiated under hypoxic conditions. *Radiat. Res,* **39,** 317 (1969).
7. H. R. Withers and K. Mason, The kinetics of recovery in irradiated colonic mucosa of the mouse. *Cancer* **34,** 896 (1974).
8. H. R. Withers, A. M. Chu and B. O. Reid (In preparation.)
9. S. Hayashi and H. D. Suit, Effect of fractionation of radiation dose on callus formation at site of fracture. *Radiology* **101**[6] 181 (1971).
10. T. L. Phillips, Split-dose recovery in euoxic and hypoxic normal and tumor cells. *Radiology* **105,** 127 (1972).
11. A. E. Howes and H. D. Suit, personal communication.
12. H. R. Withers, Capacity for repair in cells of normal and malignant tissues. In *Time & Dose Relationships in Radiation Biology as Applied to Radiotherapy,* Brookhaven National Laboratory Report 50203 (C-57), 1970, pp. 54–69.
13. A Wambersie, J. Dutreix, J. Gueulette and J. Lellough, Early recovery for intestinal stem cells, as a function of dose per fraction, evaluated by survival rate after fractionated irradiation of the abdomen of mice *Radiat. Res.* **58,** 498 (1974).

Europ. J. Cancer Vol. 11, pp. 585–587. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Combination Chemotherapy with CCNU, Vincristine and Methotrexate in Primary and Metastatic Brain Tumors*

J. HILDEBRAND†, J. BRIHAYE‡, L. WAGENKNECHT†, J. MICHEL† and Y. KENIS†

†*Service de Médecine et Laboratoire d'Investigation Clinique and* ‡*Service de Neurochirurgie et Unité de Neurologie Médico-Chirurgicale, Institut Jules Bordet,* 1000 *Bruxelles, Belgium*

PREVIOUSLY, we have reported preliminary results on the antitumoral effect of combination therapy with 1-(2-chloroethyl-3-cyclohexyl-1-nitrosourea) (CCNU) given orally every 6th week: 100 mg/m^2, vincristine (VCR) and methotrexate (MTX) given at respective dosages of 2 mg/m^2 and 24 mg/m^2, both once a week for 4 weeks and subsequently once every 4 weeks [1]. This study has now been completed and the purpose of this communication is to report its final results. Of 65 patients selected for the study, 53 were treated according to the protocol and received adequate follow-up. The criteria for patients' selection and details concerning the administration of the drugs and dexamethazone (DXM) have been previously described [1].

The first purpose of this trial was to study the rate and duration of objective remissions, defined as clinical improvement persisting 6 weeks after complete discontinuation of DXM. The results are given in Table 1. Eight of 22 patients with primary brain tumors responded to the treatment. The rate of response appeared to be higher in patients with more slowly growing primary tumors than in those with malignant glioma, but the duration and the quality of the remissions were comparable in the two groups. Objective improvement was also observed in 5 of 11 cases with metastases of breast carcinoma but the length of the remissions was significantly shorter compared to patients with primary tumors. Only one patient of 20 with metastases from lung carcinoma responded to chemotherapy. He died after an 80-week remission and the recurrence of brain metastases was confirmed by pathological examination.

Although the study was not designed to evaluate the survival, it appeared that patients with malignant glioma and brain metastases who responded to the treatment, survived longer that those who failed to respond (Table 1).

The second aim of this investigation was to study the modifications of EEG and brain scan in patients who responded to treatment. Table 2 shows that the correlation between the three parameters was fairly good. Thus, during the neurological remission, EEG improved in 11 patients and remained unchanged in two. Brain scan was improved in 8 and was unchanged in three. In no case did we notice a a worsening of the EEG or of the brain scan in patients with improving neurological status. When clinical symptoms of relapse occurred, EEG worsened in all patients but one and brain scan in all but two.

Bone marrow toxicity shown in Table 3 was related to the number of CCNU courses. It was less prominent in patients with primary brain tumors. Thrombopenia was the most frequent feature. All patients who received more than 9 mg of VCR showed some degree

Accepted 15 May 1975.

*Supported by a grant from the 'Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite de Belgique' and Grant No. 3.5459.75 from the 'Fonds de la Recherche Scientifique Médicale' (F.N.R.S.—Belgium).

of peripheral neorupathy but distal and symmetrical weakness was seen in only 6 patients who received 15 mg of VCR or more. No death could be attributed to drug toxicity.

To summarize, these data extend our preliminary results and confirm several previous investigations concerning the therapeutic effects of nitrosoureas, used alone or in combination, in brain tumors. The studied combination therapy is also active in metastases of breast carcinoma but has practically no effect on metastases of lung cancer.

Table 1. *Antitumoral effect of CCNU-VCR-MTX combination*

Tumors	Number of cases	Rate	Remissions Duration (wk)		Survival from beginning of chemotherapy (wk) Patients with remission		Patients without remission	
			Range	Mean	Range	Mean	Range	Mean
Primary								
Malignant glioma	16	4/16	6–85	37	18–87	53	4–16	12
Other glioma*	6	4/6	24–61	41	39–88	69	16–80	48
Metastases								
Breast	11	5/11	4–33	16	28–45	35	4–50	16
Lung	20	1/20	—	80	—	92	1–15	5

*(5 were astrocytoma, grade I–II, and 1 ependymoma).

Table 2. *Changes of EEG and brain scan during clinical remission and relapse*

Diagnosis	During remission		During relapse	
	EEG	Brain scan	EEG	Brain scan
Primary brain tumors	NC	NC	NC	NC
	Im	Im	PR	PR
	Im	NP	PR	PR
	NC	Im	PR	PR
	Im	Im	PR	PR
	Im	NP	PR	PR
	Im	Im	PR	PR
Metastases	Im	Im	PR	PR
	Im	Im	PR	NC
	Im	NC	NP	NP
	Im	NC	PR	PR
	Im	Im	PR	PR
	Im	Im	PR	PR

Abbreviations:
Im: improved; NC: no change; NP: not performed; PR: progression.

Table 3. *Bone marrow toxicity of CCNU-VCR-MTX combination*

Tumors	Number of courses with CCNU	Number of patients			
		Total	With thrombopenia*	With granulopenia†	With anemia‡
Primary	1 to 3	9	3	1	1
	4 to 7	6	2	1	0
	8 or more	7	5	3	1
Secondary	1 to 3	24	10	5	0
	4 to 7	5	5	4	4
	8 or more	2	2	0	0

*Platelets < 100,000/mm^3
†Leucocytes < 3 000/mm^3
‡Hemoglobin < 10 g/100 ml.

REFERENCES

1. J. HILDEBRAND, J. BRIHAYE, L. WAGENKNECHT, J. MICHEL and Y. KENIS, Combination chemotherapy with 1-(2-chloroethyl-3-cyclohexyl-1-nitrosourea) (CCNU), vincristine and methotrexate in primary and metastatic brain tumors. A preliminary report. *Europ. J. Cancer* **9,** 627 (1973).

Europ. J. Cancer Vol. 11, pp. 589–591. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Effect of Irradiation on Immune Cell-Mediated Cytolysis of Tumour Cells*

TAEWHAN KIM, CHANG W. SONG and SEYMOUR H. LEVITT
Department of Therapeutic Radiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455, *U.S.A.*

IN ORDER to investigate the combined effect of radiation and cell-mediated immune reaction under situations similar to clinical radiotherapy, we performed the present study in which a mixture of target tumor cells and immune lymphocytes were irradiated and the destruction of tumor cells was studied *in vitro*. It was found that the effect of immune lymphocytes plus X-irradiation was at least additive in causing damage to the tumor.

The tumor cells used were ascitic P-815-X2 mastocytoma of DBA/2J mice. Tumor cells growing in medium RPMI 1640 supplemented with 10% fetal calf serum were labeled with ^{125}I-iododeoxyuridine (^{125}IUdR) by incubating $3{\cdot}5 \times 10^5$ tumor cells/ml with 0·004 μCi/ml of ^{125}IUdR at 37° for 4 hr in a plastic culture tube with gentle rocking. The labeled cells were washed carefully [1] and suspended in the medium. The radioactivity of these labeled tumor cells was 500–700 cpm/1×10^5 cells when counted with a well-type γ-counter. Immune splenic lymphocytes were obtained from C57BL male mice which were inoculated with 3×10^7 of the mastocytoma cells 9–11 days previously [1, 2]. The labeled tumor cells and the lymphocytes were mixed with a concentration of 1×10^5 tumor cells and 2×10^5 lymphocytes per ml. Half of this mixture was irradiated with 250 rads of X rays in a single exposure. The radiation factors were 220 kV, 15 mA, 290 rads/min and 1 mm Al filter. The remainder was sham-irradiated and used as a control. Immediately after the irradiation, the mixture was transferred into plastic culture tubes, 2 ml each, and incubated at 37° with 5% CO_2 in air with continuous rocking (5 cycles/min). At different time intervals, the radioactivities in the incubation mixture and that in the supernatant were counted and the extent of damage to the tumor cells was calculated from the percent ^{125}IUdR release as follows [1]:

$$\%\ ^{125}\text{IUdR release} = \frac{^{125}\text{IUdR activity in 1 ml of supernatant} \times 2}{^{125}\text{IUdR activity in 2 ml of incubation mixture}} \times 100$$

As shown in Fig. 1 and Table 1, there was a 22·9% release of ^{125}IUdR at the end of 48 hr incubation in the mixture of ^{125}IUdR labeled tumor cells and control lymphocytes, (T+ Con. Lym.). This non-immunologic release could be due to spontaneous death of the tumor cells during the *in vitro* culture and also to toxicity of ^{125}IUdR [3, 4]. Studies with various doses of ^{125}IUdR for labeling, however, indicated that the toxicity of ^{125}IUdR with the concentration we used in the present study played an insignificant role in the non-immunologic release of ^{125}IUdR (Fig. 2). The simplicity and objectivity of the ^{125}IUdR release method are some of the many advantages of this method over currently available assay

Accepted March 1975.

*Supported by research grants from the Minnesota Division of the American Cancer Society, Minnesota Medical Foundation and USPHS (CA-08832).

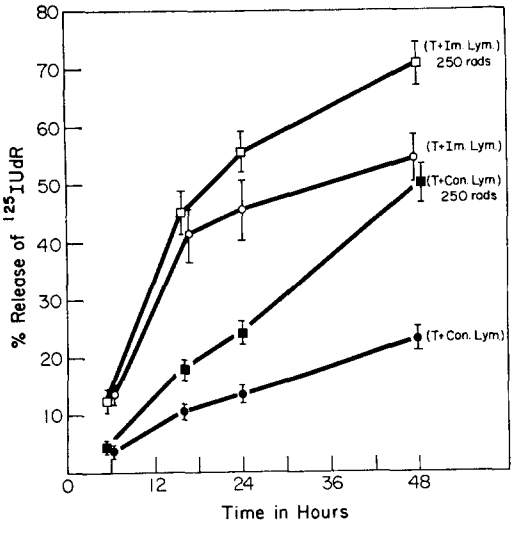


Fig. 1. *Percentage of release of* ^{125}*IUdR labeled target cells (P-815-X2 mastocytoma cells of DBA/2J mice) mixed with control lymphocytes of allogenic immune lymphocytes from C57BL mice* in vitro. *Each point is the mean of* 11–13 *experiments, and the vertical bars show s.e. of the mean.*

Table 1. *Percentage of release of* ^{125}*IUdR at* 48 *hr*

	Total release	*Net release
(T + Con. Lym.)	22·9 ± 1·9†	
(T + Con. Lym.) 250 rads	50·0 ± 3·4	27·1
(T + Im. Lym.)	54·4 ± 3·9	31·6
(T + Im. Lym.) 250 rads	70·7 ± 3·5	47·8

*Net % release of ^{125}IUdR was obtained by subtracting 22·9% by non-immunologic reaction (T + Con. Lym.), from the total % release.
†Mean ± s.e. of the mean.

systems in measuring the cytolysis of tumor cells *in vitro* [5–8].

At the end of 48 hr incubation, the net ^{125}IUdR release by the immune lymphocytes was 31·6% of the total ^{125}IUdR. The ^{125}IUdR release in the mixture of tumor cells and control lymphocytes that had been irradiated with 250 rads at the beginning of incubation, (T + Con. Lym.) 250 rads, steadily increased until the termination of incubation at 48 hr resulting in a net release of 27·1%. The net ^{125}IUdR release in the irradiated incubation mixture of tumor cells and immune lymphocytes, (T + Im. Lym) 250 rads, was 47·8% at 48 hr. Our previous study [2] indicated that X-irradiation with 250 rads decreased the cytotoxicity of immune lymphocytes by about 17%. Since the immune lymphocytes in the irradiated mixture of tumor cells and lymphocytes in the present study also received 250 rads of X rays, the cytolysis of the tumor cells in this mixture could be regarded as a combined result of radiation damage to the tumor cells and immune reaction of irradiated immune lymphocytes, whose cytotoxicity was 83% of the unirradiated lymphocytes. A calculation using the formula described by Scott [9] for the combined effect of two agents suggested that the 47·8% release of ^{125}IUdR in the irradiated mixture of tumor cells and immune lymphocytes was an additive effect of radiation damage and irradiated immune lymphocytes to the tumor cells. Further study is in progress in our laboratory in order to have a more detailed insight into the combined effect of irradiation and cell-mediated immune reaction on tumor cells, and also to elucidate the role of host immune response in radiotherapy of cancer.

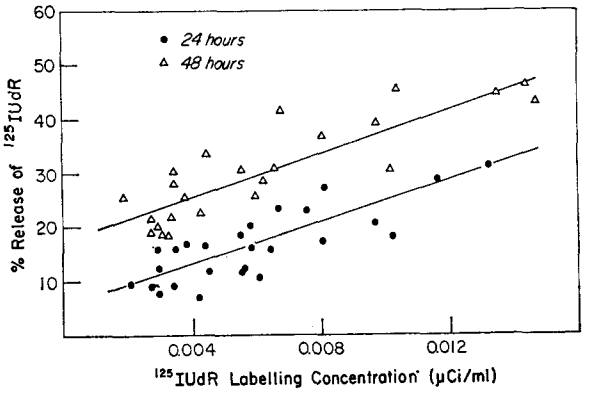


Fig. 2. *Non-immumologic release of* ^{125}*IUdR from* ^{125}*IUdR labeled target cells (P-815-X2 mastocytoma cells of DBA/2J mice)* in vitro *as a function of the concentration of* ^{125}*IUdR after incubating the labeled cells with control lymphocytes for* 24 *hr and* 48 *hr is shown. Target cell concentration at the time of labeling was* $3·5 \times 10^5$ *cells/ml. The specific activity of* ^{125}*IUdR was* 8·3 *uCi/ug. The back extrapalation of the* 48 *hr release curve indidicates that* 17 *of the* 22·91% *of non-immunologic release (Fig.* 1) *was not due to the toxicity of* ^{125}*IUdR.*

REFERENCES

1. C. W. Song and S. H. Levitt, Combined effect of X-irradiation and cell-mediated immune reaction on tumor cells *in vitro*. *Radiology* **111,** 213 (1973).
2. C. W. Song, Presented at the conference of "Interaction of Radiation and Host Immune Defense Mechanism in Malignancy" (March 1974, Greenbrier, W.Va.), *BNL* 50418 (*Brookhaven National Laboratory Report*, 1974) p. 220. National Technical Information Service, Springfield, Va. U.S.A. (1974).

3. K. G. HOFER and W. L. HUGHES, Radiotoxicity of intranuclear tritium, ^{125}Iodine and ^{131}Iodine. *Radiat. Res.* **47,** 94 (1971).
4. D. D. PORTEUS, The toxicity of ^{125}IUdR in cultured mouse BP8 tumor cells. *Brit. J. Cancer* **25,** 594 (1971).
5. A. M. COHEN, J. F. BURDICK and A. S. KETCHAM, Cell mediated cytotoxicity: Assay using ^{125}I-iododeoxyuridine labeled target cells. *J. Immunol.* **107,** 895 (1971).
6. B. P. LEMEVEL and S. A. WELLS, JR., A microassay for the quantitation of cytotoxic antitumor antibody: Use of ^{125}I-iododeoxyuridine as a tumor cell label. *J. nat. Cancer Inst.* **50,** 803 (1973).
7. R. K. OLDHAM and R. B. HERBERMAN, Evaluation of cell-mediated cytotoxic reactivity against tumor associated antigens with ^{125}I-iododeoxyuridine labeled target cells. *J. Immunol.* **111,** 1862 (1973).
8. R. C. SEEGER and J. J. T. OWEN, Measurement of tumor immunity *in vitro* with ^{125}iododeoxyuridine labeled target cells. *Transplantation* **15,** 404 (1973).
9. O. C. A. SCOTT, The parameters of combined therapy. In *Research in Radiotherapy: Approaches to Chemical Sensitization.* Edited by R. KALLMAN, p. 117. Natl. Res. Council Comm. on Nuclear Science, Publ. 888, Washington, D.C. (1961).

Europ. J. Cancer Vol. 11, pp. 593–595. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Adriamycin in Wilms's Tumor Previously Treated with Chemotherapy

F. FOSSATI BELLANI, M. GASPARINI and G. BONADONNA
Istituto Nazionale Tumori, Milan, Italy

THE ANTITUMOR effect of actinomycin D (Act D) and of vincristine (VCR) in the management of advanced Wilms's tumor is well established [1, 2] and the cure rate of this disease has been significantly improved by a multidisciplinary therapeutic approach [3]. However, the optimal combined therapeutic modality for the different stages of Wilms's tumor deserves further evaluation. At present, controlled clinical studies are in progress in USA and in Europe to establish the definite role of Act D plus VCR as adjuvant treatment for local (stages I and II) and local plus regional (stage III) disease [4].

The new antitumor antibiotic adriamycin (14-hydroxydaunomycin, ADM) was entered into Phase II study in 1969. The preliminary report from our Institute included partial complete remissions in all 5 patients with advanced Wilms's tumor [5]. Similar observations were reported by Tan *et al.* [6] in 3 of 5 children, by O'Bryan *et al.* [7] in 3 of 4, and by Massimo [8] in 2 of 2, respectively.

The aim of this report is to update our series of patients and to emphasize the effective role of ADM in Wilms's tumor. The group includes a total of 9 children with a median age of 4·4 yr (range: 1·8–8·1). All patients were referred to our Institute only after the occurrence of distant metastases. At the time of initial diagnosis, 8 had either local or local plus regional extent, and for this reason they underwent radical surgery plus postoperative irradiation. One patient had, upon entry, bilateral tumor associated with pulmonary metastases. Seven of the 8 children with stage I–II disease also received adjuvant chemotherapy (Act D 4, VCR 1, Act D plus VCR 2). Treatment was administered in full conventional doses for a median of 5 months (range: 2–11) before relapse. When distant metastases were detected, 3 of 7 children were given further chemotherapy with standard drugs (Act D 2, VCR plus CTX 1) for 5 months. Also, the patient entered with advanced disease (metastatic stage V) was treated conventionally with Act D for 1 month before progressive disease became evident. Therefore, only one child was started on ADM without being previously managed with chemotherapy.

ADM was administered through a rapid i.v. injection according to two different dose schedules: (A): 16–24 mg/m^2 for 3 days and cycles repeated after a 7-day rest period; (B): 25 mg/m^2 for 3 days and cycles repeated every 3 weeks. Table 1 presents both type and duration of response related to total dose administered as well as to schedule. Two patients showed progression and both received a low total dose (60–80 mg/m^2). Two children achieved a moderate degree of regression (25–50%), while in 5 patients a good partial ($\geqq$ 50%) or a complete tumor response was observed. The duration of response appears directly proportional to the degree of response. The type of tumor regression is apparently not related to either of the schedules employed. Although the longest regressions were achieved with schedule B, it should be recalled that in 1 patient, the complete disappearance of pulmonary metastases was subsequently consolidated with irradiation of both lungs.

Accepted 7 April 1975.

Requests for reprints: Dr. F. Fossati Bellani, Via Venezian, 1, Milan 20133—Italy.

Table 2 shows that in all 4 children in whom lung metastases were the only apparent site of involvement, a CR or a PR ≧ 50% was obtained compared to liver involvement where only 1 of 3 children achieved a significant response. No recalcification of osteolytic lesions was observed in one child.

Table 1. Response related to dose and schedule

No. of patients	Response	Duration (months)	Total dose (mg/m²)	Schedule
2	Progression		60, 80	A, A
2	PR < 50%	1, 3	165, 220	A, B
2	PR ≧ 50%	4, 4	140, 175	A, A
3	CR	5, 9, 30	140, 540, 590	A, B, B

Table 2. Response related to sites of metastases

No. of patients	Site of metastases	Type of response			
		none	< 50%	≧ 50%	CR
4	Lungs			2	2
3	Liver		2		1
1	Bone	1			
1	Abdomen + lungs	1			

Generally, treatment was well tolerated, and side effects were moderate and reversible. Loss of hair to almost complete alopecia was observed in 8 of 9 children, leukopenia in 7 of 9 (in none did leukocytes fall below 1500/mm³). No patient showed thrombocytopenia or cardiac toxicity. The mean total dose administered was 230 mg/m² (range: 60–590).

Although the present series is limited, the fact that CR plus PR ≧ 50% was observed in 5 of 9 children with a total of 7 of 9 responders is encouraging. The results are particularly noteworthy if one recalls that 8 of 9 children were not responding to conventional chemotherapy. Therefore, ADM appears to be a new useful drug in the treatment of Wilms's tumor, and should be tested in a larger series of patients. First, the agent could be considered in the treatment of all children relapsing after adjuvant therapy with either Act D or Act D plus VCR. In patients with pulmonary metastases, the drug can be combined with radiation therapy, provided the total dose does not exceed 450 mg/m². In fact, Gilladoga *et al.* [9] have observed that prior irradiation to the heart area can lower the potential cardiotoxic dose of ADM. Finally, the drug could be combined sequentially with Act D and VCR either in the management of stage IV previously untreated Wilms's tumor or in the adjuvant therapy of stage III disease. In fact, in stages III and IV of Wilms's tumor, the conventional treatment has not resulted in an improved survival as obtained in the early stages [10]. In both situations, our present approach is to alternate monthly courses of Act D (15γ/Kg, days 1 to 5) plus VCR (1·4 mg/m²/week) with ADM (60 mg/m², day 1) plus VCR (1·4 mg/m²/week).

REFERENCES

1. W. W. Sutow, Chemotherapy in Wilms's tumor. *Cancer* **32,** 1150 (1973).
2. T. J. Vietti, M. P. Sullivan, M. E. Haggard, T. M. Holcomb and D. H. Berry, Vincristine sulfate and radiation therapy in metastatic Wilms's tumor. *Cancer* **25,** 12 (1970).
3. R. B. Everson, J. F. Fraumeni, Jr. and M. H. Myers, Improved survival in Wilms's tumor. *Lancet* **2,** 1290 (1970).
4. G. D'Angio, G. B. Becwith, H. Bishop, N. Breslow, P. Feigl, W. Goodwin, A. Evans, L. Pickett, L. Sinks, W. Sutow, M. Tefft and G. Wolff, The National Wilms's Tumor Study (NWTS): Preliminary results. *Proc. Amer. Soc. clin. Oncol.* abstr. 272, 68 (1974).

5. G. Bonadonna, S. Monfardini, M. De Lena, F. Fossati Bellani and G. Beretta, Clinical trials with adriamycin. Results of a three-year study. *Int. Symposium on Adriamycin.* (Edited by S. K. Carter, A. Di Marco, M. Ghione, I. H. Krakoff and G. Mathé) p. 139. Springer-Verlag, Berlin (1972).
6. C. Tan, E. Etcubanas, N. Wollner, G. Rosen, A. Gilladoga, J. Showel, M. L. Murphy, I. H. Krakoff, Adriamycin. An antitumor antibiotic in the treatment of neoplastic disease. *Cancer* **32,** 9 (1973).
7. R. M. O'Bryan, J. K. Luce, R. W. Talley, J. A. Gottlieb, L. H. Baker and G. Bonadonna, Phase II evaluation of adriamycin in human neoplasia. *Cancer* **32,** 1 (1973).
8. L. Massimo, Adriblastine. *Symposium International.* Paris, December 19, p. 43 Roger Bellon (1973).
9. A. C. Gilladoga, C. Tan, F. S. Philips, S. S. Sternberg, C. Tang, N. Wollner and M. L. Murphy, Cardiac status of 40 children receiving adriamycin (ADM) over 495 mg/m^2 and animal studies. *Proc. Amer. Ass. Cancer Res.* **15,** 107 (1974).
10. C. A. Perez, H. A. Kaiman, J. Keith, W. B. Mill, T. J. Vietti and W. E. Powers, Treatment of Wilms's tumor and factors affecting prognosis. *Cancer* **32,** 609 (1973).

Europ. J. Cancer Vol. 11, pp. 597–598. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer — *August, 1975*

S. B. Pollack and K. Nelson: Evidence for two factors in sera of tumor-immunized mice which induce specific lymphoid cell-dependent cytotoxicity: IgG_2 and a rapidly appearing factor not associated with IgC or IgM.

H. W. Taylor and W. Lijinsky: Tumor induction in rats by feeding aminopyrine or oxytetracycline with nitrite.

R. B. Herberman, M. E. Nunn and D. H. Lavrin: Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity.

R. B Herberman, M E. Nunn, H. T. Holden and D. H. Lavrin: Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells.

N. A. Wivel, G. H. Smith and H. L. Ozer: Comparison of intracytoplasmic A particles and intracisternal A particles.

H. Betuel, M. Camoun, N. E. Day, R. Ellouz and G. de Thé: The relationship between nasopharyngeal carcinoma and the HL-A system among Tunisians.

G. Bataillon, H. Pross and G. Klein: Comparative *in vitro* sensitivity of two methylcholanthrene-induced murine sarcoma lines to humoral and cellular immune cytotoxicity.

H. F. Stich and B. A. Laishes: The response of Xeroderma pigmentosum cells and controls to the activated mycotoxins aflatoxins and sterigmatocystin.

J. W. Blasecki and S S Tevethia: Restoration of specific immunity against SV40 tumor-specific transplantation antigen to lymphoid cells from tumor-bearing mice.

R. H. C. San and H. F. Stich: DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens.

K. Nelson, S. B. Pollack and K. E. Hellström: Specific anti-tumor responses by cultured immune spleen cells: II. Culture supernatants induce specific anti-tumor cytotoxicity by non-immune lymphoid cells *in vitro*.

D. Viza and J. Phillips: Identification of an antigen associated with malignant melanoma.

W. Lijinsky and H. W. Taylor: Carcinogenicity of methylated nitrosopiperidines.

S. Cornain, C. Carnaud, D. Silverman, E. Klein and M. F. Rajewsky: Spleen cell reactivity against transplanted neurogenic rat tumors induced by ethylnitrosourea: Uncovering of tumor specificity after removal of complement receptor bearing lymphocytes.

G. Rocchi, G. Tosato, G. Papa and G. Ragona: Antibodies to Epstein–Barr virus-associated nuclear antigen and to other viral and non-viral antigens in Hodgkin's disease.

H. Fujitani and V. Holoubek: Nuclear proteins of rat liver and of an aminoazo dye induced hepatoma.

K. S. Wise, S. E. Allerton, G. Trump, D. Powars and J. W. Beierle: A fetuin-like antigen from human nephroblastoma.

D. P. Stevens, I. R. Mackay and K. J. Cullen: Carcinoembryonic antigen in an unselected elderly population: A four year follow-up.

L. Levin, J. E. McHardy, O. M. Curling and C. N. Hudson: Tumour antigenicity in ovarian cancer.

N. A. Sheth, K. J. Ranadive, J. N. Suraiya and A. R. Sheth: Circulating levels of prolactin in human breast cancer.

I. Lasnitzki, R. H. Whitaker and J. F. R. Withycombe: The effect of steroid hormones on the growth pattern and RNA synthesis in human benign prostatic hyperplasia in organ culture.

R. A. Hawkins, B. Freedman, A. Marshall and E. Killen: Oestradiol-17B and prolactin levels in the peripheral plasma from male, female and tumour-bearing rats.

M. Y. Gordon and P. J Lindop: Further studies on the humoral regulation of granulopoiesis in leukaemic mice.

J. L. Millar, B. N. Hudspith and N. M. Blackett: Reduced Lethality in mice receiving a combined dose of cyclophosphamide and busulphan.

J. S. Tobias, L. M. Parker, M. H. N. Tattersall and E. Frei III: Adriamycin/cyclophosphamide and adriamycin/melphalan in advanced L1210 leukaemia.

A. K. Adatia: Cytotoxicity of cyclophosphamide in the rat incisor.

I. Lasnitzki, D. R. Bard and H. R. Franklin: 3-methylcholanthrene uptake and metabolism in organ culture.

H. Reznik-Schuller and U. Mohr: The ultrastructure of N-dibutylnitrosamine induced pulmonary tumours (adenocarcinomata) in European hamsters.

Europ. J. Cancer Vol. 11, pp. 599–607. Pergamon Press 1975. Printed in Great Britain

R17934–NSC 238159: A New Antitumor Drug—I. Effect on Experimental Tumors and Factors Influencing Effectiveness*

GHANEM ATASSI and H. J. TAGNON

Chimiothérapie expérimentale, Service de Médecine interne et Laboratoires d'Investigation clinique, Institut Jules Bordet, Brussels, Belgium†

Abstract—*R17934–NSC238159: methyl [5-(2-thienyl carbonyl)-1H-benzimidazol-2yl] carbamate, a new antitumor agent was found active against lymphoid L1210 leukemia, lymphocytic P388 leukemia, melanotic melanoma B16 and Lewis Lung carcinoma.*

This activity was influenced by the vehicle used in the preparation of the suspension and the route of administration of the drug, and to a lesser extent by the schedule of administration. The low toxicity of therapeutically active doses singles out this compound for possible clinical application.

INTRODUCTION

R17934, methyl (5-(2-thienyl carbonyl)-1H-benzimidazol-2yl) carbamate (Fig. 1), mol. wt = 301·31, is a new synthetic antitumor drug appearing as an off-white to pinkish amorphous powder melting with decomposition around 256°C. It is soluble in formic acid, benzaldehyde, dimethyl sulfonide and in mixture of chloroform–methanol (2 vol $CHCl_3$, 1 vol methanol) but very slightly soluble in water and most organic solvents.

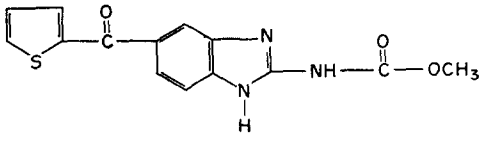


Fig. 1. *R17934-NSC* 238159*: Methyl* [5-2-*thienyl-carbonyl*)-1*H-benzimidazol-2-yl*] *carbamate.*

Accepted 29 July 1975.

*Supported in part by a grant from the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA), Brussels, Belgium and in part by the National Cancer Institute, NIH, Bethesda, Maryland, USA.

†This Service is a member of the European Organization for Research on Treatment of Cancer (EORTC).

This drug has demonstrated an inhibitory effect on the growth of mammalian cells cultured *in vitro* and it was shown to interfere with the structure and function of microtubules [1]. Therefore, we undertook to study its antitumor activity in experimental animals. The results are presented in this report.

MATERIAL AND METHODS

The compound R17934 and the solvent MSV (1 g Tween 80; 2·2 g Na_2HPO_4 12 H_2O; 25 g Na_2HPO_4 H_2O; 0·50 g Polyvinyl Piralidone; 50 mg methylparacept +5 mg ethylparacept; 100 ml H_2O) were supplied by Janssen Pharmaceutica (Beerse, Belgium) who is the manufacturer of the drug.

A suspension of the drug in MSV and the solution in polyethylene glycol 400 were also supplied by Janssen Pharmaceutica.

The Klucel (hydroxypropylcellulose, 0·3% in saline) was supplied by the National Cancer Institute (Bethesda, Maryland).

Suspension of the drug in MSV, saline and Klucel were prepared in our laboratory as follows:

(Non-micronized R17934 was used only in the

first experiment (Table 3); in all other experiments, the micronized form of the drug was used.)

Suspension in MSV. R17934 was ground in a mortar and MSV was slowly added until suspension was achieved. This suspension was kept under constant stirring for an hour before microscopic examination to verify homogeneity and absence of aggregates. This preparation was kept at room temperature.

Suspension in saline. The drug was ground as before and a small volume of saline added with one drop of Tween 80 for 30 ml saline. The remaining saline was then added. The suspension was stirred for 1 hr before verifying the absence of aggregates under the microscope.

Suspension in Klucel. Klucel was slowly added to the ground drug under constant agitation and the suspension was stirred continuously as mentioned before.

EXPERIMENTAL ANIMALS AND TUMORS

BDF_1 mice (DBA/2 × C57B16), C57B16 (supplied by Charles Rivers Laboratories) were used.

The following tumors were used:
L1210 lymphoid leukemia, P388 lymphocytic leukemia, melanotic melanoma B16 and Lewis Lung carcinoma originally obtained from Mr. I. Wodinsky (Arthur D. Little, Inc., Cambridge, Mass.) and Ependymoblastome obtained from Hazleton Laboratories, Inc., Vienna, Virginia. These tumors are maintained in our laboratory by serial transfer in mice.

1. *Toxicity*

Intraperitoneal toxicity of R17934 was determined on BDF_1 mice by the administration of one injection of the drug suspended in MSV for acute toxicity and five injections during five consecutive days for subacute toxicity.

2. *L1210 lymphoid leukemia*

L1210 acites was aspirated from DBA/2 mice and a suspension was made in Hank's balanced solution; 10^5 cells in 0·1 ml solution were injected intraperitoneally (i.p.) to obtain the ascitic form of leukemia, intravenously (i.v.) to obtain the blood form of leukemia or subcutaneously (s.c.) to obtain the solid tumor form of leukemia into BDF_1 male mice (weighing from 21 to 26 g) or into BDF_1 female mice (weighing from 18 to 21 g) as indicated in the tables. Animals were randomized into test groups and control groups. Early treatment started 24 hr after implantation of the leukemic cells was administered i.p., i.v. and orally for the ascitic form and i.p. and i.v. only for the two other forms. Three schedules of administration were used: (1) a total of three injections on days 1, 5 and 9; (2) a total of nine daily injections; (3) a single injection. The optimal schedule of treatment for L1210 ascite leukemia was investigated. The route and timing of treatment is specified on each table. The influence of the vehicles on the therapeutic activity of L1210 ascitic form was also studied while treating i.p. the ascitic leukemia.

3. *P388 lymphocytic leukemia*

P388 acites was aspirated from DBA/2 mice and a suspension was made in Hank's balanced solution; 10^6 cells in 0·1 ml solution were inoculated i.p. into BDF_1 mice weighing from 26 to 30 g.

Treatment was started 24 hr after inoculation of the leukemic cells and administered i.p. once a day on days 1, 5 and 9. The influence of the vehicles was also studied here.

4. *Melanotic melanoma B16*

One half ml of tumor suspension from a homogenate of 1 g of tumor (obtained from C57B16 mice) in 9 ml cold Hank's balanced solution was implanted i.p. in three experiments and s.c. in two others into BDF_1 mice weighing 20–27 g. In the first schedule, treatment was started i.p. 24 hr after inoculation of the melanoma B16 and continued every four days for a total of three doses with the R17934 suspension in saline and in the second experiment with the R17934 suspension in MSV. In the second schedule, treatment was started 24 hr after inoculation and continued every five days for a total of five i.p. injections, MSV being used as a vehicle. In the s c. melanoma B16, the drug was administered i.p. every 5 days from day 1 to day 21 after tumor inoculation in the first experiment and i.v. during five consecutive days in the second experiment. Doses of R17934 are indicated in the tables.

5. *Lewis lung carcinoma*

The tumor was excised from C57B16 mice and a homogenate was made. Male BDF_1 mice weighing 22–26 g were inoculated i.m. in the left posterior leg with 2×10^6 cells in cold Hank's balanced solution. Treatment was started i.p. 24 hr after tumor implantation and continued on days 7, 12 and 23 in one experiment and on days 7, 14 and 21 in a second experiment. In a third experiment, the animals were treated every 3 days from day 1 to 19.

Table 1. Acute toxicity of R17934 in BDF1 mice treated i.p. one injection

Dose mg/kg/injection	Number of animals injected	dead	Day of death
400	6	6	2, 2, 2, 3, 3, 4
300	6	5	2, 3, 4, 4, 4
200	6	5	4, 4, 4, 4, 5
150	6	3	4, 5, 8.
100	6	2	6, 7
80	6	0	

The drug was inoculated as a suspension in MSV

Table 2. Subacute toxicity of R17934 in BDF1 mice treated i.p. for 5 consecutive days

Dose mg/kg/injection	Number of animals injected	dead	Day of death
80	6	6	5, 5, 5, 5, 6, 7
70	5	5	1, 4, 5, 10, 15
60	6	5	5, 6, 7, 8, 9
50	5	3	7, 7, 7
35	5	0	
25	5	0	

The drug was inoculated as a suspension in MSV

In a fourth experiment, the treatment was delayed until day 12 and continued on days 16 and 20 after tumor inoculation.

6. *Ependymoblastoma*

This tumor is propagated by s.c. implantation of $2 \times 2 \times 8$ mm fragment in C57B16 mice. In one experiment, a fragment of this size was implanted in BDF_1 mice s.c. In a second experiment, a fragment of 1 mm^3 was implanted intracerebrally (i.c.) in the parietal zone of the right cerebral hemisphere.

RESULTS

1. *Toxicity of R17934*

Tables 1 and 2 show the 14 days survival data after the administration i.p. of various dosages of the drug to intact BDF_1 mice. The acute LD_{50} (14 days) was found to be approximately 135 mg/kg as a single dose i.p. The subacute LD_{50} of the drug in BDF_1 mice treated i.p. for five days was approximately 48 mg/kg.

2. *Antileukemic effect of R17934*

This study was carried out on animals bearing L1210 and P388 leukemia. The number of animals in the tests and control groups is indicated in the tables as well as the vehicles and schedules of treatment used (see Tables 3–10). The therapeutic effect of R17934 on ascitic L1210 leukemia is shown in Table 3. The drug was administered as a suspension in

Table 3. Effect of R17934 on the survival of BDF1 mice bearing L1210 ascites

Dose mg/kg/inj.	Schedule + route of treatment	BWC day 5 (g)	MST (days)	ILS %
	1st Experiment			
200	days 1, 5, 9 i.p.	-1.3	14.8	51
100	idem	-1.7	12.8	31
50	idem	0	12.7	30
40	days 1 to 9 i.p.	-2.5	12.3	26
20	idem	-1.7	11.6	18
10	idem	-0.6	10.0	2
Untreated Control		+1.3	9.8	
	2nd Experiment			
100	days 1, 5, 9 i.v.	-3.0	9.0	2
70	idem	-1.3	12.0	36
50	idem	+0.2	13.0	48
Untreated Control		+2.1	8.8	

Male mice (18-25 g) were inoculated i.p. with 10^5 L1210 leukemia cells on Day 0.
R17934 was suspended in saline + Tween 80 in the first experiment and in MSV in the second experiment.
MST : Median Survival Time of 7 treated mice and 18 untreated mice in the first experiment and of 10 treated mice and 28 untreated mice in the second experiment.
BWC : Body Weight Change on Day 5 (grams).
ILS : Percentage increase in survival over controls.

Table 4. Influence of the suspension on therapeutic activity against L1210 leukemia ascites form

Dose mg/kg/inj/day	ILS% in Saline + T	ILS% in Klucel	ILS% in MSV
70	55	23	69
50	49	27	45
35	22	21	24
Vehicle alone (0.1 ml/10 g)	0	0	0

Male mice (18-21 g) were inoculated i.p. with 10^5 leukemia cells on day 0.
Animals were treated i.p. on days 1, 5 and 9.
ILS : percentage increase in survival time over controls.

saline in the i.p. treatment and as a suspension in MSV in the i.v. treatment. Survival was significantly prolonged in animals with a schedule of three injections on days 1, 5 and 9 i.p or i.v. The drug was less active when the mice were treated daily from day 1 to day 9 after tumor inoculation.

The influence on the antileukemic effect of the vehicle used to prepare the suspension of R17934 was also investigated. The survival of the animals treated on days 1, 5 and 9 after tumor inoculation with the suspension of the drug in MSV was longer than the survival of mice treated with the suspension of the drug in saline or Klucel at the same dose level and with the same schedule (Table 4).

Table 5. Influence of the suspension on therapeutic activity against P388 ascitic leukemia

Dose mg/kg. inj/day	ILS% (surv.) in Saline		in Klucel		in MSV.	
	1st exp	2nd exp	1st exp	2nd exp	1st	2nd exp.
100	138 (2/6)	103	70	85	139 (3/7)	166 (3/7)
75		94	74	76	195 (2/7)	143 (1/7)
50	86	85	74	64	104 (1/7)	103
MSV alone 0.1 ml/10 g					0	
Klucel alone 0.1 ml/10 g			0			
Saline alone 0.1 ml/10 g	0					

Male mice were inoculated i.p. with 10^6 leukemia cells on day 0.
Animals were treated i.p. on days 1, 5 and 9.
ILS : Percentage increase in survival time over control
(surv.) : survivors/total on Day 30.

The enhanced therapeutic effectiveness of the suspension in MSV was much more apparent on the leukemia P388 (Table 5): here, a good percentage of long term survivors equivalent to cures was obtained when the schedule of treatment days 1, 5 and 9 was used. The therapeutic effectiveness of R17934 was also demonstrated when the L1210 cells were inoculated i.v. or s.c. and when the drug as a suspension in MSV was administered i.v. or i.p. on days 1, 5 and 9 after tumor inoculation (Tables 6 and 7).

Four different schedules of treatment were compared in the case of L1210 leukemia blood form (2nd experiment, Table 6). The therapeutic effectiveness was slightly higher with the schedule days 1, 5 and 9 as compared with the single dose on day 1, with schedule day 1 and 9 and with schedule day 1 to day 4.

When a solution of R17934 in polyethylene glycol 400 (PEG 400) was used (Table 8), the therapeutic effectiveness of the i.p. treatment decreased with all schedules as compared with the treatment with the MSV suspension. However, it increased when the solution was administered orally, on days 1, 5 and 9 after i.p. L1210 cells inoculation, but not to such an extent as to make this mode of administration preferable to others. Various schedules of administration were investigated next.

Table 9 shows that R17934 increased the survival time in L1210 leukemia over a wide range of treatment schedules but the difference between the various schedules was not significant. The oral administration of R17934 in MSV appeared therapeutically ineffective (Table 9). Three different schedules of treatment were also compared in advanced L1210 leukemia where treatments were started on day 5 after tumor inoculation. There was a slight advantage for the days 5, 9 and 13 i.p. schedule treatment over the single dose treatment and the day 5 and 13 schedule treatment (Table 10).

3. *Therapeutic effect of R17934 on melanoma B16*

In BDF_1 mice inoculated i.p. with melanoma B16, the suspension of R17934 in saline administered i.p. on days 1, 5 and 9 after tumor implantation induced a significant increase of survival time over control with 20% long term

Table 6. Effect of R17934 on life span of BDF1 mice bearing L1210 leukemia blood form

Dose mg/kg/inj./day	Schedule + route of treatment	BWC day 5 (g)	MST days	ILS %
	1st experiment			
100	days 1, 5, 9 i.v.	-2.6	12.5	69
70	idem	-1.3	13.5	82
50	idem	+0.4	9.5	28
MSV alone		+1.7	7.2	
Untreated control		+2.3	7.4	
	2nd experiment			
90	day 1 only i.v.	-1.8	7.0	
80	idem	-1.2	11.0	47
70	idem	-2.3	11.0	47
90	days 1 and 9 i.v.	-2.6	10.7	43
80	idem	-1.3	9.7	29
70	idem	-3.2	11.0	47
70	days 1, 5 and 9 i.v.	-2.9	12.2	63
60	idem	0	11.7	56
50	idem	-0.5	12.9	72
40	day 1 to day 4 i.v.	-1.9	12.0	60
30	idem	-0.3	8.7	16
20	idem	-3.1	8.4	12
Untreated control		+0.9	7.5	
	3rd experiment			
100	days 1, 5 and 9 i.p.	-1.8	8.7	21
70	idem	-2.1	9.0	25
50	idem	+0.3	8.7	21
Untreated control		+0.9	7.2	

Female mice (18-19 g) in the first experiment and male mice (18-20 g) in the two other experiments were inoculated i.v. with 10^5 leukemia cells on day 0. The drug was suspended in MSV solvent.
BWC: body weight change on Day 5.
MST: median survival time of 7 treated mice in the first and second experiments and 10 and 27 untreated mice respectively and of 10 treated and 18 untreated mice in the third experiment.
ILS: Percentage increase in survival time over control.

survival when the dose of 100 mg/kg was used (Table 11). The suspension of the drug in MSV administered i.p. every 5 days from day 1 to day 21 after tumor inoculation induced an increase in survival time over control of 92% and 96% respectively for the doses of 70 mg/kg and 60 mg/kg (Table 12) while the same suspension was unable to induce an increase in life span when the melanoma B16 was transplanted s.c. (Table 13). The 50 mg/kg dose administered i.p. in melanoma B16 i.p. induced a 55% increase in life span over control, but a dose of 70 mg/kg administered i.p. every 4 days produced chronic toxicity (Table 12). In the case of melanoma B16 transplanted s.c., the schedule of treatment with R17934 in MSV

Table 7. Effect of R17934 on the life span of BDF1 mice bearing L1210 leukemia solid form

Dose mg/kg/inj/day	Schedule + route of treatment	MST days	ILS. %
	1st experiment		
100	days 1, 5 and 9 i.v.	15.5	52
70		16.5	62
50		13.0	27
MSV alone (0.1 ml/10 g)		10.0	
Untreated control		10.2	
	2nd experiment		
100	days 1, 5 and 9 i.p.	13.0	30
70		12.7	27
50		11.7	17
Untreated control		10.0	

Female mice (18-20 g) in the first experiment and male mice (18-21 g) in the second experiment were inoculated s.c. with 105 leukemia cells on Day 0. The drug was suspended in MSV.

MST: median survival time of 7 treated mice and 10 untreated mice in the first experiment and 10 treated mice and 18 untreated mice in the second experiment.

ILS: percent increase in survival time over control.

Table 8. Effect of a solution of R17934 in PEG 400 on the life span of BDF1 mice bearing L1210 leukemia ascites form

Schedule + route of treatment	Dose range mg/kg	Optimal dose mg/kg/inj/day	ILS %
Days 1, 5, 9 i.p.	40- 70	40	5
Days 1 to 9 i.p.	10- 30	30	40
Day 1, q3h i.p.	5- 20	10 (x8)	38
Days 1 and 9 q3h, i.p.	5- 20	10 (x8)	48
Days 1, 5, 9 q3h, i.p.	5- 20	5 (x8)	28
Days 1, 5, 9 p.o.	75-200	200	32

Mice (20 - 23 g) were inoculated with 10^5 leukemia cells i.p. on day 0.

ILS: percent increase in survival time over control.

Table 9. Influence of schedule and route of administration on life span of BDF1 mice bearing L1210 leukemia ascites form

Treatment		Dose range mg/kg	Optimal dose mg/kg/day	ILS %	Survival/total day 60	
Route	Schedule				Leukemic mice	Normal mice
i.p.	once, Day 1	100-160	100		0	4/7
i.p.	q3h, Day 1	7.5 (x8) - 20 (x8)	15 (x8)	77	0	3/7
i.p.	qd, Days 1,9	80-140	80	82	0	7/7
i.p.	q3h, Days 1,9	7.5 (x8) - 20 (x8)	10 (x8)	58	0	5/7
i.p.	qd, Days 1,5, 9	30-100	70	79	0	7/7
i.p.	q3h, Days 1, 5, 9.	7.5 (x8) - 20 (x8)	7.5 (x8)	69	0	1/7
i.p.	qd, Days 1-5	10- 50	30	54	0	4/7
i.p.	qd, Days 1-9	10- 50	30	57	0	4/7
p.o.	qd, Days 1,5 9	300-600	600	4	0	7/7
p.o.	qd, Days 1-9	100-400	300	9	0	6/7

BDF1 male mice were inoculated i.p. with 10^5 leukemic cells on Day 0.
10 mice were used as treated mice and 60 as controls.
ILS: percent increase in life span over control.
The normal mice (non leukemic) received the same treatment as the leukemic mice.

Table 10. Influence of schedule of treatment on therapeutic effectiveness against advanced L1210 leukemia

Dose mg/kg	Schedule + route of treatment	MST days	ILS %
160	Day 5, i.p.	13.5	48
140	idem	12.5	37
120	idem	12.5	37
160	Days 5, 13 i.p.	14.0	54
120	idem	14.0	54
100	idem	11.5	26
100	Days 5,9,13 i.p.	16.5	81
70	idem	14.2	56
50	idem	13.0	43
Untreated		9.1	

10^5 leukemia cells were inoculated i.p. on Day to BDF1 male mice.
MST: median survival time of 7 treated mice and 18 controls.
ILS: percent increase in life span over control.

Table 11. Effect of R17934 on life span of BDF1 mice bearing melanoma B16 i.p.

Dose mg/kg/day	Schedule + route of treatment	MST days	ILS %	Surv. day 60 / total
	Days 1, 5, 9 i.p.			
150		16.0	neg	
100		46.0	108	2/10
50		39.7	80	0
Untreated		22.1		

BDF1 male mice (23-27 g) were inoculated i.p. with 0.5 ml tumor homogenate on day 0.
MST: median survival time of 10 treated mice and 27 controls.
ILS: percent increase in survival time over control.
The suspension of R17934 was prepared with saline and Tween 80.

solvent administered i.v. for five consecutive days induced 11% increase in life span with 50 mg/kg and was toxic at doses of 60 mg/kg and 70 mg/kg (Table 13).

4. *Effect of R17934 in MSV on tumor growth, lung metastases and survival of mice bearing Lewis Lung carcinoma i.m.*

The i.p. treatment started the day after tumor implantation and repeated on days 7, 12, 17 and 23 or repeated on days 7, 14 and 21 with 50 mg/kg and 70 mg/kg doses of R17934 prolonged survival time by 30% over control (Table 14) and strongly inhibited tumor growth and the formation of lung metastases (Table 15), even when treatment was delayed to day 12 after tumor implantation and repeated on days 16 and 20. However, the i.p.

Table 12. Effect of R17934 in MSV solvent on life span of BDF1 mice bearing melanoma B16 i.p.

Dose mg/kg	Schedule + route of treatment	MST days	ILS %
	1st experiment		
70	Days 1, 6, 11 and 21 i.p.	43.9	92
60	idem	45.0	96
50	idem	37.5	64
Untreated		22.9	
	2nd experiment		
100	Days 1, 5 and 9 i.p.	6	neg
70	idem	11.5	neg
50	idem	25.5	55
Untreated		16.5	

BDF1 male mice (20-24 g) were inoculated i.p. with 0.5 ml tumor homogenate on Day 0.

MST: median survival time of 10 treated mice and 27 untreated controls in the first experiment and of 7 treated mice and 33 untreated controls in the second experiment.

ILS: percent increase in survival time over control.

Table 13. Effect of R17934 in MSV on survival time of BDF1 mice bearing melanotic melanoma B16 s.c.

Dose mg/kg/day	Schedule + route of treatment	MST days	ILS %
	1st experiment		
70	Days 1, 6, 11, 16 and 21 i.p.	24.3	3
60	idem	24.7	5
50	idem	24.6	5
Untreated		23.5	
	2nd experiment		
70	Days 1 to 5 i.v.	12.5	neg
60	idem.	17.5	neg
50	idem	23.5	11
Untreated		21.2	

BDF1 male mice (18-23 g) were inoculated s.c. with 0.5 ml tumor homogenate on Day 0.

MST: median survival time of 10 treated mice and 20 untreated mice in the first experiment and 9 treated mice and 18 controls in the second experiment.

ILS: percent increase in survival time over control.

Table 14. Effect of R17934 in MSV on survival of mice bearing Lewis Lung carcinoma i.m.

Dose mg/kg	Schedule + route of treatment	MST days	T/C %	(ILS) %
	1st experiment			
70	Days 1, 7, 12, 17 and 23 i.p.	36.7	134	34
60	idem	36.0	136	31
50	idem	35.0	135	30
Untreated		27.4		
	2nd experiment			
70	Days 1, 7, 14, 21 i.p.	35.5	130	30
Untreated		27.3		
	3rd experiment			
70	Days 1, 4, 7, 10, 13, 16, 19 i.p.	20.7	85	neg
50	idem	18.2	74	neg
35	idem	24.3	99	neg
Untreated		24.5		

BDF1 male mice (22-26 g) were inoculated i.m. with 2.10^6 tumor cells.

T/C: median survival time of treated mice/ median survival time of untreated mice.

ILS: percent increase in survival time over controls.

Table 15. *Effect of* R17934 *on Lewis Lung carcinoma and lung metastases*

Treatment schedule + dose (mg/kg)	Aver. Body Wght Day animal is sacrified (g)	Aver. Tum Weight (g)	Mice with Tum / Nber of mice in the group	Mice with Met / Mice with Tum	Aver. Nb Met per mouse	Aver. Wght Met per mouse with Met (mg)
			1st experiment			
Days 1, 7, 12 17 and 23						
70	24.0	1.09	1/ 9	0/ 1	0	0
60	24.2	2.35	7/ 9	0/ 7	0	0
50	24.2	4.91	9/ 9	0/ 9	0	0
Untreated	27.2	9.04	15/15	14/15	4	25.41
			2nd experiment			
Days 1, 7, 14 and 21						
70	25.03	3.85	6/ 9	5/ 9	1.33	7.02
Untreated	26.2	10.82	10/10	10/10	9	162.75
			3rd experiment			
Days 12, 16, 20						
70	17.0	4.7538	5/ 5	0/ 5	0	0
50	20.8	7.0095	6/ 6	3/ 6	0.83	0.89
Untreated	24.88	11.2248	15/15	15/15	7	98.80

BDF1 male mice (18-26 g) were inoculated i.m. with 2.10^6 tumor cells on Day 0.
R17934 was administered as a suspension in MSV solvent i.p. Animals were sacrified on Day 24.
Tum: tumor; Met: metastases; Wght: weight; Nber: number.

treatment started 24 hr after tumor implantation and repeated every three days until day 19 resulted in chronic toxicity: the animals treated with 70 mg/kg and 50 mg/kg doses had a shorter median survival time (MST) than the untreated group and the ratio of the MST of the treated group over the MST of the control group = T/C 85 indicated a toxic effect. The dose 35 mg/kg did not increase survival time with this schedule of treatment.

Table 16. *Effect of* R17934 *on survival of mice bearing ependymoblastoma i.c.*

Doses mg/kg/inj	BWC (g)	MST days	ILS %
70	-2.3	18.5	neg
50	-1.7	22.3	6
Untreated	+0.7	21.0	

C57B16 male mice were tranplanted i.c. with 1 mm3 tumor fragment on Day 0.
Animals were treated i.p. on Days 1;7 and 12.
BWC: Body Weight Change on Day 5.
MST: median survival time of 8 treated mice and 12 controls.
ILS: percent increase in survival time over control.

Table 17. *Effect of* R17934 *on survival of mice bearing ependymoblastoma s.c.*

Dose mg/kg/inj	BWC (g)	MST days	ILS %
70	-0.7	29.5	16
50	-0.4	28.0	10
Untreated	+0.4	25.5	

BDF1 male mice (20-25 g) were transplanted s.c. with 25 mm3 tumor fragment on Day 0.
Animals were treated i.p. on Days 1, 5, 9 and 13.
BWC: Body Weight Change on Day 5
MST: median survival time of 7 treated mice and 33 controls.
ILS: percent increase in survival time over control.

5. *Therapeutic effect of* R17934 *on brain murine tumor. Ependymoblastoma*

The i.p. treatment on days 1, 7 and 12 after the transplantation of the tumor s.c. was unable to increase survival time of treated animals over control animals (Table 16). The inefficacy of the i.p. treatment on days 1, 5, 9 and 13 after the i.c. tumor implantation is demonstrated in Table 17.

DISCUSSION

Our data show that the R17934 in MSV solvent has a high antitumor activity in four out of five different tumors used in our laboratory. The therapeutic effectiveness was not influenced by the schedules of treatment used here in the case of L1210 leukemia while the choice of the vehicle used for the preparation of the suspension appeared to be critical. Indeed, the therapeutic activity of R17934 as a suspension in Klucel (hydroxypropylcellulose 0·3 ml) was reduced to about 50% as compared to the suspension in MSV. Care in preparation of the suspensions was very important. Lower doses of micronized R17934 were as active, if not more, (70 mg/kg) than the higher doses of the non-micronized form (200 mg/kg) in the same experimental conditions.

The outcome of therapy was not influenced by the route of inoculation of L1210 leukemia

cells. The suspension in MSV administered i.p. or i.v. was active against L1210 whether inoculated i.p., i.v. or s.c. However, the outcome was influenced by the route of administration of the drug: following intraperitoneal inoculation of the L1210 cells, the drug appeared more effective by i.p. administration than by i.v. administration in increasing the survival time of leukemic animals. Also following intravenous or subcutaneous inoculation of the leukemic cells, the drug was therapeutically more active when intravenous treatment was used instead of intraperitoneal treatment. This may be explained by better contact between L1210 cells and drug when the same route of injection is used for both cells and drug and the higher rapidity to reach the s.c. tumor when the drug is administered i.v.

Oral administration of R17934 as a suspension in MSV solvent was ineffective and this may be the result of lack of absorption by the gastro-intestinal tract. The solution of R17934 in PEG 400 showed a moderate therapeutic activity, presumably explained by better absorption.

In solid tumors such as melanoma B16, therapeutic results were influenced by the site of inoculation of the tumor. R17934 was therapeutically active against melanoma B16 inoculated i.p. and treated i.p. or i.v. The intervals between treatment were found to be important: when relatively high therapeutically active doses were used, interval between treatments should be prolonged.

In Lewis Lung carcinoma, the efficacy of the treatment depended on the number of injections and the interval between injections to avoid toxicity.

R17934 appeared to have no therapeutic activity on the Ependymoblastoma s.c. or i.c. in the conditions of our experiments. Therefore, we were not able to conclude that this drug crosses the meningeal barrier. The drug will have to be investigated on L1210 leukemia inoculated i.c

Acknowledgements—The technical help of Mrs. F. Bournville-Lefort and Mr. C. Schaus was greatly appreciated. The authors also wish to thank Mrs. D. Maréchal-De Bock for reviewing and typing the manuscript.

REFERENCE

1. M. De Brabander, R. Vandeveire, F. Aerts, M. Borgers, L. Desplenter and P. A. Janssen, R17934, a new synthetic antitumor drug interfering with microtubules: effects on mammalian cells cultures *in vitro*. *Cancer Res.* (Submitted for publication).

Europ. J. Cancer Vol. 11. pp. 609–614. Pergamon Press 1975. Printed in Great Britain

R17934-NSC238159: A New Antitumor Drug—II. Effect on Mitotic Cycle of L1210 Leukemia Cells *In Vivo* and Synergism with Cytosine Arabinoside (NSC 63878)*

GHANEM ATASSI, C. SCHAUS and H. J. TAGNON

Chimiothérapie expérimentale, Service de Médecine interne et Laboratoires d'Investigation clinique, Institut Jules Bordet, Brussels, Belgium.†

Abstract—*R17934 methyl [5-2-thienyl carbonyl)-1H benzimidazol-2-yl] carbamate an antitumor agent effective against L1210 and several other experimental tumors was observed to produce arrest of mitosis in metaphase of L1210 leukemia cells* in vivo *in a way very similar to vincristine. In combination with cytosine arabinoside, therapeutic synergism was observed when cytosine arabinoside was administered 15 or 24 hr after R17934. In contrast, treatment with cytosine arabinoside given immediately after R17934 was found to result in an antagonistic combination. Combined treatment with cytosine arabinoside preceding the administration of R 17934 by 15–24 hr also demonstrated a synergistic effect. This synergism was less pronounced in early leukemia than when R17934 was given 15–24 hr before cytosine arabinoside but equally pronounced in the case of advanced leukemia. Maintained synergism after inverting the order of drug administration is not in agreement with the explanation invoked for this synergism based on prior metaphase induction followed by the administration of a metaphase active drug.*

INTRODUCTION

R17934 (NSC 238159): methyl [5-(2-thienyl carbonyl)-1H benzimidazol-2-yl] carbamate was found to interfere with the structure and function of microtubule of mammalian cells cultured *in vitro* [1]. These effects are seen also with colchicine, vinblastine and vincristine [2–5].

On the other hand, we have demonstrated the therapeutic activity of R17934 against a wide range of experimental tumors [6].

In this report we will describe the effect of this drug on the L1210 leukemia cell cycle *in vivo* and the therapeutic activity resulting from the combination schedule treatment of this drug with a phase dependent drug cytosine arabinoside as compared with the therapeutic activity of R17934 alone or cytosine arabinoside alone.

MATERIAL AND METHODS

The L1210 leukemia ascites form is maintained in our laboratory in DBA/2 mice by weekly intraperitoneal (i.p.) inoculation. BDF_1 mice (C57B16 female × DBA/2 male)

Accepted 29 July 1975.

*Supported in part by a grant from the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA), Brussels, Belgium and in part by the National Cancer Institute, NIH, Bethesda, Maryland, U.S.A.

†This Service is a member of the European Organization for Research on Treatment of Cancer (EORTC).

mice, weighing 19–23 g were used in these investigations.

R17934 as a suspension in MSV vehicle was obtained from Janssen Pharmaceutica, Beerse, Belgium. Cytosine arabinoside (NSC 63878) was obtained from the National Cancer Institute, NIH, Bethesda, Maryland, USA and dissolved in saline. The drugs were injected i.p. intermittently in a volume of 0·1 ml/10 g body weight.

After the administration of R17934, leukemic ascites was withdrawn with a G22-1 1/2 needle at intervals and duplicate smears were prepared from each leukemic mouse. The smears were stained with May Grünwald stain and the mitotic indices were determined microscopally by counting 500 cells per slide.

For purposes of comparison in certain experiments, vincristine was used as a drug having the same effect on mitosis as R17934.

RESULTS

Effect of in vivo *treatment with R17934 on the mitotic cycle of L1210 leukemia cells*

Groups of BDF_1 mice inoculated with 10^5 leukemia cells on day 0 were treated i.p. on day 4 with a single dose of respectively 200 mg/kg and 70 mg/kg in one experiment and with 50, 30 and 10 mg/kg of R17934 in a second experiment.

Smears of ascites cells from treated mice and from untreated controls were made hourly for 24 hr after treatment by R17934. In the untreated control mice (Fig. 1) 1% to 3% of cells on smear were found to be in metaphase over the 24 hr observation period. In mice treated with R17934 (70 mg/kg) cells in mitosis increased to 31·8% in 8 hr. After the 8th hour there was a rapid decrease reaching 5% in 9 hr then a moderate decrease in the number of dividing cells to the control level.

On smears from mice injected with 200 mg/kg, distinction between cells in division and necrosed cells (cells with a divided nucleus) was difficult to establish.

In another experiment, the lowest doses which modified the number of metaphases in L1210 leukemia cells was studied: the results are summarized in Fig. 1. It is to be noted that at the doses of 70 mg/kg, 50 mg/kg, 30 mg/kg and 20 mg/kg, we always obtained approximately the same peak. With a 10 mg/kg dose, approximately 7·5 of the cells were in metaphase.

In repeating the same experiment with 1 mg of vincristine instead of R17934, all other conditions remaining unchanged, the number of cells in metaphase was found to reach 28% maximally, suggesting an identity of action between R17934 and vincristine (Fig. 2).

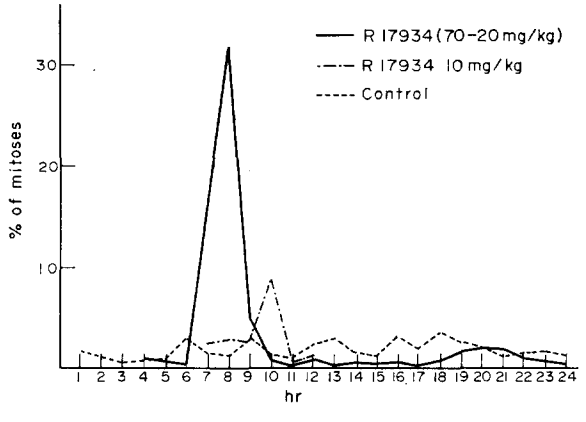


Fig. 1. *Effect of* in vivo *treatment of R17934-NSC* 238159 *on mitotic cell cycle in mice bearing L1210 leukemia.*

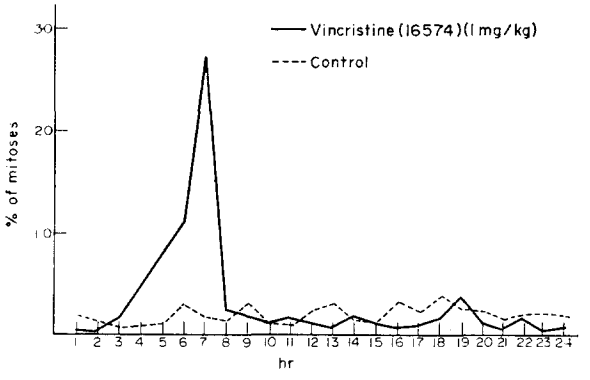


Fig. 2. *Effect of* in vivo *treatment with vincristine NSC* 67574 *on leukemic cell cycle in mice bearing L1210 leukemia.*

Therapeutic effect of i.p. treatment with R17934 in combination with cytosine arabinoside (Ara C) on early L1210 leukemia ascites form

The peak metaphase arrest of L1210 leukemia cells was found to occur between 8 and 10 hr after treatment with R17934 (Fig. 1). Therefore, two experiments were conducted in which leukemic mice were randomized into treated groups of seven animals and a single control group of 38 animals. In this first experiment, treatment was started on day 1 after tumor cells inoculation with R17934 at the doses of 70 mg/kg and 50 mg/kg every 4 days followed 15 hr later by 1200, 833 or 416 mg/kg of cytosine arabinoside. In this first experiment, a group of mice was treated with 833 mg/kg of cytosine arabinoside given on day 1 followed 15 hr later by 70 mg/kg of R17934 (Table 1).

Table 1. Therapeutic effect of i.p. treatment with R17934 (R) in combination with cytosine arabinoside (ARA C) on early L1210 leukemia ascites form

Agent mg/kg	Interval	MST (days)/range 1st Exp.	MST (days)/range 2nd Exp.	ILS % 1st Ex.	ILS % 2nd Exp	Surv. Day 60 1st Exp.	Surv. Day 60 2nd Exp.
Untreated Control		8.1/ 8- 9	8.2/ 8- 9				
R17934 (70)		20.5/11-22	16.5/12-34	153	101	0	0
(50)		12.8/12-15		58		0	
Ara C (1200)		20.5/18-22	17.5/10-21	153	113	0	0
(833)		17.5/16-20	16.5/15-20	116	102	1	0
(416)		15.7/14-16	14.8/14-16	94	80	0	0
VCR (1)			12.1/12		48		0
(0.5)			11.2/10-12		37		0
R17934 (70) + Ara C (1200)	15	18.5/12-18	13.5/ 5-21	128	65	3	1
R17934 (70) + Ara C (1200)	24		19.0/ 9-23		132		0
R17934 (70) + Ara C (833)	15	24.5/ 5-24	21.5/18-26	202	162	3	2
R17934 (70) + Ara C (833)	24		13.5/ 8-24		65		2
R17934 (70) + Ara C (416)	15	>60 / 26	>60 / 13			4	5
R17934 (70) + Ara C (416)	24		24.5/19-36		199		2
R17934 (50) + Ara C (1200)	15	26.5/23-30		227		1	
R17934 (50) + Ara C (833)	15	21 /20-25		159		0	
R17934 (50) + Ara C (416)	15	22.5/19-26		178		1	
Ara C (833) + R17934 (70)	15	12.5/ 8-13		54		2	
Ara C (1200) + R17934 (70)	24		22.5/11-28		177		2
Ara C (833) + R17934 (70)	24		22.0/11-22		168		2
Ara C (416) + R17934 (70)	24		22.0/20-22		168		2
VCR (1) + Ara C (833)	24		17.2/16-22		110		0
VCR (0.5) + Ara C (833)	24		17.5/15-20		113		0

BDF1 female mice (20-22 g) in the first experiment and male mice (17-19 g) in the second experiment were inoculated i.p. with 10^5 L1210 leukemia cells on Day 0.

R17934 was injected i.p. every 4th day (Day 1, 5 and 9).
Ara C was also injected i.p. every 4th day, 15 or 24 hours later.

MST: median survival time of 7 treated mice and 38 untreated mice in both experiments

ILS: increase in survival time over untreated controls.

In the second experiment, the leukemic mice received on day 1 after tumor implantation 70 mg/kg of R17934 followed by 1200, 833 or 416 mg/kg of Ara C at 15 or 24 hr intervals after R17934. On the other hand, three groups of leukemic mice were treated with 1200, 833 or 416 mg/kg of Ara C followed 24 hr later by 70 mg/kg of R17934. In this second experiment, a group of mice was treated with 1 or 0·5 mg/kg of vincristine (VCR) followed 24 hr later by 833 mg/kg of Ara C. The results of these two experiments are summarized in Table 1. The median survival time (MST) of untreated control mice was 8·1 days in the first experiment and 8·2 days in the second. The MST of mice treated with R17934 alone at 50 and 70 mg/kg doses was 16·5 and 20·5 days respectively in the first experiment and 16·5 days for the dose 70 mg/kg in the second. The MST of mice treated with Ara C alone at doses ranging from 416 to 1200 mg/kg was 15·7–20·5 days. The MST of mice treated with the combination of the drugs: 70 mg/kg of R17934 followed 15 hr later by 416 mg/kg of Ara C was higher than the highest MST obtained by the treatment with any of the drugs alone.

We also obtained, with this combination, a high percentage of long term survivals. Four mice out of seven in the first experiment and five mice out of seven in the second experiment were alive on day 60. When the interval between the two drugs was 24 hr, a lower increase in life span was obtained and only two mice out of seven were alive on day 60. When Ara C was injected before and R17934 was injected 15 or 24 hr later, there was also an increase in the number of long term survivors, but never as many as in the inverse combina-

tion (approximately 50% fewer) and the increase in life span with different combination doses was never as high as the optimal doses when R17934 was injected first.

Also in this second experiment, for purposes of comparison, a combination of vincristine and Ara C was used under the same experimental conditions. In this case, we registered a relative increase in life span of the mice treated with the combination as compared to the mice treated with each of the drugs alone but there was no long term survival.

Therapeutic effect of i.p. treatment with R17934 (R) in combination with cytosine arabinoside (Ara C) on advanced L1210 leukemic ascites form

Two other experiments were conducted to determine the influence of time interval for cytosine arabinoside administration after the administration of R17934 in mice bearing L1210 leukemia and to investigate whether the combination remains synergistic in advanced L1210 leukemia. Animals were divided into a treated and an untreated control group. R17934 was given every 4 days after tumor implantation from day 4 to day 12 at the doses of 70 and 50 mg/kg in the first experiment. Treatment with cytosine arabinoside was started at 0, 8, 10, 12 and 15 hr intervals following R17934 at the doses of 400 and 200 mg/kg.

In the second experiment, 70 mg/kg of R17934 was given every four days from day 4 to day 12 and treatment with Ara C at the doses of 1200, 833, 600 and 416 mg/kg was started at 5-, 8-, 15- and 24-hr intervals following R17934 administration. In this experiment, a group of mice received 416 mg/kg of Ara C administered on days 4, 8 and 12 after tumor inoculation and 70 mg/kg of R17934 administered 15 hr later. The results of these experiments are shown in Table 2. The MST of the untreated control mice was 8·7 days in the first experiment and 8·6 days in the second experiment. The median survival time of mice treated with each of the drugs alone was as follows: 14·2 and 11 days respectively at the doses of 70 and 50 mg/kg of R17934; 10·9 or 10 days respectively at the doses of 400 and 200 mg/kg of cytosine arabinoside. The MST of the mice treated with the combination was higher than the MST of the mice treated with each drug separately, provided there was an interval of a few hours between the inoculation of the two drugs. When there was no interval, the combination became antagonistic and the MST of the mice treated with the combination was lower than the MST of the mice treated with each drug separately.

When Ara C was administered before R17934, the MST of this group of mice was similar to the MST of the group of mice treated with R17934 followed by Ara C at the same doses and same interval.

DISCUSSION

The fact that R17934 is a specific antitubuline has been demonstrated by De Brabander *et al.* [1] with different murine cells and human embryonal skin fibroblasts. The effect on *in vitro* cultured mammalian cells may be compared with that produced by colchicine and the vinca alkaloid [2, 3, 7–10]. The effect of R17934 *in vivo* on the mitotic cycle of L1210 leukemia was similar to the effect of vincristine (VCR) as may be seen from our study and is found comparable to the effect of vinblastine [11].

In this study it was observed that maximum relative metaphase arrest of L1210 leukemia cells *in vivo* occurred 8 or 10 hr after R17934 administration. The accumulation of cells in metaphse took approximately 3 hr which is similar to the effect of vincristine in our study and in the findings of Valdamudi and Goldin [11].

Optimal doses of R17934 yielded a good increase in the survival time of leukemic mice over the untreated controls but long term survivors were not observed.

Cytosine arabinoside has been shown to interrupt the DNA synthesis of growing cells [4, 12] and a therapeutic synergism was observed in combination treatment with vinblastine as a mitotic cell inhibitor administered 15–24 hr before cytosine arabinoside [11].

In our investigations, a very high synergism was also found when cytosine arabinoside was administered 15 hr after R17934 in early L1210 leukemia but this therapeutic synergism was less pronounced when Ara C was administered first. In the case of advanced L1210 leukemia, the therapeutic synergism was lower than in early leukemia. Synergism remained unchanged whether R17934 was administered before or after Ara C provided they were given at the same doses and with the same interval. When both drugs were administered simultaneously, an antagonism was observed, which may be explained by cumulative toxicity. It appeared sufficient to increase the interval between the administration of each drug to abolish the cumulative toxicity and demon-

Table 2. Therapeutic effect of i.p. treatment with R17934 (R) in combination with cytosine arabinoside (ARA C) on advanced L1210 leukemia ascites form

Agent mg/kg	Interval	MST (days)/range 1st Exp.	MST (days)/range 2nd Exp.	ILS % 1st Ex	ILS % 2nd Exp	Surv. Day 60 1st Ex.	Surv. Day 60 2 Exp.
Untreated Control		8.7/ 8-11	8.6/ 8- 9				
R17934 (70)		14.2/12-18	14.0/13-16	63	63	0	0
R17934 (50)		11.0/ 9-14		26		0	
Ara C (1200)			21.5/18-25		150		0
Ara C (833)			18.8/16-20		119		1
Ara C (600)			17.7/16-20		106		0
Ara C (416)			14.0/12-18		63		0
Ara C (400)		10.9/10-12		25		0	
Ara C (200)		10.0/ 9-12		15		0	
R17934 (70) + Ara C (1200)	15		27.5/19-35		220		1
R17934 (70) + Ara C (833)	5		25.5/22-30		197		0
R17934 (70) + Ara C (833)	8		24.0/20-29		179		0
R17934 (70) + Ara C (833)	15		29.6/28-33		244		0
R17934 (70) + Ara C (833)	24		28.5/26-36		231		0
R17934 (70) + Ara C (600)	15		22.8/19-32		165		0
R17934 (70) + Ara C (416)	15		24.2/17-30		181		0
R17934 (70) + Ara C (400)	0	13.5/12-16		55		0	
R17934 (70) + Ara C (400)	8	16.6/13-20		91		0	
R17934 (70) + Ara C (400)	10	18.9/15-22		117		0	
R17934 (70) + Ara C (400)	12	18.4/17-20		111		0	
R17934 (70) + Ara C (400)	15	18.6/15-24		114		0	
R17934 (70) + Ara C (200)	0	12.9/12-21		48		0	
R17934 (70) + Ara C (200)	8	17.5/14-21		101		0	
R17934 (70) + Ara C (200)	10	18.5/16-21		113		0	
R17934 (70) + Ara C (200)	12	17.5/13-20		101		0	
R17934 (70) + Ara C (200)	15	19.4/13-25		123		0	
R17934 (50) + Ara C (400)	0	12.2/11-13		40		0	
R17934 (50) + Ara C (400)	8	15.6/13-18		79		0	
R17934 (50) + Ara C (400)	10	17.0/15-19		95		0	
R17934 (50) + Ara C (400)	12	17.4/17-18		100		0	
R17934 (50) + Ara C (400)	15	17.2/17-18		98		0	
R17934 (50) + Ara C (200)	0	12.1/12-13		39		0	
R17934 (50) + Ara C (200)	8	13.5/12-16		55		0	
R17934 (50) + Ara C (200)	10	13.9/13-17		60		0	
R17934 (50) + Ara C (200)	12	15.1/12-17		74		0	
R17934 (50) + Ara C (200)	15	15.1/13-19		74		0	
Ara C (416) + R17934 (70)	15		23.5/11-27		173		0

BDF1 female mice (19-23 g) were inoculated i.p. with L1210 ascitic leukemia cells (10^5 cells/0.1 ml).

R17934 was injected i.p. every 4th day, Days 4, 8 and 12.

Ara C was injected i.p. every 4 th day.

MST: median survival time of 8 treated mice in both experiments and 56 untreated mice in the first experiment and 35 untreated mice in the second experiment.

ILS: percent increase in survival time over control.

strate a synergic effect. A 15 hr interval appeared optimal. The fact that a synchronization of the leukemic cells by metaphase arrest and subsequent treatment in S-phase resulted in therapeutic enhancement was well demonstrated in early leukemia but this phenomenon was not as clear enough in the case of advanced L1210 leukemia.

Acknowledgements—The technical help of Mrs. F. Bournonville-Lefort was greatly appreciated. The authors also wish to thank Mrs D. Maréchal-De Bock for reviewing and typing the manuscript.

REFERENCES

1. M. De Brabander, R. Vandeveire, F. Aerts, M. Borgers, L. Desplenter and P. A. Janssen, R17934, a new synthetic antitumoral drug interfering with microtubules. Effects on mammalian cells cultures *in vitro*. *Cancer Research.* (Submitted for publication).
2. A. N. Bhisey and J. J. Freed, Altered movement of endosomes in colchicine-treated cultured macrophages by colchicine or vinblastine. *Exp. Cell. Res.* **64,** 430 (1971).
3. A. N. Bhisey and J. J. Freed. Ameboid movement induced in cultured macrophages by colchicine or vinblastine. *Exp. Cell. Res.* **64,** 419 (1971).
4. M. Y. Chu and G. A. A. Fischer. Proposed mechanism of action of 1-B-D-arabinofuranosyl-cytosine as an inhibitor of the growth of leukemic cells. *Biochem. Pharmacol.* **11,** 423 (1962).
5. J. H. Cutts, Changes in mitosis in ascitic tumors and normal marrow induced by vincaleucoblastine *in vivo*. *Canad. Cancer Conf.* **4,** 363 (1961).
6. G. Atassi and H. J. Tagnon, R17934—NSC 238159: A new antitumor drug—I. Antitumor effect on experimental tumors and factors influencing this effectiveness. *Europ. J. Cancer* **11,** 559–607 (1975).
7. W. A. Greasy and M. E. Markiw. Biochemical effects of vinca alcaloids—II. A comparison of the effect of colchicine, vinblastine and vincristine on the synthesis of ribonucleic acids in Ehrlich ascites carcinoma cells. *Biochim. Biophys. Acta* **87,** 601 (1964).
8. E. F. Dag, T. J. Starr and K. K. Church, Micromelei fermentation in tissue cultured cells treated with colchicine. *Proc. Soc. Exp. Biol. Med.* **III,** 248 (1962).
9. J. J. Freed and M. M. Lebowitz, The association of a class of salatory movements with microtubules in cultured cells. *J. Cell. Biol.*, **45,** 334 (1970).
10. E. Robbins and N. K. Gonatas, Histochemical and ultrastructural studies on HeLa cells cultures exposed to spindle inhibitors with special reference to interphase cells. *J. Histochem. Cytochem.* **12,** 704 (1964).
11. Valdamudi and A. Goldin, Influence of mitotic cycle inhibitors on the anti-leukemic activity of cytosine arabinoside in mice bearing L1210 leukemia. *Cancer Chem. Reports, Part I,* **55,** 5 (1971).
12. J. H. Kim and M. L. Eidinoff, Action of 1 B-D-arabinofuranosylcytosine on the nucleic acid metabolism and viability of HeLa cells. *Cancer Res.* **25,** 698 (1965).

Europ. J. Cancer Vol. 11, pp. 615–618. Pergamon Press 1975. Printed in Great Britain

Relationship Between Plasma Carcinoembryonic Antigen and Prognosis in Women with Breast Cancer*

D. Y. WANG†, R. D. BULBROOK†, J. L. HAYWARD‡, J. C. HENDRICK§ and P. FRANCHIMONT§

†*Dept. of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, P.O. Box* 123, *Lincoln's Inn Fields, London, WC2A* 3*PX.*

‡*Breast Unit, Guy's Hospital, London, SE*1 9*RT.*

§*Laboratoire de Radioimmunologie, Hopital de Baviere—Institut de Medecine, Liège* 4000, *Belgium.*

Abstract—*The concentration of CEA was measured in plasma from normal women, patients with early breast cancer (before and after mastectomy) and advanced breast cancer. Assays were performed on coded plasma of unknown origin and only when the estimations had been completed was the code broken and the results analysed.*

The data showed that CEA levels were not of diagnostic value since the proportion of normal women with measurable CEA (35%) *was similar to that found in women with early breast cancer (before or after mastectomy). It was also observed that about* 30% *of subjects had an increased CEA level after mastectomy. The proportion of patients with advanced breast cancer with positive CEA titres was* 69%.

Patients after mastectomy with CEA levels above 2·5 *ng/ml had a significantly* ($P < 0{\cdot}001$) *faster recurrence rate than similar patients with CEA titres below this level. At two years after mastectomy the disease had recurred in* 65% *of the former group compared to* 20% *of the latter. Pre-operative levels of CEA did not correlate with recurrence rates.*

THERE is now sufficient evidence that many patients with cancers at various sites have an increased level of carcinoembryonic antigen (CEA) in their blood [1, 2]. CEA is not, however, diagnostic since undetectable levels can be found in patients with cancer while raised levels may be found in some normal subjects [2–5]. Thus, early hopes that the measurement of CEA might prove a diagnostic test for the presence of cancerous tissue have not been realized.

However, measurements of CEA concentrations have been claimed to be of value in monitoring the treatment of tumours of the colon and gastro-intestinal tract [2, 6] and Laurence *et al.* [7] have reported that the level of CEA is correlated with metastatic spread in women with breast cancer. CEA measurements therefore, might be of value in monitoring the treatment and in the prognosis of women with breast cancer.

The present study was designed to see whether the measurement of CEA was of value in predicting recurrence rates after mastectomy. At the same time, the CEA levels in the patients were compared with those in a normal population.

MATERIAL AND METHODS

Subjects

The 52 normal healthy women in this study were volunteers resident on the Island of Guernsey. These were totally unselected women from a general population. The patients with

Accepted 6 June 1975.

*Supported by a grant from the World Health Organization, Geneva, Switzerland and by Grant No. 20305 from the "Fonds de la Récherche Scientifique Medicale" (FRSM), Brussels, Belgium.

early breast cancer consisted of 71 women from whom blood was taken one day before mastectomy and 84 from whom blood was taken 10 days after mastectomy. Of these pre- and post-mastectomy samples, 56 of each were taken from the same patient. The advanced breast cancer group comprised 51 women. This latter group had either local (Stage 3) or widespread (Stage 4) recurrence of the disease. The mean ages and S.D. of the controls, early breast cancer (before and after mastectomy) and advanced breast cancer groups were $51{\cdot}7 \pm 7{\cdot}8$, $50{\cdot}8 \pm 9{\cdot}9$, $49{\cdot}3 \pm 10{\cdot}4$ and $50{\cdot}0 \pm 9{\cdot}0$, respectively. All patients were being treated at Guy's Hospital, London.

Blood was taken into heparinised tubes and after centrifugation the plasma was stored at $-20°C$. Aliquots of plasma, packed on solid CO_2, were air-freighted to Belgium, where they arrived still frozen. All blood samples were coded and as a consequence CEA was determined on plasma whose origin was unknown.

Method for CEA assay

All dilutions were made in Sorensen phosphate buffer, pH 7·5, 0·05 M, containing 0·5% bovine albumin. The incubation volume was 0·4 ml containing 50 pg of labelled CEA, anti-CEA serum (diluted 1:2000) and either a standard preparation of CEA (the Hoffman-La Roche material) or 0.1 or 0·05 ml of the unknown sample. In the tubes used for the establishment of the standard curve, 0·1 ml of a pool of normal human serum was added so that the concentration of human serum proteins would be the same in all tubes. After incubating for 4 days at 4°C, 0·1 ml of rabbit serum (diluted 1 in 200) and 0·1 of anti-γ-globulin serum (prepared in our laboratory and diluted 1–34) were added to the incubation medium. These concentrations correspond to the zone of equivalence allowing complete precipitation of the CEA antibody complex. Thirty-six to 48 hr later the precipitate was separated by centrifugation. It was washed twice with buffer and counted in a Phillips spectrometer. For each level of non-labelled CEA and for each serum, incubations were carried out in triplicate.

RESULTS

CEA levels in normal women and patients with breast cancer

The concentration of plasma CEA for normal women and patients with early and advanced breast cancer are shown in Fig. 1. There appears to be no difference between the titre of CEA in normal women and women with early breast cancer, before or after mastectomy. Although there were no consistent or significant changes in CEA concentration associated with mastectomy, it was observed that CEA levels increased in 17 out of 57 (30%) of patients after mastectomy (cf 43% in series reported by Chu and Nemoto [8]. The percentage above 2·5 ng/ml is about 33% for all these categories. In the group of patients with advanced breast cancer there is a marked increase in the number with elevated CEA concentrations; 69% having levels greater than 2·5 ng/ml.

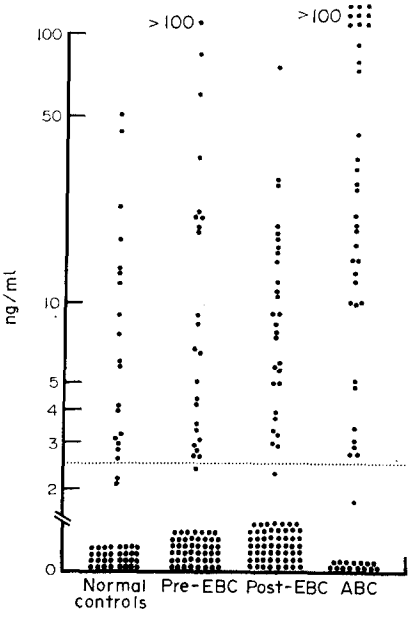


Fig. 1. Plasma CEA levels in normal controls and women with breast cancer. The four groups of women shown are as follows: normal controls, women with early breast cancer before mastectomy (pre-EBC) and after mastectomy (post-EBC), and patients with advanced breast cancer (ABC). The dotted line refers to 2·5 ng/ml CEA.

Prognosis

The relation between CEA levels (both before and after mastectomy) and recurrence rates were examined. Values of CEA lower than 2·5 ng/ml were below the limit of sensitivity of the assay. The recurrence rates of women with values for CEA below 2·5 ng/ml (before and after mastectomy) were compared with the rates for women with values higher than this cut-off level.

The recurrence rate for women with low values of CEA before mastectomy was not significantly different from that for women with high values and the measurements were therefore

of no prognostic value. However, after mastectomy in 81 patients in whom follow-up data were available, women with CEA levels of more than 2·5 ng/ml had a significantly higher recurrence rate than those with values of less than 2·5 ng/ml (Fig. 2). In the latter group, some 20% had a recurrence of the disease in two years compared with a 65% recurrence rate in women with high CEA levels.

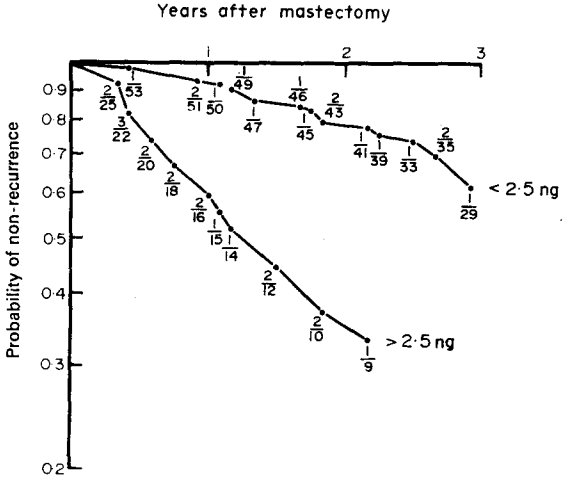


Fig. 2. Relationship between post-mastectomy CEA levels and recurrence rate of breast cancer. The recurrence rates of women with early breast cancer with < 2·5 ng/ml CEA are compared with the rate for similar women having > 2·5 ng/ml CEA (both measured after mastectomy). The numerator of the fractions refer to the number of patients whose disease has recurred whilst the denominator refers to the number of patients free of the disease. The recurrence rates for the two groups are significantly different (P < 0·001).

DISCUSSION

The proportion of patients with localized breast cancer who had CEA values greater than 2·5 ng/ml was about 33%, a figure which applied to women both before and after mastectomy. This compares with 10% of 10 similar patients who were studied by Reynoso *et al.* [9] and 36% of 59 patients investigated by Laurence *et al.* [7].

The present study confirms that there is a larger proportion of high CEA values in patients with advanced and progressive metastatic disease. In this study 69% fall into this group. This is comparable to the figures quoted by Reynoso *et al.* [9], (60%), Concannon *et al.* [4] (74%), Laurence *et al.* [7], (80%) and MacSween *et al.* [10] (about 75%). Lo Gerfo claimed that all 21 patients with metastatic breast cancer had positive CEA values [11].

In the present study, 35% of normal controls had values of plasma CEA exceeding 2·5 ng/ml. This contrasts with no positive CEA levels in the normal control population studied by Lo Gerfo *et al.* [11], Laurence *et al.* [7] and Reynoso *et al.* [9]. However, Concannon *et al.* [4] found 16 of 100 healthy volunteers and Meeker [5] reported 11% of 176 normal controls to have measurable CEA levels. Nevertheless, the high figure of 36% reported in this study is surprising, although it should be pointed out that there was no positive check on the state of health of the volunteers. The number of positive CEA levels is increased in heavy cigarette smokers [12] and our volunteers come from an area with one of the highest cigarette consumption rates in the world.

The main finding in this paper is the close relationship between plasma CEA concentration and the rate of recurrence of breast cancer after mastectomy. It is of interest that the post-operative levels give more prognostic information than the pre-operative concentrations. It may be that the post-operative levels are a measure of residual tumour mass, the latter being closely associated with prognosis.

Attempts to associate the actual concentration of plasma CEA with recurrence rates were unsuccessful in this study and this agrees with the report of Concannon *et al.* [13]. They found that the levels of CEA in patients with bronchiogenic carcinoma did not correlate well with survival.

Finally, it will be necessary to examine the prognostic value of post-mastectomy CEA measurements within stage and grade categories. This will require more patients than were examined in this study and the work is now in progress. As the results stand at the moment they indicate that post-operative CEA levels could be used to identify a third of patients after mastectomy who have 65% probability of recurrence within 2 years of operation.

REFERENCES

1. D. M. P. Thomson, J. Krupey, S. O. Freedman and P. Gold, The radioimmunoassay of circulating carcinoembryonic antigen of the human digestive system. *Proc. nat. Acad. Sci.* **64,** 161 (1969).
2. D. Holyoke, G. Reynoso and T. M. Chu, Carcinoembryonic antigen (CEA) in patients with carcinoma of the digestive tract. *Ann. Surg.* **176,** 559 (1972).

3. D. J. R. Laurence and A. M. Neville, Foetal antigens and their role in the diagnosis and clinical management of human neoplasms: a review. *Brit. J. Cancer* **26,** 335 (1972).
4. J. P. Concannon, M. H. Dalbow and J. C. Frich, Carcinoembryonic antigen (CEA) plasma levels in untreated cancer patients and patients with metastatic disease. *Radiology* **108,** 191 (1973).
5. W. R. Meeker, R. Kashmiri, L. Hunter, W. Clapp and W. O. Griffen, Clinical evaluation of carcinoembryonic antigen test. *Arch. Surg.* **107,** 266 (1973).
6. P. Dhar, T. Moore, N. Zamcheck and H. Z. Kupchik, Carcinoembryonic antigen (CEA) in colonic cancer. *J. Amer. med. Assoc.* **221,** 31 (1972).
7. D. J. R. Laurence, U. Stevens, R. Bettelheim, D. Darcy, C. Leese, C. Turberville, P. Alexander, E. W. Johns and A. M. Neville, Role of plasma carcinoembryonic antigen in diagnosis of gastrointestinal, mammary, and bronchial carcinoma. *Brit. med. J.* **3,** 605 (1972).
8. T. M. Chu and T. Nemoto, Evaluation of carcinoembryonic antigen in human mammary carcinoma. *J. nat. Cancer Inst.* **51,** 1119 (1973).
9. G. Reynoso, T. M. Chu, D. Holyoke, E. Cohen, T. Nemoto, J.-J. Wang, J. Chuang, P. Guinan and G. P. Murphy, Carcinoembryonic antigen in patients with different cancers. *J. Amer. med. Assoc.* **220,** 361 (1972).
10. J. M. MacSween, N. L. Warner, A. D. Bankhurst and I. R. MacKay, Carcinoembryonic antigen in whole serum. *Brit. J. Cancer* **26,** 356 (1972).
11. P. Lo Gerfo, J. Krupey and H. J. Hansen, Demonstration of an antigen common to several varieties of neoplasia. *New Eng. J. Med.* **285,** 138 (1971).
12. D. P. Stevens and I. R. MacKay, Increased carcinoembryonic antigen in heavy cigarette smokers. *Lancet,* **ii,** 1238 (1973).
13. J. P. Concannon, M. H. Dalbow, G. A. Liebler, K. E. Blake, C. S. Weil J. W. Cooper, The carcinoembryonic antigen assay in bronchogenic carcinoma. *Cancer (Philad.)* **34,** 184 (1974).

Europ. J. Cancer Vol. 11, pp. 619–622. Pergamon Press 1975. Printed in Great Britain

Metastatic Growth of Human Tumour Xenografts in Thymectomised Irradiated Mice Reconstituted with Syngeneic Bone Marrow Cells

C. R. FRANKS*, L. R. BOULGER, A. J. GARRETT, DIANA BISHOP, DIANE REESON and F. T. PERKINS†

Division of Viral Products, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB.

Abstract—*Human tumour xenografts were grown by s.c. implantation in thymectomised irradiated mice reconstituted with syngeneic bone marrow cells. Sixty-three tumour specimens were implanted into 462 mice, and tumour survivals of 1 to 20 weeks were recorded. At autopsy, a comprehensive macroscopic and microscopic examination was made. Eighteen cases of metastasis were observed from 5 different tumour types.*

INTRODUCTION

THE GROWTH of human tumours in thymectomised irradiated mice reconstituted with syngeneic bone marrow cells (T− B+) has been studied extensively [1–4]. In all these investigations using the T− B+ mouse, the tumours were implanted either in the renal capsule [1, 2], or subcutaneously [3, 4]. There has not been a single recorded case of metastatic growth in any of these studies. In 1974, Franks *et al.* reported that a human melanoma implanted in a mouse had metastasised to a para-aortic node [5]. Since then, metastatic growth from 3 other tumour types has been reported [6]. This report includes another 14 cases of metastases, making a total of 18 cases. The analysis is based on autopsy examination of 462 mice, into which 63 tumour specimens were implanted.

Accepted 27 May 1975.

*ICRF Breast Cancer Unit, Guy's Hospital, London, SE1 9RT.

†Chief, Biological Standardization, W.H.O., 1211 Geneva 27, Switzerland.

MATERIAL AND METHODS

HeLa tumours

HeLa cells were originally obtained from the Central Public Health Laboratories at Colindale. Thereafter, the cells were passaged in the laboratory; they were shown to be free from mycoplasma. Each mouse received $2{\cdot}5 \times 10^5$ cells s.c., when the HeLa cells were given as a suspension, or, at least 3 mm^3, when transplanted from a mouse carrying a solid HeLa tumour. One hundred and sixty implantations were made from 12 HeLa samples.

Human tumours

Tumour specimens were obtained at operation from patients with primary cancer. These were transported to the laboratory in a medium containing Eagle's minimum essential medium, 2% calf serum, 200 units ml^{-1} of penicillin, and 100 $\mu g\ ml^{-1}$ of streptomycin sulphate. At least 3 mm^3 pieces of tumour were implanted s.c. in the iliac region of the mouse. Three hundred and two implantations were made from 51 tumour specimens.

Mice

Female CBA mice bred in the specific

pathogen-free colony at the National Institute for Medical Research were used.

Immune deprivation

A method similar to that of Miller *et al.* (1963) [7] was used. The mice were thymectomised at 4 weeks of age, and 3 weeks later were given a lethal dose of 900R whole body irradiation from a ^{60}Co source. Within 5 hr of irradiation, each mouse received 10^7 syngeneic bone marrow cells i.v.

Measurement of tumours

The vertical and transverse diameters of the solid tumour implants were measured immediately after implantation, and thereafter once a week, using vernier calipers. As soon as a palpable tumour appeared after the inoculation of a cell suspension, the vertical and transverse diameters were also measured. Where possible, the same person took all the measurements for a particular tumour specimen.

Autopsy examination

When the implanted tumours threatened the life of the mouse, or showed signs of regression, the mouse was killed. Histological sections were made of the following:

(1) Tumour implant (2) Lungs
(3) Liver (4) Spleen
(5) Kidneys (6) Ovaries
(7) Lymph nodes
(8) Vertebral column and long bones

RESULTS

Survival of tumour implants

Of the 160 mice implanted with the 12 HeLa tumour specimens, only 94 mice showed evidence of a palpable tumour at one week (Table 1). By eight weeks, 40 mice had palpable tumours, and, at 20 weeks, only 7 of the original 160 mice had palpable tumours. Thirteen cases of metastatic growth were found at autopsy.

Of the 302 mice implanted with 51 tumour specimens from patients with primary cancer, 13 mice with 2 caecal, 3 rectal, and 5 anal carcinoma implants were still palpable at 20 weeks. Three mice had palpable melanomas (Table 1). At 16 weeks, just under half of the mice, implanted with 17 specimens from patients with breast cancer, had palpable tumours, and 3 of the 18 mice implanted with colonic carcinomas also had palpable tumours. Of the remaining 6 tumour types investigated, only the mice implanted with squamous cell carcinomas had palpable tumours beyond 8 weeks. Mice with gastric and basal cell carcinomas had detectable tumours at 8 weeks, and the remaining 3 tumour types, mainly carcinomas of the tongue, pancreas, and bronchus did not grow beyond the first week following implantation. In all the specimens in Table 1, the histological analysis at autopsy, and the comparison of the implant with the original tumour specimen removed from the patient, confirmed the presence of the specific tumour specimen being studied. It also demonstrated 5 cases of metastatic growth.

Metastatic growth of tumours

Metastases were found at autopsy in mice into which 2 melanomas, a colon, a rectal, and a squamous cell carcinoma had been implanted. In addition, 13 cases of metastases, following implantation with HeLa, were also found (Table 2). Nine out of 18 cases were to lymph nodes; 8 out 18 to lung, and 1 out 18 to spleen.

Table 1. *Survival of s.c. implanted tumours in T− B+ mice*

Tumour type	Tumour implants	Mice implants	Mice with metastases	Mice takes 1 wk	4 wk	8 wk	12 wk	16 wk	20 wk
HeLa	12	160	13	94	59	40	24	21	7
Melanoma	17	84	2	67	40	21	6	5	3
Colon	3	18	1	12	8	4	3	3	
Breast	3	17	0	11	15	8	7	6	
Caecum	6	61	0	42	28	13	7	5	2
Rectum	5	27	1	25	13	6	5	3	3
Anus	7	43	0	37	21	16	8	5	5
Sq cell ca	2	12	1	11	2	2	1		
Tongue	2	7	0	2					
Pancreas	1	8	0	8	1				
Bronchus	1	8	0	7					
Stomach	3	15	0	15	7	4			
Basal cell ca	1	2	0	1	1	1			

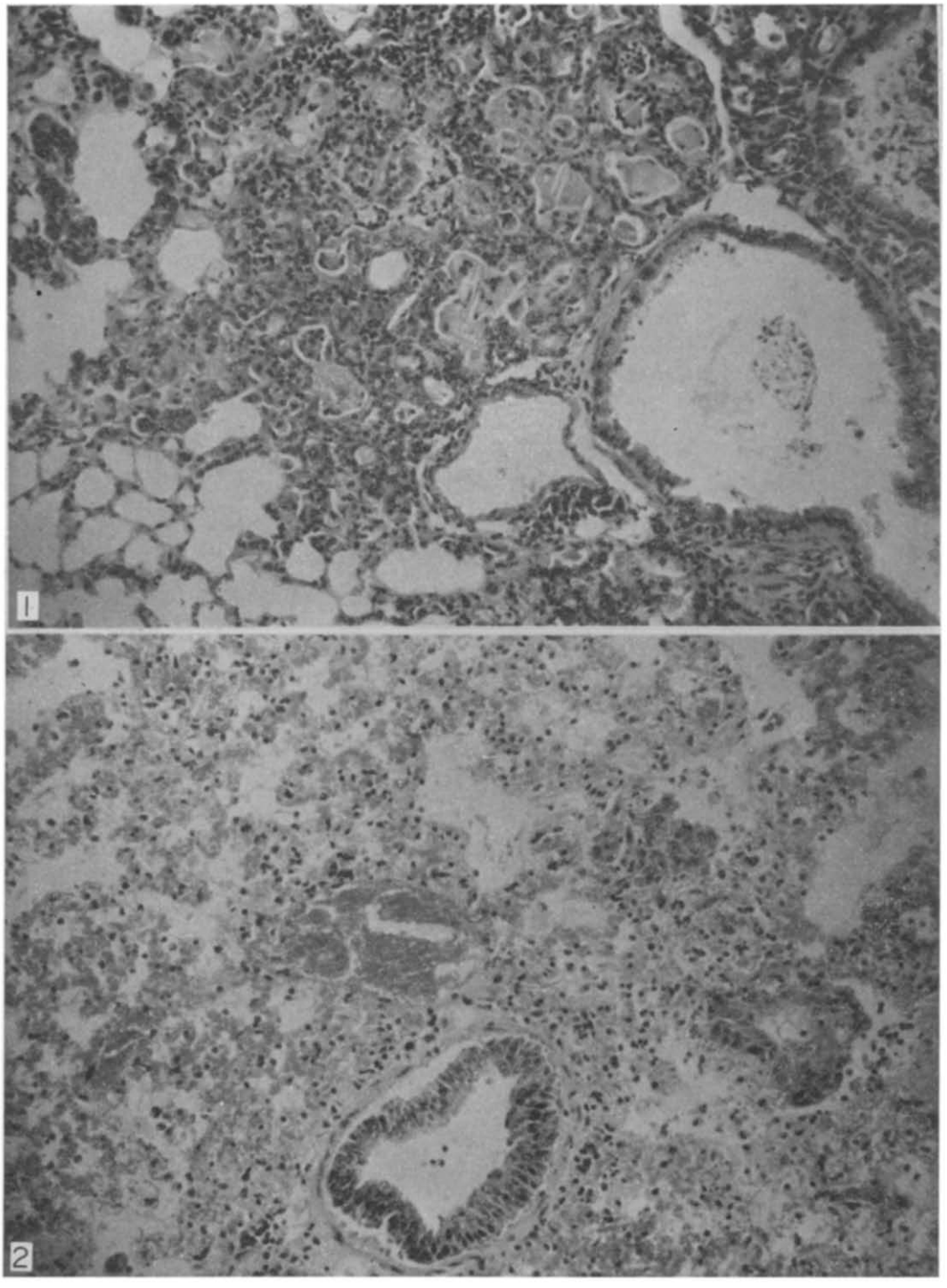


Fig. 1. *Metastatic growth of carcinoma of rectum to mouse lung.* × 80.

Fig. 2. *Metastatic growth of carcinoma of colon to mouse lung.* × 80.

(to face page 620)

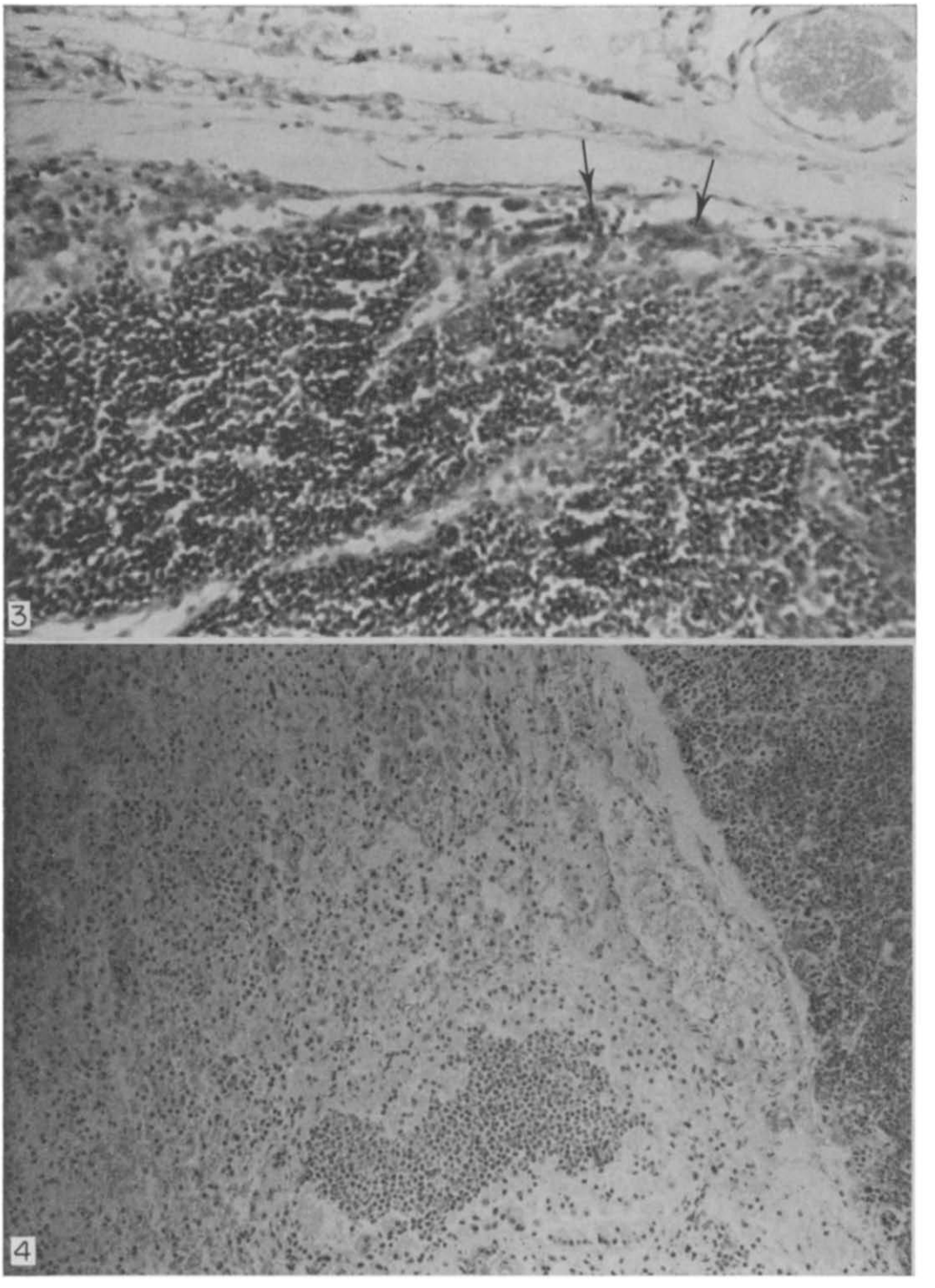


Fig. 3. *Metastatic growth of melanoma to mouse para-aortic node.* × 200.

Fig. 4. *Metastatic growth of HeLa tumour to mouse lung.* × 40.

Table 2. Metastatic growth of s.c. implanted tumours in T− B+ mice

Tumour type	Day killed after implantation	Passage	Site of metastases
Melanoma	19	1st	Lymph node (no type)
Melanoma	69	1st	Para-aortic node
Colon	146	1st	Lung
Rectum	47	1st	Lung
Sc Ca Pinna	98	1st	Para-aortic node and mesentery
HeLa	38	1st	Lung
HeLa	46	1st	Inguinal node
HeLa	64	1st	Axillary node
HeLa	66	1st	Lung
HeLa	84	1st	Lung
HeLa	84	1st	Inguinal node
HeLa	91	1st	Lung
HeLa	106	2nd	Inguinal node
HeLa	124	6th	Lung
HeLa	154	1st	Para-aortic node
HeLa	161	1st	Spleen
HeLa	161	1st	Lung
HeLa	199	3rd	Para-aortic node

These occurred from day 19 to day 199 after implantation. Fig. 1–4 show metastatic growth of the rectum, colon, a melanoma, and HeLa tumours.

DISCUSSION

It is not entirely clear why metastatic growth of tumour xenografts is now occurring. Although this is a recent finding in T− B+ mice, Cobb has observed metastatic spread in immune deficient hamsters [8]. In these studies, 3·9% of the mice demonstrated metastases, and 2·3% occurred from HeLa tumours. In mice treated with antilymphocyte serum, HeLa tumours never metastasise [9]. Why HeLa should demonstrate such radically different behaviour in two similar experimental groups of mice, both of which were immune incompetent to a greater or lesser extent, is not possible to say.

Of the 12 primary carcinomas investigated, metastases were found only in 1·1% of implanted mice. Clearly, in the T− B+ mouse, these tumours do not retain their human metastatic characteristics. Were this the case, metastases would be found in a high percentage of mice, because many of the tumours studied have a high tendency to metastasise in humans.

Metastases do not appear to be related to any particular growth period of a tumour in the mouse, nor are they governed by the number of passages of an individual tumour. Most of the metastatic growth observed occurred during the first passage, and varied from day 19 to day 199, these being the days the mice were killed. It is of course possible that metastases may have developed long before the mice were withdrawn from the experiment. Five of the tumours included in this study were in mice killed after the 20 week study period. The only significant change that has been made recently is the use of mice from the specific pathogen-free unit at the National Institute of Medical Research. This was done to guarantee pathogen-free mice unlike the outbred stock of mice which often died following thymectomy.

In view of the rarity of metastatic growth of tumour xenografts in T− B+ mice, it is possible that the process of transplantation of primary tumours to mice represents essentially the process of metastasis. As such, when carcinomas are removed from their primary human environment and transplanted to mice, they no longer behave as primary tumours, and therefore lose the human characteristic of being able to metastasise, being metastases themselves now. This hypothesis, however, does not account for the rare finding of mouse metastases from implanted human tumours, nor does it explain why HeLa tumours metastasise in T− B+ mice, and not in mice treated with antilymphocyte serum. It is an interesting finding, which needs further study.

REFERENCES

1. J. E. Castro, Human tumours grown in mice. *Nature* (*New Biol.*) **239,** 83 (1972).
2. J. E. Castro, A method of *in vivo* maintenance of human prostatic tissue in immunosuppressed mice. *Brit. J. Urol.* **45,** 163 (1973).
3. C. R. Franks, F. T. Perkins and J. Thornton Holmes, Subcutaneous growth of human tumours in mice. *Nature* (*Lond.*) **243,** 91 (1973).
4. L. M. Cobb and B. C. V. Mitchley, Growth of human tumours in immune deprived mice. *Europ. J. Cancer* **10,** 473 (1974).
5. C. R. Franks, F. T. Perkins and J. Thornton Holmes, Growth of human tumours in immune suppressed mice. *Proc. roy. Soc. Med.* **68,** 287 (1975).
6. C. R. Franks, F. T. Perkins, L. R. Boulger and J. Thornton Holmes, Maintenance of human tumours in immune deficient mice. *Brit. J. Cancer* **31,** 260 (1975).
7. J. F. A. P. Miller, Sheila M. A. Doak and A. Marjorie Cross, Role of the thymus in recovery of the immune mechanism in the irradiated adult mouse. *Proc. Soc. exp. Biol. Med.* **112,** 785 (1963).
8. L. M. Cobb, Metastatic spread of human tumour implanted into thymectomised antilymphocyte serum treated hamsters. *Brit. J. Cancer* **26,** 183 (1972).
9. C. R. Franks, Krystyna Curtis and F. T. Perkins, Long-term survival of HeLa tumours in mice treated with antilymphocyte serum. *Brit. J. Cancer* **27,** 390 (1973).

Europ. J. Cancer Vol. 11, pp. 623–626. Pergamon Press 1975. Printed in Great Britain

High Dose Intravenous Bleomycin in the Treatment of Advanced Lymphomas

SUSAN P. HUBBARD, BRUCE A. CHABNER, GEORGE P. CANELLOS, ROBERT C. YOUNG and VINCENT T. DEVITA, JR.

Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014, *U.S.A.*

Abstract—*Aggressive therapy with high dose intermittent courses of bleomycin (25 mg/M² daily for five consecutive days) was evaluated in patients with advanced rapidly progressive lymphomas in an attempt to exploit the relative marrow-sparing effects of this drug in patients and to maximize responses early in treatment by reaching a high total dose level over a short time interval. Toxicity was similar to that observed in patients given the lower conventional doses in other studies, but, as could be expected, occurred earlier and at total doses averaging* 125–150 *mg/M².*

In spite of the rapid administration of a full therapeutic dose, the response rate in this small group of patients did not seem to be enhanced.

INTRODUCTION

BLEOMYCIN, an antitumor antibiotic introduced into clinical trials in 1966 [1], has demonstrated antitumor activity in patients with Hodgkin's disease and non-Hodgkin's lymphomas [2–4]. Its relative lack of myelosuppressive toxicity has made bleomycin an attractive agent for use in patients with compromised bone marrow function.

The conventional schedules of bleomycin administration have been either a weekly or twice weekly regimen of 5 to 15 mg/M² per dose. However, pharmacologic data have suggested that alternative schedules providing more prolonged plasma levels of the drug might offer a significant advantage in enhancing tumor cytotoxicity [8, 9]. We have treated a group of patients with advanced lymphoma with a new dose schedule of daily i.v. injections of 25 mg/M² for five consecutive days and herein report our observations concerning toxicity and antitumor response.

MATERIAL AND METHODS

Twenty-two patients with advanced lymphomas received five-day courses of i.v. bleomycin at 25 mg/M²/day, repeated at monthly intervals. Twelve patients had Hodgkin's disease and 10 had non-Hodgkin's lymphomas. Seventeen patients were males; 5 were females. The median age of the group of patients was 29 (range 15–68), and the median duration of disease prior to therapy with bleomycin was 15·5 months (range 3–84 months). Nineteen of these 22 patients had documented stage IV disease. Nine patients had bone marrow involvement with lymphoma. One had suspected marrow involvement on the basis of unexplained pancytopenia and another had myelofibrosis. All patients with lymphomas were refractory to intensive combination chemotherapy with MOPP, CVP, or C-MOPP. The majority of patients had received significant radiotherapy in addition to chemotherapy. Thrombocytopenia (< 100,000 cells/mm³) and/or granulocytopenia (< 2000 cells/mm³) were present at the time that treatment was initiated in 19 of 22 patients as a result of marrow involvement or prior myelosuppressive therapy. Only one patient receiving therapy with bleomycin had entirely normal peripheral blood counts.

Measurable disease was present in all patients and each was evaluated weekly for evidence of drug response and toxicity.

Accepted 15 May 1975.

A partial remission was defined as a greater than 50% reduction of all measurable disease with a duration in excess of one month. A minor response was defined as a clinically useful response which represented less than 50% improvement or lasted less than one month.

All biopsy and autopsy examinations were reviewed by the Laboratory of Pathology of the National Cancer Institute. Post mortem examinations were performed and reviewed in 21 out of 22 patients. One patient remains alive with active disease.

RESULTS

All patients admitted to the study were considered evaluable. Two of 22 patients with malignant lymphomas who received five-day courses of i.v. bleomycin achieved a partial remission lasting 2 and 3 months respectively. Both patients had Hodgkin's disease; one was Stage IIB, the other Stage IV_{M+H+}. Both had failed therapy with MOPP and BCNU. Three patients had greater than 50% tumor regression lasting less than one month. Seven additional patients achieved a clinically useful objective response of short duration. Four of these transient responses were seen in patients with Hodgkin's disease, and 3 were in patients with non-Hodgkin's lymphomas.

Toxicity

A primary objective of the study was to evaluate the toxicity of this intensive schedule. The most common side effect encountered was fever. Thirteen of 22 patients had febrile reactions with each dose of drug, occurring within 10 hr of administration. Temperature elevations frequently reached 40°C. Shaking chills accompanied episodes of fever in 5 patients. Nausea, vomiting, and general malaise were also observed frequently, although the intensity and duration of these reactions varied widely among the patients. Prolonged nausea occurred in three patients but did not necessitate drug discontinuation.

Mucocutaneous

Manifestations of bleomycin-related skin toxicity occurred in 6 of 22 patients receiving the five-day schedule. Hyperpigmentation, dryness, pruritis, dermatographia, palmar erythema and alopecia were the changes observed, appearing 7–10 days after the start of therapy. In 3 patients, small ulcerations occurred in areas of diffuse erythema. Therapy was not curtailed in any of these patients on the basis of skin toxicity. Four patients developed mild mucositis. Moderately severe dysphagia which developed in one patient represented an exacerbation of pharyngitis related to prior radiation to cervical nodes.

Pulmonary

Evidence of pulmonary fibrosis secondary to bleomycin given in this manner was observed in 3 patients, and in one, a 54-year-old male with IVB_{H+M+} Hodgkin's disease, proved fatal. This patient, who had received extensive prior chemotherapy, had an excellent partial response lasting three months with complete disappearance of incapacitating fevers and night sweats, clearing of bone marrow involvement on two sequential bone marrow biopsies, and progressive improvement in abnormal liver function tests with each of his three cycles of bleomycin therapy. After receiving a total dose of 350 mg/M^2 of bleomycin (662 mg), the patient noted the onset of dyspnea, fever and sweats. A patchy bilateral interstitial infiltrate was seen on chest X-ray at this time. Routine cultures and pulmonary needle aspiration were negative for pathogens. The patient was treated with 40 mg of prednisone daily for presumed bleomycin pulmonary toxicity. He died of pulmonary insufficiency one month after the onset of symptoms. At autopsy, his lungs demonstrated severe pulmonary fibrosis with hyperplasia of alveolar lining cells and marked focal squamous metaplasia most severe in peripheral pulmonary parenchyma, characteristic of bleomycin lung toxicity [5, 6]. Review of autopsy material available in twenty-one autopsied cases from this series revealed two additional patients with incidental pulmonary pathologic findings consistent with bleomycin toxicity. Both had focal pulmonary fibrosis in addition to pulmonary lymphoma and acute bronchopneumonia. Both had been treated with the five day schedule to total doses of 100 mg/M^2 (204 mg) and 125 mg/M^2 (245 mg).

Acute allergic reactions in the form of acute bronchospasm occurred in two patients. The first patient developed acute dyspnea and intense bilateral bronchospasm two hours following his first dose of 25 mg/M^2 of bleomycin. A second less intense bronchospastic reaction occurred following the second dose and responded to aminophylline. Aminophylline was administered prior to subsequent doses of bleomycin and no additional reactions occurred. The second patient had exhibited shortness of breath following each daily dose,

and developed acute dyspnea, periorbital edema, and bronchospasm at a total dose of 200 mg/M^2 which resulted in discontinuation of bleomycin therapy. Neither of these patients had pathologic evidence of bleomycin lung toxicity at autopsy.

Hepatic

Transient hepatic abnormalities were seen in two patients receiving five day courses of intravenous bleomycin. One patient became jaundiced during two consecutive five day courses of therapy. Both jaundice and liver function abnormalities cleared during the week following each of the two cycles he received. The other patient developed transient liver function abnormalities during his second cycle of therapy which also resolved spontaneously following the five day course, but did not recur with subsequent courses.

Hematologic

Myelosuppression, defined as greater than 50% reduction in peripheral WBC and platelet counts was seen in 14 instances (9 patients) following five daily doses of 25 mg/M^2. In 12 of 14 instances the baseline values prior to therapy were abnormal. No episodes of bleeding or infection developed as a result of bleomycin therapy.

DISCUSSION

Because administration of bleomycin is associated with relatively little marrow toxicity, it has been used in the treatment of pancytopenic patients with advancing lymphoma refractory to other therapy. Low dose daily therapy with bleomycin has been studied in lymphomas and response rates in the range of 40–50% have been reported [3, 7]. However, the quality and duration of responses to bleomycin alone have been disappointing even when maintenance has been given to those patients who initially responded well.

The intensive five-day course of bleomycin at a dose of 25 mg/M^2 was selected to determine whether this schedule would result in more rapid regression of disease and greater responses than achieved with conventional biweekly or weekly schedules which are generally employed. There are no data on the therapeutic or toxic effects of daily high-dose administration of bleomycin.

Pharmacologic studies have suggested that a daily high-dose schedule might have enhanced effectiveness. The data have demonstrated rapid disappearance of drug from plasma, with an initial half-life of 16 to 45 min [8]. Tissue culture studies have further indicated that the drug has its greatest cytotoxic effect during the G_2 phase of the cell cycle and that cell kill is maximized with prolonged exposure to the drug [9]. In addition, assays of tissue inactivation, distribution, and clearance suggest that rapid disappearance of measurable drug activity relates to the ability of various biologic tissues to inactivate the antibiotic [8]. These findings have provided a rationale for the use of intermittent high-dose schedules or multiply daily doses to produce greater therapeutic effects.

Although skin toxicity occurred with five-day courses of bleomycin, it was not the dose-limiting skin toxicity frequently encountered with chronic biweekly administration of 15 mg/M^2 [2–4]. Mucocutaneous toxicity with this regimen was not associated with unacceptable morbidity, and in no instance did it necessitate interruption of therapy. However, several episodes of serious allergic reactions were encountered with this schedule. Severe bronchospasm requiring treatment occurred in 2 patients. Prolonged severe nausea and vomiting was also seen in 3 patients. Fever and shaking chills were encountered in 5 patients, and this side effect prompted one patient to refuse his fifth dose of drug. Fatal pulmonary fibrosis developed in one patient at a total dose of 350 mg/M^2 (662 mg).

Although bleomycin can be given in short high-dose intermittent courses with acceptable toxicity, these results suggest that the low response rate and associated morbidity encountered do not show any therapeutic advantage for this schedule over conventional low dose administration of bleomycin.

REFERENCES

1. H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, New antibiotics, bleomycin A and B. *J. Antibiol. Tokyo* **19,** 200 (1966).
2. T. Ohnuma, O. Selawry, J. F. Holland, V. T. De Veta, Jr., D. P. Shedd, H. H. Hansen and F. M. Muggia, Clinical study with bleomycin: tolerance to twice weekiy dosage. *Cancer (Philad.)* **30,** 914 (1972).

3. A. Yagoda, B. Mukherji, C. Young, E. Etcubanas, C. Lamonte, J. R. Smith, C. T. Tan and I. H. Krakoff, Bleomycin, an antitumor antibiotic. *Ann. intern. Med.* **77,** 861 (1972).
4. R. H. Blum, S. K. Carter and K. Agre, A clinical review of bleomycin—A new antineoplastic agent. *Cancer (Philad.)* **31,** 903 (1973).
5. M. De Lena, A. Guzzon, S. Monfardini and G. Bonadonna, Clinical, radiologic, and histopathologic studies on pulmonary toxicity induced by treatment with bleomycin (NSC–125066). *Cancer Chemother. Rep.* **56,** 343 (1972).
6. F. Perez-Guerra, L. E. Harkleroad, R. E. Walsh and J. J. Costanzi, Acute bleomycin lung. *Amer. Rev. resp. Dis.* **106,** 909 (1972).
7. R. A. Rudders, Treatment of advanced malignant lymphomas with bleomycin. *Blood* **40,** 317 (1972).
8. T. Ohnuma, J. F. Holland, H. Masuda, J. A. Waligunda and G. A. Goldberg, Microbiological assay of bleomycin: inactivation, tissue distribution, and clearance. *Cancer (Philad.)* **33,** 1230 (1974).
9. B. Drewinko, J. K. Novak and S. C. Barranco, The response of human lymphoma cells *in vitro* to bleomycin and 1,3-Bis(2-chloroethyl)-1-nitrosourea. *Cancer Res.* **32,** 1206 (1972).

Europ. J. Cancer Vol. 11, pp. 627–632. Pergamon Press 1975. Printed in Great Britain

Impaired Responsiveness to Folinic Acid Protection in Methotrexate-resistant L5178Y Cells*

J. H. GOLDIE, S. I. HARRISON, L. A. PRICE and BRIDGET T. HILL

Department of Medicine, St. Michael's Hospital and the University of Toronto, Toronto, Canada.

Abstract—*The ability of folinic acid to prevent lethal effects of pyrimethamine and methotrexate on methotrexate-sensitive and methotrexate-resistant L5178Y lymphoblasts in tissue culture has been studied. In the methotrexate-sensitive cell line the addition of equimolar amounts of folinic acid significantly reduces the lethal effect of methotrexate and completely abolishes the lethal effect of pyrimethamine. In the methotrexate-resistant sub-line, however, equimolar amounts of folinic acid failed to prevent the cytotoxic effects of either methotrexate or pyrimethamine. Further studies showed that folinic acid competed with pyrimethamine for uptake into the sensitive cells but did not prevent the uptake of pyrimethamine into the resistant cells. The inability of folinic acid to prevent pyrimethamine influx into methotrexate-resistant cells may account possibly for the failure of folinic acid to reverse pyrimethamine toxicity in this cell-line.*

INTRODUCTION

PROTECTION against the cytocidal effects of folic acid antagonists by folinic acid (5-formyltetrahydrofolic acid), both *in vivo* and *in vitro*, has been reported by numerous workers [1, 2, 3]. Goldin *et al.* [3] demonstrated that delayed administration of folinic acid following treatment with methotrexate (MTX) of mice bearing the L1210 leukemia protected against the toxic effects of the MTX without significantly reducing therapeutic effects. Simultaneous administration of folinic acid and MTX prevented toxicity but resulted in significant loss of therapeutic activity. Borsa and Whitmore [2] showed that the simultaneous addition of folinic acid to L-cells exposed to MTX appreciably reduced toxic effects on cell viability.

Whether all mammalian cells can be expected to show the same sensitivity to folinic protection is uncertain; in particular, the ability of folinic acid to protect tumour cell lines, showing varying degrees of resistance to MTX, has not been established. In this report, we found that an MTX-resistant subline of L5178Y lymphoblasts exhibited a markedly impaired responsiveness to folinic acid protection, as compared with the parent MTX-sensitive line.

We have shown previously that both MTX and pyrimethamine (PRM) act as potent folic acid antagonists against L5178Y lymphoblasts *in vitro* [4]. In this study, we have determined the effects of simultaneous addition of equimolar folinic acid with either MTX and PRM on the colony—forming ability of two sublines of L5178Y lymphoblasts. In addition, the effects of folinic acid on the extent of uptake of isotopically labelled PRM and MTX by both cell lines was measured.

The sublines of L5178Y cells used in these experiments were (i) an MTX-sensitive line, which is inhibited by continuous exposure to 10^{-8} M MTX and (ii) an MTX-resistant line, which can grow in 10^{-6} M MTX but is inhibited by drug concentrations of 5×10^{-6} M. Resistance in this cell line has been previously found to be related to impaired MTX trans-

Accepted 18 March 1975.

*Supported by the Ontario Cancer Treatment and Research Foundation, Grant No. 286.

port [5] with a two-fold difference in uptake between sensitive and resistant cells (see Results).

For the present studies, drug concentrations were selected sufficient to produce a significant cell kill (greater than one log reduction in viability) even in the drug resistant line.

MATERIAL AND METHODS

Chemicals and reagents

MTX and folinic acid were obtained from Lederle Products Limited, Montreal, Quebec. PRM (non-radioactive) and ^{14}C-PRM (specific activity 14·7 mCi per mole) were obtained as gifts from the Burroughs Wellcome Co., courtesy Dr. C. A. Nichol, who tested the materials for radiochemical purity. ^{3}H-MTX (specific activity 250 mCi per mmole) was purchased from Amersham Searle Corporation, Illinois, U.S.A. and checked for radiochemical purity by paper chromatography in (*a*) 0·5% sodium carbonate solution and (*b*) *n*-butanol: pyridine: water (1:1:1).

Cell cultures

The two sublines of L5178Y lymphoblasts were grown in suspension, in Fisher's Medium for leukemic mouse cells (Grand Island Biological Supplies, New York, U.S.A.), supplemented with 10% fetal calf serum (Flow Laboratories Limited, Maryland, U.S.A.). The viability assays were performed according to the method of Chu and Fisher [6] as modified by us [4]. The cells were incubated for varying times, with a range of drug concentrations, then washed free of drug before being added at a concentration of 200 per ml to cloning media. This was tissue culture media supplemented with dilute agar (0·15%) and heavily-irradiated (1500 rads) L5178Y cells. The irradiated cells were required to provide conditioning factors to enhance cloning efficiency, which ranged between 50 and 75%. Background colonies from irradiated cells alone were negligible. Treated cultures were expressed as a percentage of the control, which was given the value of 100%. The mean and s.d. of the colony counts of 5 replicate cultures were computed to attain the survival curves which were produced by plotting the percent surviving fraction against time on a semi-logarithmic scale.

Transport studies

L5178Y lymphoblasts selected from cultures in logarithmic growth at a density of approximately 2×10^6 cells/ml were counted and removed from suspension by centrifugation at 350 *g* at room temperature for 3 min. The cells were resuspended at a concentration of 2×10^6 cells/ml in Hank's balanced salt solution pH 7·4 (Grand Island Biologicals Ltd., N.Y., U.S.A.) at 37°C. Drugs were added to aliquots of these cell suspensions, resulting in a final drug concentration of 5×10^{-5} M and a radioisotope concentration of 0·625 μCi/ml and 2·5 μCi/ml of ^{14}C-PRM and ^{3}H-MTX respectively. Incubations were carried out at 37°C in a water bath with gentle shaking. Previous experiments had established that after a 30 min incubation, a steady-state concentration of both PRM [7] and MTX [5] was achieved in both cell lines. Therefore, aliquots of cell suspension were removed at 30 min after drug addition and rapidly chilled by immersion in ice and dilution $\times 5$ with ice-cold Hank's balanced salt solution. The cells were removed from suspension by centrifugation at 500 *g* for 2 min, washed once in ice-cold Hank's solution and twice with ice-cold 0·15 M NaCl. The resultant cell pellets were solubilized in 100 ml of N.NaOH by resuspension at room temperature and neutralized with HCl prior to mixing with 10 ml Aquasol (New England Nuclear, Dorval, Quebec). Radioactivity was determined using a Nuclear Chicago Scintillation Counter Unilux II Model No. 6853 at 63% efficiency for ^{14}C and 40% efficiency for ^{3}H.

The efflux of MTX from L5178Y cells at 37°C was determined by pre-incubating cells with drug for 30 min, using the conditions described above. The cells were then pelleted by centrifugation at 350 *g* for 2 min at room temperature, and resuspended to the same volume in fresh Hank's solution without drug. Aliquots were removed at various later time intervals and their radioactive content determined.

RESULTS

Figure 1(a) shows the effects of 10^{-4} molar MTX on the survival of the L5178Y MTX-sensitive cell line following 6, 24, and 48 hr drug exposure, in the presence and absence of equimolar folinic acid. Figure 1(b) shows the effects of the same drug concentration on the MTX-resistant line. 10^{-4} molar MTX by itself significantly reduced cell viability in both cell lines, resulting in a 2·6 log kill for the sensitive line, and a 1·5 log kill for the resistant cells after 48 hr. The simultaneous addition of equimolar folinic acid with the MTX had no effect on the response of resistant L5178Y cells

to MTX. However, the lethal effects of MTX on the sensitive cells were considerably reduced by exposure to the combination of MTX and folinic acid, with only a 0·8 log kill after 48 hr.

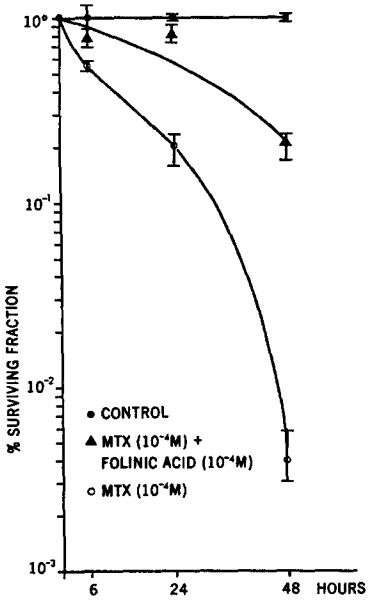


Fig. 1(a). Effect of 6-, 24- and 48-hr exposure to 10^{-4} molar MTX, and 10^{-4} molar MTX+10^{-4} molar folinic acid on the colony-forming ability of L5178Y MTX-sensitive cells. Percentage surviving fraction plotted against duration of drug exposure. Each point represents the mean of 5 determinations.

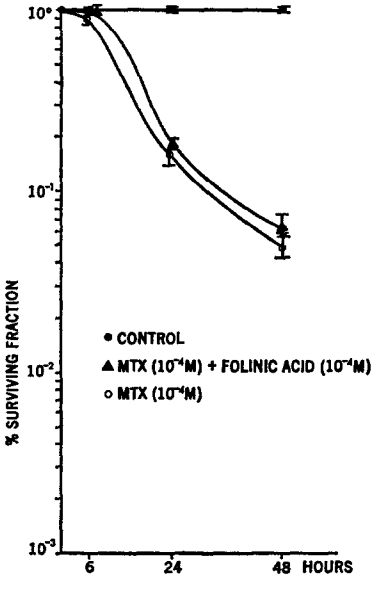


Fig. 1(b). As in 1(a), except L5178Y MTX-resistant cells employed.

Figures 2(a), (b) show the effects of PRM alone (10^{-5} molar) and PRM+10^{-5} molar folinic acid on the colony-forming ability of MTX-sensitive and MTX-resistant cells. PRM produced approximately a 1·5 log kill in both cell lines following a 48 hr exposure. The simultaneous addition of equimolar folinic acid abolished the reduction of cell viability in the MTX-sensitive cells. In the resistant cells, however, no protection by folinic acid was observed.

Table 1 shows the effect of folinic acid on the steady-state level of intracellular MTX and PRM. The presence of folinic acid markedly reduced the level of PRM achieved in the

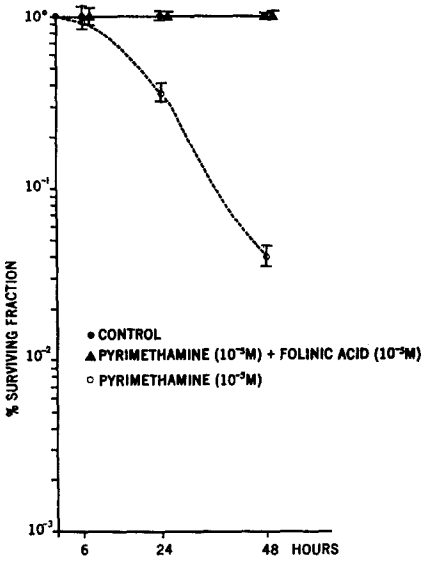


Fig. 2(a). Effect of 6-, 24- and 48-hr exposure to 10^{-5} molar PRM and 10^{-5} molar PRM+10^{-5} molar folinic acid on colony-forming ability of L5178Y MTX-sensitive cells. Percentage surviving fraction plotted against duration of drug exposure. Each point represents the mean of 5 determination.

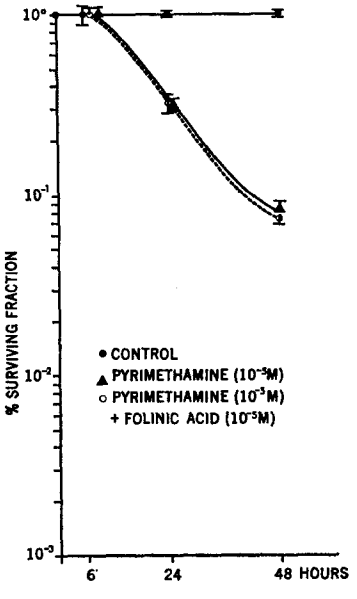


Fig. 2(b). As in 2(a) except L5178Y MTX-resistant cells employed.

Table 1. The effect of folinic acid on the steady-state level of intracellular antifolates in L5178Y lymphoblasts†*

Cell line	Concentration of folinic acid (M)	Extent of uptake of methotrexate (%)‡	Extent of uptake of pyrimethamine (%)‡
Methotrexate-sensitive	5×10^{-6}	98	56
	10^{-5}	96	27
	10^{-4}	72	—
Methotrexate-resistant	10^{-5}	96	98
	2×10^{-5}	82	88
	10^{-4}	65	76

*Folinic acid (5-formyltetrahydrofolate) was obtained from Lederle Products, Cyanamid of Canada, Montreal, P.Q. as the calcium salt.

†Uptake of ^{3}H-methotrexate and ^{14}C-pyrimethamine were measured following a 30 min exposure of cells in culture (2×10^{6} cells/ml) to concentrations of 10^{-5} M methotrexate and 10^{-5} M pyrimethamine by the method described above.

‡Extent of uptake in the absence of added folinic acid = 100%.

sensitive cells, but had no effect on PRM uptake into the resistant cells. The results also show that in both cell lines folinic acid is without effect on the steady-state level of MTX after a 30 min incubation.

Figure 3 shows the effect of folinic acid on the extent and rate of uptake of PRM into the sensitive and resistant cells. No effect is seen in the resistant cells, but both are reduced in the sensitive cells.

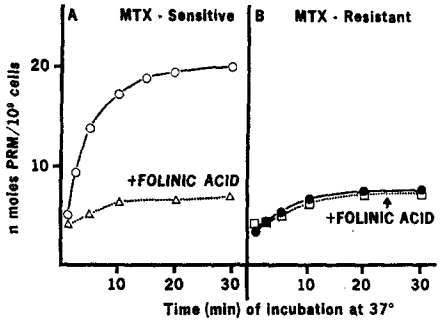


Fig. 3. Effect of simultaneous addition of folinic acid (10^{-5}M) on rate and extent of influx of ^{14}C-PRM into MTX-sensitive and MTX-resistant cells expressed in nmoles of drug per 10^{9} cells as a function of time of incubation at 37°C. PRM = 10^{-5}M. Solid line = PRM alone. Dotted line = PRM + folinic acid.

Table 2 shows the efflux of MTX from the sensitive cells which had been preloaded with either MTX alone or a combination of MTX + folinic acid. The addition of folinic acid did not significantly alter the efflux of MTX from the sensitive cells. Virtually identical results were seen with the L5178Y MTX-resistant cells. Attention is drawn to the fact that here, for comparative purposes, the extent of uptake of MTX is expressed as 100% in both cell lines, but there was in fact a two fold difference in uptake in the sensitive vs resistant cells (S = 0·91 nmoles/10^{9} cells; R = 0·48 nmoles/10^{9} cells), a finding which confirms previous observations [5, 8].

DISCUSSION

The data presented above indicate that there are significant differences in sensitivity to folinic acid protection in two sublines of L5178Y lymphoblasts. The viability curves indicate that the lethal effect of PRM may be completely reversed, and that of MTX partially reversed, by simultaneous addition of equimolar folinic acid to the MTX-sensitive L5178Y cells (Fig. 1(a), 2(a)). Figures 1 (b), 2(b) show the inability of equimolar folinic acid to protect the MTX-resistant cell line exposed to cytotoxic concentration of either MTX or PRM.

Several mechanisms have been implicated in the folinic acid reversal of MTX toxicity [9]. These include: (a) competitive inhibition of MTX uptake by folinic acid, (b) enhanced rate of efflux of MTX from the cells, and (c) repletion of intracellular folate pools. In attempting to explain the observed phenomenon of differential sensitivity to folinic acid protection in these two cell lines, we have examined the effect of folinic acid on MTX and PRM uptake. The uptake of both MTX [10] and PRM [7] has been shown to be due to an active transport process. From Table 1 and Fig. 3, it appears that folinic acid competes effectively for PRM uptake into the sensitive cells, but competes poorly for PRM uptake into the resistant cells. The inability of folinic acid to inhibit PRM influx into the resistant cells might account for the failure of folinic acid

Table 2. Efflux of MTX from "preloaded" MTX-sensitive L5178Y cells (%)†*

Cell line	Drugs used to "preload"‡	Duration of re-incubation at 37°/(min)				
		1	2·5	5	10	25
MTX-sensitive	MTX	81	56	39	31	28
	MTX + folinic acid	75	51	34	29	26
MTX-resistant	MTX	80	67	60	56	50
	MTX + folinic acid	75	66	58	51	47

*Extent of uptake of MTX under preloading conditions ≡ 100%.

†Cells (2×10^6/ml) were preincubated with drugs for 30 min at 37° in Hank's balanced salt solution pH 7·4 to achieve a steady-state intracellular concentration, prior to centrifugation and resuspension in fresh medium without drugs. Aliquots were removed at the times indicated.

‡Concentrations of MTX and folinic acid were equimolar ≡ 10^{-5} molar.

to prevent toxicity in this cell line. Another possible interpretation of these data is that folinic acid displaces PRM from loose intracellular binding sites in MTX-sensitive cells, but has no comparable effects on MTX-resistant cells.

Table 1 shows that, in contrast to the situation seen with PRM, folinic acid is a poor competitor of MTX uptake in both lines of L5178Y cells. This observation is in contrast to the findings of numerous workers [9, 11] and our own unpublished data, which indicates appreciable competition between folinic acid and MTX for uptake in the L1210 leukemia. Therefore, in the L5178Y system, the observed failure of folinic acid to prevent MTX toxicity in resistant cells is unlikely to be due to an inhibition of MTX uptake.

The results in Table 2 indicate that folinic acid was without effect in enhancing efflux of MTX in both lines of L5178Y cells. This suggests that the basis for the lack of response to folinic acid protection by the L5178Y-MTX resistant cells is not related to impaired MTX efflux in this cell line.

Our results show that in addition to impaired MTX transport, the MTX-resistant cells have a reduced sensitivity to the protective effects of folinic acid against anti-folate induced toxicity. In addition, folinic acid does not prevent the uptake of PRM into resistant cells, although it does so in sensitive cells. This may be the basis for impaired folinic acid protection from PRM toxicity in this cell line, though alternative explanations are possible. Our results do not explain the failure of folinic acid to protect against MTX toxicity in the resistant cells.

One possibility, not investigated in this present study, is that there is also impaired folinic acid transport in MTX-resistant cells. Reduced affinity for folinic acid by the transport system in the resistant cells could account for the observed results with both PRM and MTX, by virtue of producing an intracellular folate level insufficient to overcome the effect of the drug. The level of intracellular folate may be of crucial importance in determining whether cell killing takes place, particularly if there is competition between drug and reduced folate for certain loci of drug action as has been postulated by Borsa and Whitmore [12].

CONCLUSION

This study indicates that at least one line of MTX-resistant cells has impaired responsiveness to folinic acid protection. If this property is exhibited by any human tumour cells, then a possible area of clinical application presents itself. Such neoplasms could be treated with concurrent administration of folinic acid and a folic acid antagonist, with possible preferential rescue of normal cell renewal systems, such as gut and bone marrow, thus increasing the therapeutic index of the drug therapy. This "simultaneous rescue" or "protection" strategy might permit the maintenance of a longer duration of drug level than is possible using the method of "delayed rescue" with folinic acid [13–15]. We have undertaken clinical trials using this approach with the folic acid antagonist methodichlorophen with preliminary results indicating the feasibility of the technique [16].

REFERENCES

1. A. Goldin, J. M. Venditti, I. Kline and N. Mantel, Eradication of leukaemic cells (L1210) by methotrexate and methotrexate plus citrovorum factor. *Nature* (Lond.) **212,** 1548 (1966).
2. J. Borsa and G. F. Whitmore, Studies relating to the mode of action of methotrexate. II. Studies on sites of action in L cells *in vitro*. *Molec. Pharmacol.* **5,** 303 (1969).
3. A. Goldin, J. M. Venditti, S. R. Humphreys, D. Dennis and N. Mantel, Studies on the management of mouse leukaemia (L1210) with antagonists of folic acid. *Cancer Res.* **15,** 742 (1955).
4. J. H. Goldie, M. E. Furness and L. A. Price, Comparison of the effects of methotrexate and pyrimethamine on L5178Y lymphoblasts in cell culture. *Europ. J. Cancer* **9,** 709 (1973).
5. K. R. Harrap, B. T. Hill, M. E. Furness and L. I. Hart, Sites of action of amethopterin: Intrinsic and acquired drug resistance. *Ann. N.Y. Acad. Sci.* **186,** 312 (1971).
6. M. Chu and G. A. Fischer, The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukemic cells (L5178Y). *Biochem. Pharmacol.* **17,** 753 (1968).
7. B. T. Hill, L. A. Price, S. I. Harrison and J. H. Goldie, Studies on the transport and distribution of diaminopyrimidines in L5178Y lymphoblasts in cell culture. *Biochem. Pharmacol.* **24,** 535 (1975).
8. G. A. Fischer, Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolite in L5178Y leukemic cells. *Biochem. Pharmacol.* **11,** 1233 (1962).
9. I. D. Goldman, The characteristics of the membrane transport of Amethopterin and the naturally-occurring folates. *Ann. N.Y. Acad. Sci.* **186,** 400 (1971).
10. D. Kessel and T. C. Hall, Amethopterin transport in Ehrlich ascites carcinoma and L1210 cells. *Cancer Res.* **27,** 1539 (1963).
11. A. Nahas, P. F. Nixon and J. R. Bertino, Uptake of 5-formyltetrahydrofolate by L1210 mouse leukemia cells and its effect on uptake of methotrexate. *Proc. Amer. Ass. Cancer Res.* **10,** 64 (1969).
12. J. Borsa and G. F. Whitmore, Studies relating to the mode of action of methotrexate. III. Inhibition of thymidylate synthetase in tissue culture cells and in cell-free systems. *Molec. Pharmacol.* **5,** 318 (1969).
13. M. Levitt, M. B. Mosher, R. C. DeConti, L. R. Farber, R. T. Skeel, J. C. Marsh, M. S. Mitchell, R. J. Papac, E. D. Thomas and J. R. Bertino, Improved therapeutic index of methotrexate with "Leucovorin Rescue". *Cancer Res.* **33,** 1729 (1973).
14. C. B. Pratt, DeW. Roberts, E. C. Shanks and E. L. Warmath, Clinical trials and pharmacokinetics of intermittent high dose methotrexate—"Leucovorin Rescue" for children with malignant tumours. *Cancer Res.* **34,** 3326 (1974).
15. J. H. Goldie, L. A. Price and K. R. Harrap, Methotrexate toxicity: correlation with duration of administration, plasma levels, dose and excretion pattern. *Europ. J. Cancer* **8,** 409 (1972).
16. L. A. Price, B. T. Hill and J. H. Goldie, Evaluation of Methodichlorophen as an anti-cancer agent: a preliminary report. *Brit. Med. J.* In press (1975).

Europ. J. Cancer Vol. 11, pp. 633–637. Pergamon Press 1975. Printed in Great Britain

Immunosurveillance of Spontaneous Lung Adenomagenesis in Mice*

MARIA I. COLNAGHI

Division of Experimental Oncology A, Istituto Nazionale per lo Studio e la Cura dei Tumori
20133 *Milano, Italy.*

Abstract—*BALB/c mice repeatedly injected with syngeneic or allogeneic lymphoma cells blocked by mitomycin-C had a statistically significant decrease of lymphoma and increase of lung adenoma spontaneous incidence in comparison to untreated controls. When tested for the number of plaque-forming spleen cells, animals inoculated with mitomycin-C-treated cells revealed a severe immunodepression suggesting a role of the immunosurveillance in adenomagenesis. Also, one group of mice injected with untreated allogeneic normal immunocompetent cells revealed an increased lung adenoma incidence most likely due to a graft versus host phenomenon; this result seems to exclude the fact that a direct carcinogenic effect of mitomycin-C on lung tissue could be responsible for the increased adenomagenesis.*

INTRODUCTION

A NUMBER of experiments are presently on record on the role of immune surveillance in lung adenomagenesis in mice. Thymectomy or antilymphocytic serum have been shown to facilitate the spontaneous or chemically-induced onset of lung tumors [1–5], and it seems that lung adenoma development and impairment of the cellular immune response are closely related [6]. However, no antigenicity of lung adenomas was detected by *in vivo* tests [7, 8] whereas *in vitro* studies of cell-mediated response produced evidence that lung adenomas possess tumor-associated antigens [9] whose strength was dependent on the immune status of the host in which the tumor arose [10].

In the course of a series of experiments on antigenicity of murine lymphomas, BALB/c mice were immunized, to obtain anti-lymphoma antisera, with untreated allogeneic normal lymphoid or lymphoma cells, or with syngeneic or allogeneic lymphoma cells blocked by mitomycin-C or by X-rays. Mitomycin-C-treated cells were injected either after a single centrifugation, for gross removal of the chemical, or after repeated washings. We found that mice inoculated with unwashed mitomycin-C-treated cells or with untreated lymphoid cells of one of the allogeneic strains used, had a significantly higher lung adenoma incidence than untreated mice. It was subsequently found that, when injected without repeated washing, mitomycin-C caused a severe immunodepression.

Accepted 30 May 1975.

*This work was supported by a grant from Consiglio Nazionale delle Ricerche, Rome.

MATERIAL AND METHODS

Mice

BALB/c, C57BL/He and C3Hf female inbred mice, maintained in our laboratory by brother × sister mating, were used.

Tumors

The thymic lymphosarcomas utilized for immunizations were induced by urethan in mice of the three strains and maintained by s.c. transplant in preirradiated syngeneic mice. Only tumors that arose in female mice were used.

Inoculation with lymphoid cells

Cell suspensions from pooled C3Hf, C57BL or BALB/c lymphoma or normal thymus and spleen cells were injected repeatedly in young

adult female BALB/c mice at 1- to 3-week intervals, each injection being done half s.c. and half i.p. The first inoculum was about 2×10^6 cells and the following doses increased twofold. Syngeneic and, in some experimental groups, allogeneic lymphoma cells were blocked before inoculum by X-rays (10,000 R) or by mitomycin-C (Kyowa Hakko Kogyo Co., Ltd., Tokyo). In the latter case, 2×10^7 tumor cells suspended in 2 ml of Hank's balanced salt solution (HBSS) were added with 150 μg mitomycin-C, incubated for 2 hr, centrifuged and resuspended in HBSS, without any additional washing, before being injected in the appropriate dose.

Plaque-forming cells (PFC) determination

Groups of 3 BALB/c mice each were repeatedly inoculated with BALB/c or C3Hf mitomycin-C treated lymphoma cells or with C3Hf untreated lymphoid cells, and were injected with SRBC 7 days after the 1st, 3rd, 5th, and 9th immunizing inoculum or 40 days after the 9th inoculum, to test for the PFC capacity of their spleens. The number of PFC was determined following the technique of Jerne *et al.* [11]. Four days after immunization with SRBC, the spleen was removed and the nucleated cells of each spleen were counted and then processed in agar plates. Three plates were prepared from each spleen and the number of PFC was calculated as a mean of the 3 plates.

Recording of tumor incidence

Mice were controlled weekly, killed when in bad condition and autopsied. Lymphomas and lung adenomas were recorded and histology was made when necessary to confirm the gross diagnosis.

RESULTS

Influence of hyperimmunization with untreated or blocked cells on lymphoma and lung adenoma onset

In Table 1 the incidence of lymphomas and lung adenomas in the various groups of mice is reported. Most of the lymphomas were generalized lymphosarcomas of non-thymic origin. In comparison to untreated control mice, the lymphoma incidence was decreased in all 3 groups injected with mitomycin-C-treated cells, the difference reaching a statistically significant level in 2 groups. It should be noted that the average age at death of the untreated controls was 107 weeks, whereas in none of the mitomycin-C treated groups was it over 90. Therefore, the observed decreased incidence of lymphoma could have been due to the shorter survival time since the average age at death for lymphoma was 109 weeks in the control group and varied from 72 to 86 weeks in the 3 mitomycin-C-treated groups.

The lung adenoma incidence was much higher than in the controls in all the 3 groups injected with mitomycin-C-treated cells ($P < 0{\cdot}001$) and in the group injected with untreated C3Hf thymus and spleen cells ($P < 0{\cdot}01$). The group injected with C57BL untreated lymphoid cells also showed an increased (though not significant) incidence of lung adenomas. The 3 groups injected with X-ray-treated syngeneic or untreated allogeneic lymphoma cells were only slightly different

Table 1. *Effect of hyperimmunization of BALB/c female mice with normal or neoplastic cells of thymic origin on the spontaneous incidence of lymphoma and lung adenoma*

Injected cells	Cells treated with	No. of mice	Animals with: Lymphoma No.	%	Mean age*	Lung adenoma No.	%	Mean age*	Both tumors No.	%	Mean age*
—	—	62	27	43	109 ± 29	20	32	117 ± 22	7	11	116 ± 16
BALB/c lymphoma	X-rays	32	10	31	96 ± 16	12	37	99 ± 12	2	6	112, 120
C3Hf lymphoma	—	61	30	49	100 ± 12	26	42	101 ± 9	8	13	103 ± 12
C3Hf thymus + spleen	—	28	13	46	114 ± 13	18	64‡	112 ± 9	7	25	107 ± 4
C57BL lymphoma	—	71	36	50	99 ± 12	31	43	99 ± 7	8	11	97 ± 3
C57BL thymus + spleen	—	26	8	30	94 ± 12	13	50	96 ± 9	2	8	93, 95
BALB/c lymphoma	Mitomycin-C	48	2	4§	78 ± 8	34	70§	79 ± 5	2	4	84, 85
C3Hf lymphoma	Mitomycin-C	29	4	14†	86 ± 2	26	89§	84 ± 5	3	10	86 ± 3
C57BL lymphoma	Mitomycin-C	23	6	26	72 ± 36	18	78§	85 ± 8	3	13	86 ± 11

*Mean age at death ± S.D.
†$P < 0{\cdot}05$ (probability of statistical difference from the control).
‡$P < 0{\cdot}01$.
§$P < 0{\cdot}001$.

from the controls. The increased lung adenoma incidence observed in the mitomycin-C-treated groups was strengthened by the shorter survival time.

In all groups, some animals developed both lymphoma and lung adenoma as reported in Table 1, with no meaningful differences among the groups.

Effect of immunizations on plaque-forming spleen cells

The number of PFC in the spleen of control or experimental mice is reported in Table 2. After the 1st and the 3rd inoculum no depression was seen and an even higher immune response to SRBC occurred in all treated groups than in the untreated controls. From the 5th to the 9th inoculum the 2 groups injected with mitomycin-C treated cells showed a striking decrease of the PFC number, whereas the group injected with untreated lymphoid cells revealed only a slight, not statistically significant reduction. Forty days after the last inoculum, the mice injected with syngeneic lymphoma cells appeared to be completely recovered, whereas the allogeneic lymphoma-injected mice still showed a significantly reduced number of PFC.

DISCUSSION

Our results demonstrate that some manipulation of the immune apparatus resulting in inadequate immunocompetence may interfere with the process of spontaneous tumorigenesis. Mitomycin-C, one of the antibiotics discovered among the fermentation products of one species of *Streptomyces* [12], has been demonstrated to have chemotherapeutic effectiveness against experimental as well as human tumors and to cause in different mammalian species analogous pathologic lesions in hematopoietic tissue [13]. These effects are probably mediated by the ability of the chemical to inhibit DNA synthesis and cell division and for this property it is commonly used to block neoplastic cells for preventing their *in vivo* growth in immunizing procedures. The reduced lymphoma incidence we have observed, if not due to shorter survival, might have been caused by the mitomycin-C acting as a prophylactic chemotherapeutic agent by killing cells involved in the spontaneous neoplastic transformation.

The reduction of the splenic PFC after repeated immunizing inoculum confirmed that even the low quantity of mitomycin-C remaining in the injected cell suspensions could exert damage on the cells of the immunocompetent compartment. The consequent severe immunodepression could be responsible for the increased lung adenoma incidence, indicating that immunological surveillance operates during the spontaneous onset of this type of tumor. Cell-mediated, but not humoral, response has been found to control chemical induction of lung adenomas [3, 6]. We are unable to say what type of immune response controlled the spontaneous adenomagenesis. The direct correlation between the reduction of PFC, that is of antibody producing cells, and the increase of adenoma incidence, does not necessarily indicate that adenomagenesis is under humoral control since cells directly involved in antibody production (B cells) as cells with helper activity (T cells), which are responsible also for the cell-mediated response, both are essential for anti-SRBC response.

Another explanation for the increased number of lung adenomas, could be a specific carcinogenic activity of the chemical for lung tissue, although only induction of sarcomas has

Table 2. Plaque forming cells in spleen of BALB/c mice immunized against mitomycin-C-treated lymphoma cells or untreated thymus cells

Injected cells	No. of PFC ± SE × 1 × 10^6 nucleated spleen cells after 1	3	5	7	9	9* inocula
—	1103 ± 290	1450 ± 257	1450 ± 246	1457 ± 201	1885 ± 185	1387 ± 145
BALB/c lymphoma, Mitomycin-C-treated	1750 ± 200	1604 ± 215	32 ± 2‡	190 ± 56‡	114 ± 83§	1226 ± 203
C3Hf lymphoma, Mitomycin-C-treated	2023 ± 490	1310 ± 224	370 ± 61‡	29 ± 8§	17 ± 5§	216 ± 92‡
C3Hf thymus and spleen untreated	2140 ± 190†	2355 ± 335	1011 ± 104	1059 ± 330	1448 ± 134	1362 ± 82

*40 days after the 9th inoculum.
†$P < 0.05$ (probability of statistical difference from the control).
‡$P < 0.01$.
§$P < 0.001$.

been reported until now [14]. This seems to be excluded by the fact that an increased adenomagenesis in comparison to controls was also found in groups injected with untreated normal allogeneic immunocompetent cells, although the difference reached significance level only in mice injected with C3Hf cells. It has already been reported [15] that outbred mice repeatedly injected with lymphoid cells from mice of the same colony had an increased urethan-induced lung adenoma incidence, attributed to a graft–host reaction. It seems from our experiment that when normal immunocompetent cells were injected, a graft–host phenomenon decreasing the ability of the immune apparatus to exert a control over neoplastic transformation, may also interfere with spontaneous adenomagenesis. It is true that mice injected with C3Hf lymphoid cells had a statistically non-significant decrease of PFC. However, the cell-mediated control of carcinogenesis might be such a delicate phenomenon that it can be affected even by a weak graft–host reaction, unable to interfere with antibody production. It is interesting to note that the lung adenoma incidence was higher in BALB/c mice injected with C3Hf lymphoid cells, which can recognize 9 alien determinants of the host H-2 system, than in mice injected with C57BL cells, recognizing 6 alien determinants only.

In conclusion, our results indicate that an immunosuppression caused by mitomycin-C-treated cells, or a prolonged disturbance of the immune apparatus exerted by untreated allogeneic immunocompetent cells markedly influence lung adenomagenesis.

REFERENCES

1. N. Trainin and M. Linker-Israeli, Increased incidence of spontaneous lung adenomas in mice following neonatal thymectomy. *Israel J. med. Sci.* **7,** 36 (1971).
2. R. Ribacchi and G. Giraldo, Sui fattori intrinseci della cancerogenesi polmonare del topo: effetti della timectomia neonatale. *Lav. Anat. pat. Perugia* **26,** 127 (1966).
3. N. Trainin, M. Linker-Israeli, M. Small and L. Boiato-Chen, Enhancement of lung adenoma formation by neonatal thymectomy in mice treated with 7,12-dimethylbenz(a)anthracene or urethan. *Int. J. Cancer* **2,** 326 (1967).
4. G. Della Porta, M. I. Colnaghi and L. Parmi, Influenza della timectomia, della splenectomia e del cortisone sulla cancerogenesi. *Tumori* **56,** 121 (1970).
5. N. Trainin and M. Linker-Israeli, Influence of immunosuppression and immunorestoration on the formation of urethan-induced lung adenomas. *J. nat. Cancer Inst.* **44,** 893 (1970).
6. M. A. Lappe and R. T. Prehn, The predictive value of skin allograft survival times during the development of urethan-induced lung adenomas in BALB/c mice. *Cancer Res.* **30,** 1357 (1970).
7. R. T. Prehn, Specific isoantigenicities among chemically induced tumors. *Ann. N.Y. Acad. Sci.* **101,** 107 (1962).
8. G. Pasternak, F. Hoffman and A. Graffi, Growth of diethylnitrosamine-induced lung tumours in syngeneic mice specifically pretreated with X-ray killed tumour tissue. *Folia Biol. (Praha)* **12,** 299 (1966).
9. M. I. Colnaghi, S. Menard and G. Della Porta, Demonstration of cellular immunity against urethan-induced lung adenomas of mice. *J. nat. Cancer Inst.* **47,** 1325 (1971).
10. S. Menard, M. I. Colnaghi and G. Cornalba, Immunogenicity and immunosensitivity of urethane-induced murine lung adenomata, in relation to the immunological impairment of the primary tumour host. *Brit. J. Cancer* **27,** 345 (1973).
11. N. K. Jerne, A. A. Nordin and C. Henry, The agar plaque technique for recognizing antibody-producing cells. In *Cell Bound Antibodies.* Edited by B. Amos and H. Koprowski, p. 109. Wistar Institute Press, Philadelphia (1963).
12. S. Wakaki, H. Marumo, K. Tomioka, G. Shimizu, E. Kato, H. Kamada, S. Kudo and Y. Fujimoto, Isolation of new fractions of antitumor mitomycins. *Antib. Chemoth.* **8,** 228 (1958).
13. F. S. Philips, H. S. Schwartz and S. S. Sternberg, Pharmacology of mitomycin-C. I. Toxicity and pathologic effects. *Cancer Res.* **20,** 1354 (1960).

14. R. IKEGAMI, Y. AKAMATSU and M. HARUTA, Subcutaneous sarcomas induced by mitomycin C in mice: comparisons of occurrence, transplantability and histology, between sarcomas induced by actinomycin S and 3-methylcholanthrene. *Acta path. jap.* **17,** 495 (1967).
15. N. TRAININ and M. LINKER-ISRAELI, Increased incidence of urethan-induced lung adenomas in neonatally thymectomized mice challenged with lymphoid cells. *Cancer Res.* **29,** 1840 (1969).

Europ. J. Cancer Vol. 11, pp. 639–647. Pergamon Press 1975. Printed in Great Britain

Response of L1210 to Combinations of Cytosine Arabinoside and VM-26 or VP16-213*

GASTON RIVERA, THOMAS AVERY and DEWAYNE ROBERTS
Laboratories of Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38101, *U.S.A.*

Abstract—*Each of two promising new oncolytic drugs, 4'demethylepipodophyllotoxin 9-(4,6-O-2-thenylidene-β-D-glucopyranoside) (VM-26) and 4'-demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside) (VP 16–213), was administered simultaneously with cytosine arabinoside (Ara-C) to BDF_1 mice inoculated i.p. with L1210 ascites tumor cells. The therapeutic effectiveness of these drug combinations and the interdrug relationships contributing to the observed responses were evaluated. Sixteen combinations of VM-26 and Ara-C produced cures in eight treatment groups and, for mice that died with tumor, survival was extended 6·42 to 20·39 days (88–280%) over controls. Similarly, combinations of VP16–213 and Ara-C produced cures in 10 of 16 treatment groups and mice that died with tumor lived 7·12–21·72 days (98–298%) longer than controls. None of the drugs given singly on the same schedule cured mice or extended mean survival beyond controls by more than 9·62 days (132%). Responses obtained with combinations of VM-26 and Ara-C were qualitatively similar to those obtained with combinations of VP16–213 and Ara-C. Simultaneous administration of Ara-C, while extending survival, neither antagonized nor potentiated the response to VM-26 or VP16–213. Conversely, each podophyllum potentiated the response to Ara-C. Each 100-mg/kg increase in Ara-C extended survival by 0·6 days when the drug was administered alone, by 1·3 days when given with VM-26, and by 1·4 days when given with VP16–213. Dosage interactions between Ara-C and either podophyllum were not statistically significant. Thus, within the employed limits of dosage, small quantities of podophyllum were as effective as larger quantities in potentiating the response to Ara-C. When comparably effective levels of Ara-C and VM-26 or VP16–213 were combined, variation in Ara-C dosage accounted for approximately two-thirds of the treatment-related variation in survival. Inasmuch as survival increased as a function of increasing Ara-C dosage, this observation emphasizes the importance of high Ara-C dosage to the success of treatment with combinations of Ara-C and the epipodophyllotoxins, VM-26 and VP16–213.*

INTRODUCTION

THE epipodophyllotoxins, 4'-demethylepipodophyllotoxin 9-(4,6-O-2-thenylidene-β-D-glucopyranoside) (VM-26) and 4'-demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside) (VP16-213), are relatively new chemotherapeutic agents with antitumor properties in experimental animal systems and in man. Excellent oncolytic activity by these agents in the treatment of transplantable murine tumors has been reported [1–4], and promising results have been obtained in patients with advanced neoplasms, especially malignant lymphomas [5–13]. In an ongoing evaluation of the effectiveness and toxicity of VM-26 and VP16-213

*Accepted 15 May 1975.

This investigation was supported by Childhood Cancer Research Centre Grant CA08480 and Research Project Grant CA12732 from the National Cancer Institute, American Cancer Society Institutional Grant IN-99, and by ALSAC.

for treatment of childhood cancer, we have observed a reduction in tumor cell masses of up to 90% in 8 of 26 children with advanced leukemia refractory to conventional chemotherapy [14]. On the other hand, dosages of VM-26 lacking pronounced acute toxicity have produced delayed fatal toxicity in mice [4], and later work in our laboratory has indicated a delayed toxicity to VP16-213.

All reported studies of the antitumor activity of VM-26 or VP16-213 have dealt with the agents given singly. Because combination chemotherapy has proved highly successful against a variety of neoplasms [15–19], each of these epipodophyllotoxins was tested in combination with cytosine arabinoside (Ara-C). Ara-C, an S-phase-specific drug with demonstrable activity against L1210 leukemia, was chosen because its mechanism of action appears to differ from that of the epipodophyllotoxins. Early work showed that VM-26 inhibited tritiated thymidine uptake by P-815 cells [1]. Later studies have indicated that both VM-26 and VP16-213 not only interfere with metabolism in the late S phase but also inhibit cells in the G_2 phase of the cell cycle [20–22].

The objectives of the present studies were:

1. To determine and compare the effectiveness of combination chemotherapy with Ara-C plus either VM-26 or VP16-213.
2. To identify interrelationships between Ara-C and VM-26 or VP16-213 that would assist in explaining their enhanced or diminished effectiveness in combination.
3. To determine whether simultaneous administration of Ara-C would induce delayed toxic responses to dosages of VM-26 or VP16-213 that, when administered alone, do not produce the condition.

MATERIAL AND METHODS

BDF_1 female mice weighing from 18 to 20 g each were caged in groups of five and maintained under uniform conditions of temperature (75°F) and relative humidity (55%) with free access to food and water. All mice were inoculated intraperitoneally with 1 million L1210 ascites tumor cells. On days 1, 4 and 7 after tumor inoculation 480 mice received i.p. injections of drug. The epipodophyllotoxins were obtained from Sandoz Pharmaceuticals, Hanover, New Jersey, USA, and Ara-C from the Upjohn Co., Kalamazoo, Michigan, USA. Drugs were prepared immediately before injection and were administered individually, in rapid succession, as a volume of 0·01 ml/g of body weight. The dimethyl sulfoxide, Tween 80, saline vehicle for the epipodophyllotoxins (4) was also administered to 25 mice that served as controls. All three compounds were administered at four levels of dosage. Each decreasing dosage of Ara-C and VP16-213 was 60% of the preceding one, while reductions in VM-26 dosage each represented 70% of the previous level. The highest dosage of Ara-C (1200 mg/kg) produces maximum oncolytic response on the treatment schedule used in this study. The maximum dosages of VM-26 (4·8 mg/kg) and VP16-213 (18·0 mg/kg) are the highest that can be administered without causing death from delayed toxicity. The selected dosages of VM-26 and VP16-213 were comparable in oncolytic effectiveness to those selected for Ara-C.

Drug effectiveness was evaluated by comparing the postinoculation life span of treated mice with that of untreated controls. All mice dying during the observation period were subjected to postmortem examination. Deaths were attributed to tumor on the basis of specific autopsy findings, such as organomegaly, solid tumor masses, and peritoneal fluid with lymphoblasts. Death was attributed to acute toxicity when mice died before control mice. Surviving mice were observed 120 days for evidence of delayed toxicity.

Each two-drug experiment was designed as a 4×4 factorial and consisted of 16 treatment groups. Estimates of the mean $\pm$ one S.D. were calculated for each group [23]. Results were also partitioned into their variance components, and analyses of variance [23, 24] were performed to evaluate contributions by single drugs and by drug interaction to variation in survival time. Linear regression equations were calculated by simple or multiple sequential regression to aid presentation of data. Statistical inference was determined by the F-test, Chi-square and the *t*-test [23, 24].

RESULTS

The responses of 480 BDF_1 mice inoculated with L1210 ascites tumor cells and treated with VM-26, VP16-213 or Ara-C alone or with combinations of a podophyllum and Ara-C are presented in Table 1.

Response to individual drugs

Compared to the mean control value, 7·28 days, the life span of treated mice was extended 4·62–9·62 days (64–132%) by VM-26; 4·42–9·50 days (61–131%) by VP16-213; and 4·07–9·32 days (56–128%) by Ara-C. Each drug was increasingly effective with increasing dosage, but cures were not obtained by single-

Table 1. *Response of leukemic mice to VM-26 or VP16-213 administered individually or in combination with Ara-C*

Treatment (mg/kg)			No. of Mice	No. of Deaths Due to Tumor	Mean Survival ± 1 S.D. (Days After Inoculation)[a]	No. of Cures[b]
VM-26	VP16-213	Ara-C				
0	0	0	25	25	7.28 ± 1.67	0
1.7	0	0	10	10	11.90 ± 3.32	0
2.4	0	0	10	10	13.30 ± 1.49	0
3.4	0	0	10	10	14.20 ± 2.66	0
4.8	0	0	10	10	16.90 ± 8.25	0
0	3.9	0	10	10	11.70 ± 1.16	0
0	6.5	0	10	10	13.30 ± 1.42	0
0	10.8	0	10	10	15.00 ± 2.49	0
0	18.0	0	10	9	16.78 ± 3.23	0
0	0	259	20	20	11.35 ± 0.75	0
0	0	432	20	19	12.16 ± 1.71	0
0	0	720	20	20	15.60 ± 3.41	0
0	0	1200	20	20	16.60 ± 2.96	0
1.7	0	259	10	10	13.70 ± 3.30	0
1.7	0	432	10	10	14.80 ± 3.91	0
1.7	0	720	10	9	20.00 ± 3.74	1
1.7	0	1200	10	9	20.00 ± 4.50	1
2.4	0	259	10	10	15.50 ± 2.55	0
2.4	0	432	10	9	17.33 ± 2.29	0
2.4	0	720	10	10	18.40 ± 5.30	0
2.4	0	1200	10	6	21.33 ± 5.35	3
3.4	0	259	10	7	16.29 ± 3.86	0
3.4	0	432	10	9	16.75 ±3.81	1
3.4	0	720	10	6	18.67 ± 4.37	4
3.4	0	1200	10	6	27.67 ± 6.02	4
4.8	0	259	10	10	17.10 ± 3.35	0
4.8	0	432	10	8	17.75 ± 3.99	2
4.8	0	720	10	10	20.30 ± 9.03	0
4.8	0	1200	10	7	21.14 ± 6.74	3
0	3.9	259	10	10	14.40 ± 1.58	0
0	3.9	432	10	10	16.10 ± 2.18	0
0	3.9	720	10	8	20.25 ± 4.27	1
0	3.9	1200	10	9	20.33 ± 5.03	1
0	6.5	259	10	10	16.10 ± 2.03	0
0	6.5	432	10	10	18.50 ± 2.42	0
0	6.5	720	10	7	19.43 ± 4.86	3
0	6.5	1200	10	6	25.00 ± 6.87	4
0	10.8	259	10	10	16.80 ± 2.04	0
0	10.8	432	10	10	20.40 ± 3.72	0
0	10.8	720	10	9	24.89 ± 10.29	1
0	10.8	1200	10	3	29.00 ± 7.94	6
0	18.0	259	10	9	19.33 ± 4.03	1
0	18.0	432	10	7	17.86 ± 2.91	3
0	18.0	720	10	6	19.17 ± 4.12	3
0	18.0	1200	10	3	19.00 ± 7.94	7

Ten mice were lost during the course of these studies; 4 drug-treated mice died for unknown reasons prior to control mice and 6 because of accidental death due to handling.

[a]Mice were inoculated i.p. with 1 million L1210 ascites tumor cells 24 hr before treatment.

[b]Mice were considered cured if they had no evidence of disease on day 120 after inoculation.

drug treatment. The influence of VM-26 dosage on survival is represented by the regression equation $Y = 9{\cdot}30+1{\cdot}55X$, that of VP16-213 by the equation $Y = 10{\cdot}78+0{\cdot}35X$, and that of Ara-C by $Y = 10{\cdot}10+0{\cdot}006X$.

Response to combinations of VM-26 and Ara-C

Of 160 mice treated with VM-26 and Ara-C, 136 died with tumor, 19 were cured, and 5 died prior to controls or from accidents in handling (Table 1). After tumor inoculation, the mean life span for mice dying with tumor ranged from 13·7 to 27·7 days in 16 treatment groups indicating increases of 6·42 to 20·4 days (88–280%) over controls. Statistical analysis of variance (ANOVA) indicated that variation among treatment groups was significantly greater than within groups ($P < 0{\cdot}01$), and hence that treatment was responsible for a significant portion of the observed variation in life span. Changing Ara-C dosage was the only significant contributor ($P < 0{\cdot}01$) to treatment-induced variation in life span of mice dying with tumor (Table 2).

By multiple sequential analysis the treatment-induced variation in life-span of animals dying with tumor is expressed by the equation $Y = 10{\cdot}91+0{\cdot}89X+0{\cdot}0087X'-0{\cdot}004XX'$, in which Y represents life span in days. Dosages of VM-26 and Ara-C in mg/kg are represented respectively by X and X'.

In the preceding analysis, only data relating

Table 2. Analysis of variation in life span after treatment with Ara-C plus VM-26

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Critical value to be exceeded by "F" for significance at the indicated level of probability		
				"F"	$P<0.05$	$P<0.01$
With cured mice as 43-day survivors						
Among treatment	15	4941	329	4.93	1.75	2.19
VM-26	3	1064	355	5.30	2.68	3.95
Ara-C	3	3305	1102	16.47	2.68	3.95
VM-26 times Ara-C	9	572	64	0.95	1.96	2.56
Within treatment	139	9298	67	--	--	--
Total	154	14239	--	--	--	--
As indicated by the "F" test, response varied significantly among treatment groups, and both VM-26 and Ara-C made significant contribution to that variation.						
For mice dying with tumor						
Among treatment	15	1215	81	3.57	1.75	2.19
VM-26	3	128	43	1.88	2.68	3.95
Ara-C	3	845	282	12.42	2.68	3.95
VM-26 times Ara-C	9	242	27	1.19	1.96	2.56
Within treatment	120	2722	23	--	--	--
Total	135	3937	--	--	--	--
As indicated by the "F" test, survival varied significantly among treatment groups, and Ara-C made significant contribution to that variation.						

to mice dying with tumor were considered. However, conclusions based on such considerations may be misleading since tumor was eradicated in 19 mice. To reach more definitive conclusions on the effectiveness of treatment, cured mice were included in the following computations as 43-day survivors, the last day on which a mouse died with tumor. Mice were cured at each level of VM-26, at the three higher levels of Ara-C and in 8 of 16 treatment groups. The incidence of cures in these groups ranged from 1 of 10 to 4 of 10 mice. With these cured mice included in Table 2, ANOVA again indicated that treatment contributed significantly to variation in life span ($P < 0{\cdot}001$). Significant contributions to treatment-induced variation in life span were assigned to changing dosages of VM-26 and Ara-C ($P < 0{\cdot}01$). Contributions from dosage interactions between Ara-C and VM-26 were not statistically significant.

The influence of VM-26 and Ara-C on survival is expressed by the equation $Y = 8{\cdot}37 + 1{\cdot}42X + 0{\cdot}013X' + 0{\cdot}0001XX'$, which was obtained by multiple sequential regression of data for the two-drug combinations. Dosages of VM-26 and Ara-C in mg/kg are represented respectively by X and X', while Y represents survival in days. As expressed by this equation, each mg/kg increase in VM-26 extended survival by 1·42 days, not significantly less than the 1·55 days increase observed with VM-26 alone. In combination with VM-26, each 100 mg/kg increase in Ara-C extended life span by an additional 1·3 days, approximately twice the increase observed for Ara-C alone. According to this equation expected life span would also increase as a function of the product for Ara-C and VM-26 dosage. However, ANOVA indicated that this contribution was not statistically significant.

Response to combinations of VP16-213 and Ara-C

Of 160 mice treated with VP16-213 and Ara-C, 127 died with tumor, 30 were cured, and 3 died prior to controls or were lost from accidents in handling (Table 1). After tumor inoculation the mean life span for treated mice dying with tumor ranged from 14·4 to 29·0 days among the 16 treatment groups indicating increases of 7·1 to 21·7 days (98–298%) over controls. In Table 3 the variation among treatment groups is shown to be significantly greater than within-group variation ($P < 0{\cdot}01$). Variation in Ara-C dosage and in VP16-213 dosage each contributed to treatment-induced variation in life span ($P < 0{\cdot}01$). Contribution from dosage interaction was not significant.

By multiple sequential analysis the equation $Y = 11{\cdot}58 + 0{\cdot}37X + 0{\cdot}012X' - 0{\cdot}004XX'$ expresses response to each of the drugs and to the dosage interaction between them.

Table 3. Analysis of variation in life span after treatment with Ara-C plus VP16-213

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Critical value to be exceeded by "F" for significance at the indicated level of probability		
				"F"	$P<0.05$	$P<0.01$
With cured mice as 43-day survivors						
Among treatment	15	7373	492	6.79	1.75	2.19
VP16-213	3	1572	524	7.23	2.68	3.95
Ara-C	3	5097	1699	23.45	2.68	3.95
VP16-213 times Ara-C	9	704	117	1.62	1.96	2.56
Within treatment	141	10214	72	–	–	–
Total	156	17578	–	–	–	–
As indicated by the "F" test, response varied significantly among treatment groups, and both VP16-213 and Ara-C made significant contribution to that variation.						
For mice dying with tumor						
Among treatment	15	1322	88	4.08	1.77	2.21
VP16-213	3	249	83	3.84	2.69	3.99
Ara-C	3	691	230	10.65	2.69	3.99
VP16-213 times Ara-C	9	382	42	1.96	1.97	2.58
Within treatment	111	2400	22	–	–	–
Total	126	3722	–	–	–	–
As indicated by the "F" test, response varied significantly among treatment groups, and both VP16-213 and Ara-C made significant contribution to that variation.						

Again, Y indicates life span after tumor inoculation. The dosages of VP16-213 and Ara-C in mg/kg are indicated respectively by X and X′. In this analysis, only data relating to mice dying with tumor were considered.

Tumor cells were eradicated in 30 mice. Cured mice were observed at each dosage of VP16-213 and of Ara-C, as well as in 10 of the 16 treatment groups. The within-group incidence of cures ranged from 1 of 10 to 7 of 10 mice. With these cured mice included as 43-day survivors, ANOVA in Table 3 indicated that treatment contributed significantly to variation ($P < 0.01$). Significant contribution to treatment-induced variation in life span was assigned to variation in dosages of VM-26 and Ara-C ($P < 0.01$). Contributions from dosage interactions between Ara-C and VM-26 were not statistically significant.

The influence of combinations of VP16-213 and Ara-C on survival with cured mice as 43-day survivors is represented by the equation $Y = 9.62 + 0.38X + 0.014X' + 0.0002XX'$, which was obtained by multiple sequential regression of the data. Y is life span in days, while X and X′ respectively represent VP16-213 and Ara-C dosages in mg/kg. As expressed by this equation, each mg/kg increase in VP16-213 extended life span by 0·38 days, virtually identical to response from VP16-213 alone. As with VM-26, Ara-C was twice as effective with VP16-213 as alone. In combination with VP16-213 each 100 mg/kg increase in the dosage of Ara-C increased life span by 1·40 days. From the ANOVA in Table 3, the contribution from dosage interaction, $+0.0002XX'$, in the regression equation was not significant.

Comparison of response to Ara-C in combination with VM-26 or VP16-213

In evaluating the relative merits of combining Ara-C with VM-26 or VP16-213, we considered the incidence of cures, incidence of tumor deaths, and mean life span of mice dying with tumors. Neither the incidence of tumor eradication nor the incidence of tumor deaths was statistically significant by Chi-square analysis. For mice dying with tumor the mean life span after treatment with VM-26 and Ara-C was 18.21 ± 5.40 days while mice treated with VP16-213 and Ara-C had a mean life span of 19.18 ± 5.42 days. This variation in life span was not significant by *t*-test analysis.

DISCUSSION

In these studies, combinations of Ara-C and either VM-26 or VP16-213 were more effective than Ara-C or either epipodophyllotoxin administered individually on the same schedule. Responses to combinations of Ara-C and VM-26 were qualitatively similar to those obtained with combinations of Ara-C and VP16-213. Combining Ara-C with VM-26 or VP16-213 did not significantly change the slope constant established with the individual epipodophyllotoxin (Fig. 1). It thus appears that concomitant administration of Ara-C, while extending survival, neither potentiated nor antagonized response to increasing dosages of VM6 or VP16-213. Conversely, concomitant administration of either VM-26 or VP16-213 potentiated response to increasing dosages of Ara-C. This relationship is illustrated by the significant increase in slope constants for Ara-C (Fig. 2). Each 100 mg/kg increase in the dosage of Ara-C administered alone extended survival by 0·6 days and by either 1·3 days when administered with VM-26 or 1·4 days when given with VP16-213, if cured mice are included as 43-day survivors. Dosage interactions between Ara-C and either epipodophyllotoxin were not statistically significant (Tables 2 and 3) and, consequently, potentiation of Ara-C did not change with variation in dosage of VM-26 or of VP16-213.

Ara-C alone produced a range of oncolytic responses comparable to that obtained with VM-26 or VP16-213. When Ara-C was combined with comparably effective levels of VM-26 or VP16-213, variation in Ara-C dosage accounted for approximately two-thirds of the treatment-related variation in survival (Tables 2 and 3). Since survival increased as a function of increasing Ara-C dosage, this observation emphasizes that maintaining high dosages of Ara-C was important to the success of treatment with combinations of Ara-C and VM-26 or VP16-213. Failure of delayed toxicity to develop in these combination drug

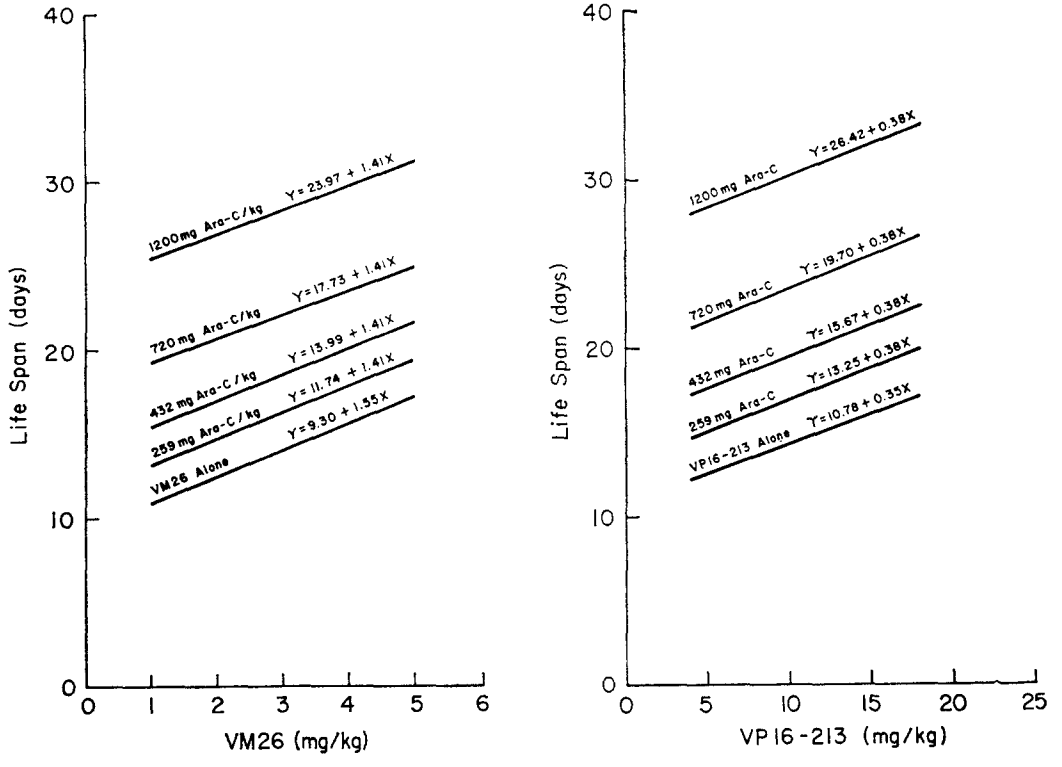


Fig. 1. Response of leukemic mice to VM-26 or VP16-213 when administered alone and with various dosages of Ara-C. Cured mice are included as 43-day survivors in the computation of mean life span. Regression equations are based on the model $y = a + bx$ in which a is the y intercept, x is podophyllum dosage (mg/kg), y is survival (days), and b is a slope constant that indicates the change in survival per mg/kg change in podophyllum dosage.

These series of lines were calculated from the general equations, $Y = 8{\cdot}37 + 1{\cdot}42X + 0{\cdot}013X' + 0{\cdot}0001XX'$ for VM-26 or $Y = 9{\cdot}62 + 0{\cdot}38X + 0{\cdot}014X' + 0{\cdot}0002XX'$ for VP16-213 by dropping the non-significant dosage interactions from the equations. In addition response to the constant dosage of Ara-C for each line was incorporated into the intercept for that line. Ara-C produced dosage-related increases in the y-intercept, but failed to produce statistically significant changes in slope constant. Increased y-intercepts indicate that progressive enhancement of survival accompanied increasing Ara-C dosages. Failure of Ara-C to produce significant changes in the slope constant indicates that response to changing podophyllum dosage is independent of Ara-C.

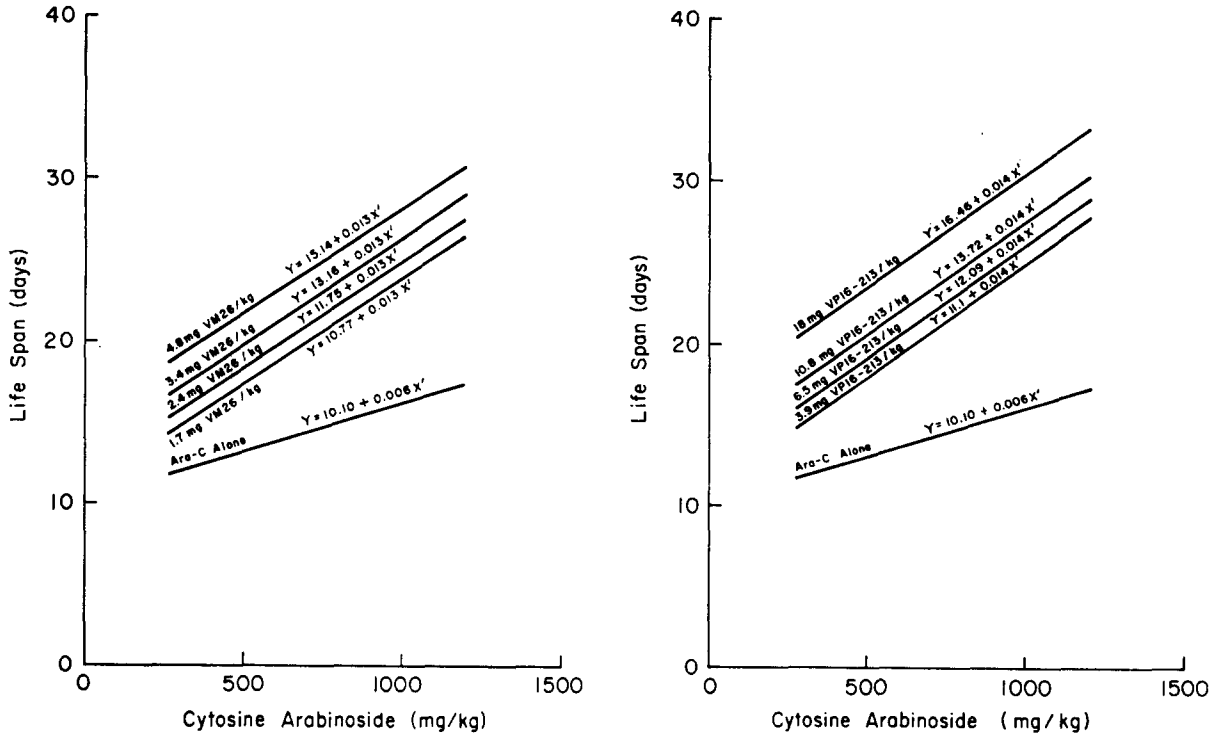


Fig. 2. Response of leukemic mice to Ara-C when administered alone and with various dosages of VM–26 or VP16–213. Cured mice were included as 43-day survivors in the computation of mean survival. Regression equations are based on the model $y = a + bx'$ *in which a is the y-intercept, x' is Ara-C dosage (mg/kg), y is survival in days, and b is a slope constant that indicates the change in survival per mg/kg change in Ara-C dosage.*

These series of lines were calculated from the general equations $Y = 8{\cdot}37 + 1{\cdot}42X + 0{\cdot}013\,X' + 0{\cdot}0001\,XX'$ *for VM–26 or* $Y = 9{\cdot}62 + 0{\cdot}38x + 0{\cdot}014X' + 0{\cdot}0002XX'$ *for VP16–213 by dropping the non-significant dosage interactions from the equations. In addition, response to the constant dosage of VM–26 or VP16–213 for each line was incorporated into the intercept for that line. Each podophyllum produced dosage-related increases in y intercept. Statistically significant increases in slope constant were produced by VM–26* ($P < 0{\cdot}01$) *and by VP16–213* ($P < 0{\cdot}005$). *Increased y intercepts indicate progressive enhancement of survival to have accompanied increasing podophyllum dosage. The larger slope constants when Ara-C was administered with VM–26 or VP16–213 indicate that response to changing Ara-C dosage is not independent of VM–26 or VP16–213 but was increased in the presence of either podophyllum. Within the employed limits of dosage, minimum podophyllum dosages produced the increased response to Ara-C as readily as did larger dosages.*

studies supports the contention that Ara-C failed to potentiate response to the epipodophyllotoxins.

The data were analyzed by ANOVA and by multiple sequential analysis for mice dying with tumor and with and without inclusion of the cured mice as 43-day survivors. Cured mice were included as 43-day survivors because this was the last day on which a mouse died with tumor after treatment with VM-26 or VP16-213 plus Ara-C. The rationale for including cured mice in the analysis is based on a number of observations.

Skipper, Schabel and Wilcox, by using the uniform relationship between inoculum size and life span of the tumor-inoculated host, demonstrated that the cure of tumor-inoculated mice by drugs was directly dosage-related to the probability of killing all the tumor cells [25]. However, a growing body of evidence indicates that after some oncolytic-drug treatments, mice die with tumor at a much later time than would be projected from survival curves after inoculation of one tumor cell. The basis for this unprojected, drug-induced increase in life span presumably results from changes in the tumor–host relationship. Drug-induced changes in the tumor which are seen on subsequent passage to other mice have been associated with increased life span in comparison with the parental cell line [26, 27]. Immune response by the host to the parental cells also leads to prolongation of life span by delaying the accumulation of a lethal number of tumor cells [28, 29].

In addition, cell cycle kinetics suggest that cells must be in a drug-sensitive stage of the division cycle before some oncolytic drugs are cytocidal. The length of time that cells remain in a drug-insensitive stage before resuming cell division and the possible effect of oncolytic drugs in delaying progression in the division cycle are poorly defined.

In designing these studies the largest dosage

of each drug was considered optimal. Higher dosages of Ara-C can be administered on our treatment schedule. However, life span of the host was not significantly increased by these higher dosages, and fatal toxicity varied from study to study. The largest dosages of VM-26 and VP16-213 are 0·6 of the lowest dosages for which in other studies we observed delayed toxicity. An increase of drug dosages to the LD_{10} level would have required that toxicity data be included in the analysis of results or would have limited the analysis to selected data. Similarly, exclusion of cured mice from the computation of mean life span does not provide for full consideration of response to treatment. A majority of the mice died within 25 days after tumor inoculation, the projected average of 18 days required for a single cell to kill an untreated mouse [25] plus 7 days of treatment. However, to assign a 25-day life span to cured animals would also require a reduction of life span for 21 mice that died with tumor at 26 days or more after inoculation. Elimination of cured mice from analysis limits the evaluation of drug effectiveness to the less responsive animals. Elimination of the groups in which one or more cured mice are observed removes 18 of 32 treatment groups. Delay of treatment until the number of tumor cells is larger increases the complication of data analysis because of possible sequestration of tumor cells in drug-inaccessible loci.

Employment of ANOVA and multiple sequential regression permits evaluation of the contribution by each drug and by dosage interactions to response. Both types of analyses with inclusion of cured mice as 43-day survivors emphasize the contribution of Ara-C in combination with VM-26 or VP16-213 to response. Extrapolation of these results to clinical studies suggests that either VM-26 or VP16-213 would be equally effective in potentiating Ara-C.

REFERENCES

1. H. Stahelin, 4′-Demethylepipodophyllotoxin thenylidene glucoside (VM-26), a podophyllum compound with a new mechanism of action. *Europ. J. Cancer* **6,** 303 (1970).
2. J. Venditti, Treatment schedule dependency of experimentally active antileukemic (L1210) drugs. *Cancer Chemother. Rep.* **55,** 35 (1971).
3. H. Stahelin, Activity of a new glycosidic lignan derivative (VP16–213) related to podophyllotoxin in experimental tumors. *Europ. J. Cancer* **9,** 215 (1973).
4. T. Avery, D. Roberts and R. Price, Delayed toxicity of 4′-demethylepipodophyllotoxin-9-(4,6-O-2-thenylidene-β-D-glucopyranoside) (NSC-122819; VM-16) in mice. *Cancer Chemother. Rep.* **57,** 165 (1973).
5. G. Trempe, M. Sykes, C. Young and I. Krakoff, Phase I trial of the podophyllotoxin derivative VM-26. *Proc. Amer. Assoc. Cancer Res.* **11,** 79 (1970).
6. P. Dombernowsky, N. I. Nissen and V. Larsen, Clinical investigation of a new podophyllum derivative, epipodophyllotoxin, 4′-demethyl-9-(4,6-O-2-thenylidene-β-D-glucopyranoside) (NSC 122819) in patients with malignant lymphomas and solid tumors. *Cancer Chemother. Rep.* **56,** 71 (1972).
7. N. Nissen, V. Larsen, H. Pedersen and K. Thomsen, Phase I clinical trial of a new antitumor agent, 4-demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside) (NSC 141549; VP16–213). *Cancer Chemother. Rep.* **56,** 769 (1972).
8. European Organization for Research of the Treatment of Cancer, Clinical screening of epipodophyllotoxin VM-26 in malignant lymphomas and solid tumors. *Brit. med. J.* **2,** 744 (1972).
9. European Organization for Research of the Treatment of Cancer, Epipodophyllotoxin VP16–213 in treatment of acute leukemias, hematosarcomas, and solid tumors. *Brit. med. J.* **3,** 199 (1973).
10. M. Goldsmith and S. K. Carter, 4′-Demethylepipodophyllotoxin-β-D-thenylidene glucoside (VM-26). A brief review. *Europ. J. Cancer,* **9,** 477 (1973).
11. G. Mathe, L. Schwarzenberg, P. Pouillart, R. Weiner, R. Oldham, C. Jasmin et C. Rosenfeld, M. Hayat, M. Schneider, J. L. Amiel, B. Ceoara, M. Steresco-Nusset et F. de Vassal (Villejuif), Essai de traitment de divers hematosarcomas par le 4′-demethylepipodophyllotoxine-β-D-thenylidene glucoside (VM-26 or EPT). *Nouv. Presse Med.* **3,** 447 (1974).

12. R. W. Sonntag, H. J. Senn, G. Nagel, K. Giger and P. Alberto, Experience with 4′-demethylepipodophyllotoxin-9-(4,6,-O-2-thenylidene-β-D-glucopyranoside); VM-26 NSC-133819 in the treatment of malignant lymphosis. *Europ. J. Cancer* **10,** 93 (1974).
13. G. Mathe, L. Schwarzenberg, P. Pouillart, R. Weiner, R. Oldham, C. Jasmin et C. Rosenfeld, M. Hayat, M. Schneider, J. L. Amiel, B. Ceoara, M. Steresco-Musset et F. de Vassal (Villejiuf), Leucemies aigues et hematosarcomes divers. Essai de traitment par un second derive de la podophyllotoxine, VP16–213. *Nouv. Presse Med.* **3,** 521 (1974).
14. G. Rivera, T. Avery and C. Pratt, Epipodophyllotoxins in childhood cancer. *Proc. XI Intern. Cancer Congr.* **4,** 802 (1974).
15. L. W. Law, Effects of combination of antileukemic agents on ALL of mice. *Cancer Res.* **12,** 871 (1952).
16. E. H. Henderson and R. Samaha, Evidence that drugs in multiple combination have materially advanced the treatment of human malignancies. *Cancer Res.* **29,** 2272 (1969).
17. R. J. Aur, J. A. Simone, O. Hustu, T. Walters, L. Borella, C. Pratt and D. Pinkel, Central nervous system therapy and combination chemotherapy of childhood lymphocytic leukemia. *Blood* **37,** 272 (1971).
18. A. Goldin, J. Sandberg, E. Henderson, J. Newman, E. Frei and J. Holland, The chemotherapy of human and animal acute leukemia. *Cancer Chemother. Rep.* **55,** 309 (1971).
19. V. DeVita and P. S. Schein, Medical progress. The use of drugs in combination for the treatment of cancer. Rationale and results. *New Engl. J. Med.* **288,** 998, (1973).
20. C. C. Huang, Y. Hou and J. J. Wang, Effects of a new antitumor agent, epipodophyllotoxin, on growth and chromosomes in human hematopoietic cell lines. *Cancer Res.* **33,** 3123 (1973).
21. A. Grieder, R. Maurer and H. Stahelin, Effect of an epipodophyllotoxin derivative (VP16–213) on macromolecular synthesis and mitosis in mastocytoma cells *in vitro*. *Cancer Res.* **34,** 1788 (1974).
22. N. Misra and D. Roberts, Inhibition by 4′-demethylepipodophyllotoxin thenylidene glucoside (VM-26) of human lymphoblast cultures in G_2 phase of the cell cycle. *Cancer Res.* **35,** 99 (1975).
23. W. J. Dixon and F. J. Massey, Jr., *Introduction to Biostatistics*, 2nd Ed., McGraw-Hill, New York (1957).
24. A. L. Delaunois, *Biostatistics in Pharmacology*, Pergamon Press, Oxford (1973).
25. H. E. Skipper, F. M. Schabel and W. B. Wilcox, Experimental evaluation of potential anticancer agents. XIII. On the criteria and kinetics associated with "curability" of experimental leukemia. *Cancer Chemother. Rep.* **35,** 1 (1964).
26. E. Bonmassar, A. Bonmassar, S. Vadlamudi and A. Goldin, Antigenic changes of L1210 leukemia in mice treated with 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxyamide. *Cancer Res.* **32,** 1446 (1972).
27. F. A. Schmid and D. I. Hutchison, Decrease in oncogenic potential of L1210 leukemia by triazenes. *Cancer Res.* **33,** 2161 (1973).
28. E. Mihich, Modification of tumor regression by immunologic means. *Cancer Res.* **29,** 2345 (1969).
29. E. Mihich and M. Kitano, Differences in the immunogenicity of leukemia L1210 sublines in DBA/2 mice. *Cancer Res.* **31,** 1999 (1971).

Europ. J. Cancer Vol. 11, pp. 649–655. Pergamon Press 1975. Printed in Great Britain

The Effect of *Fv*-2 Gene on the Incorporation of Thymidine in the Mouse Spleen

SUSUMU MITANI* and KAZUHIKO WAKABAYASHI†

**Department of Surgery and †Department of Biochemistry, Faculty of Medicine, University of Tokyo, Hongo, Tokyo 113, Japan.*

Abstract—*The incorporation of thymidine in spleen cells from congenic mice, which were either resistant or susceptible to Friend leukemia, was studied by the use of N-demethyl rifampicin. The cells from susceptible DDD mice and C57BL/6-Fv-2^s mice were found to be sensitive to N-demethyl rifampicin, while those from resistant DDD-Fv-2^r mice and C57BL/6 mice were almost insensitive. The sensitivity of spleen cells to N-demethyl rifampicin seemed to be controlled by factor(s) related to* Fv-2 *gene or a gene closely linked to* Fv-2 *gene.*

The cells from spleens of DDD mice infected with Friend virus were insensitive to N-demethyl rifampicin in contrast to the cells from DDD mice. This insensitivity was not observed on the spleen cells from mice with induced erythroblastic anemia, suggesting that the change might be due to a virus-induced transformation.

INTRODUCTION

FRIEND [1] described a leukemia which gives rise to splenomegaly in mice. Certain strains of mice, such as DDD mice, are susceptible to Friend disease, whilst other strains (i.e. C57BL/6) are insensitive. Friend virus, obtained after passages in rats or in mice of certain resistant strains, however, does not induce Friend disease [2]. This suggests the Friend leukemia virus complex contains more than one component.

Two genes are involved in the development of Friend virus leukemia [3–5]. A single gene, which was later denoted as *Fv* or *Fv*-2, was involved in the rapid development of splenomegaly and focus formation [3–5]. On the other hand, DDD-*Fv*-2^r mice did not produce rapid splenomegaly, when infected with the virus.

Though the genetic factors involved in Friend disease have now been partially clarified, only limited information is still available on the biochemical function of these genes. Since the multiplication of Friend virus was observed both on DDD and DDD-*Fv*-2^r mice [6], the action of the gene *Fv*-2 seemed to be correlated with the mechanism involved in the transformation of the infected cells or in the multiplication of the transformed cells. Cells of spleen were isolated from congenic mice with *Fv*-2^s or *Fv*-2^r gene and a study was made on the incorporation of thymidine using antibiotics. Studies were also made of the cells from mice infected with Friend virus and compared to those from mice with an induced erythroblastosis.

Accepted 26 May 1975.

*Present address: Department of Biochemistry, Faculty of Medicine, University of Tokyo. Hongo 7-3-1, Bunkyoku, Tokyo 113, Japan.

MATERIAL AND METHODS

Mice

DDD mice (genotype *Fv*-2^s/*Fv*-2^s), DDD-*Fv*-2^r mice (*Fv*-2^r/*Fv*-2^r), C57BL/6 mice (*Fv*-2^r/*Fv*-2^r), C57BL/6-*Fv*-2^s mice (*Fv*-2^s/*Fv*-2^s) were kindly donated by Dr. Odaka of The Institute of Medical Science, University of Tokyo. The DDD pair or the C57BL/6 pair were congenic with their partner except for the gene *Fv*-2 [6, 7]. The mice were 4–5 weeks old when used in these experiments.

For the induction of erythroblastic anemia, 1 mg of acetylphenylhydrazine in buffer A was injected to the mice i.p. daily for 4 days. Spleens were removed 3 days after the last injection. Spleens were found to be enlarged, dark red in colour and weighed about 0·5 g.

Friend virus and suspension of spleen cells

A 20% homogenate of spleen cells from DDD mice infected with Friend virus, was donated by Dr. Odaka. The homogenate was diluted tenfold with phosphate-buffered saline containing 0·25% gelatin and 0·2 ml injected to mice i.p.

Spleens were removed and washed with buffer A, containing 102 mM of NaCl, 15 mM of $CaCl_2$, 4 mM of KCl, 28 mM of sodium lactate and 10 mM of Tris pH 7·4. Spleens were cut at the ends and the cells were then carefully squeezed out with wide-ended forceps to make suspensions in buffer A.

The spleens of C57BL/6 mice were smaller than those of DDD mice and the cells from two or three spleens were combined for the experiments. The cell suspension was made homogeneous by pumping it 20 times through pasteur pipettes and then straining it through a 200 mesh filter to remove connective tissues and large masses. Cells were then centrifuged $2{,}000 \times g$ for 8 min and washed with 10 ml of buffer A. These procedures were repeated 3 times. Cells were resuspended and filtered to make suspensions which contained about 2×10^7 nucleated cells/ml. At least 85% of the cells were shown to be viable by testing with 0·5% trypan blue. After incubation of the cells for 1 hr at 30°C, 80% of the cells were still viable.

Incorporation of thymidine (^{3}H-TdR)

A suspension of cells (0·98 ml) was incubated with buffer A (0·02 ml), containing 2 μCi of tritiated thymidine (specific activity = 28 Ci/mM) with or without inhibitors. Rifamycin derivatives were dissolved in dimethylsulfoxide at various concentrations and 10 μl of the solution was added to the assay mixture. For the control test, dimethylsulfoxide (10 μl) was added to the reaction mixture. No significant change was detected. Actinomycin D and adriamycin were dissolved in buffer A. The assay mixture was incubated at 30°C for 1 hr with occasional stirring. The reaction was stopped by cooling with ice and the assay mixture was added with 20 μl of 10% Sarkosyl (Sodium N-lauryl sarcosinate) solution, and finally 5 ml of 10% trichloroacetic acid (TCA) was added. The precipitates were spun down, washed twice with 5% TCA and then three times with hot ethanol. The final precipitates were resuspended in 5% TCA and heated at 90°C for 15 min. The material was devided in two, half was used for the assay of DNA and the other half was assayed for radioactivity. Plots in the figures on the incorporation study were the means of the experiments in duplicate.

Isolation of DNA–membrane complex

The method used was essentially as described by Trembley *et al.* [8]. For the preparation of a discontinuous sucrose gradient, 1 ml of each 40, 25, 15 and 8·5% (w/w) of sucrose–TMK buffer was successively layered in the ultracentrifuge tube (Hitachi RPS 40A rotar). TMK buffer consisted of 0·01 M Tris buffer pH 7·4, 0·01 M magnesium acetate and 0·1 M KCl. A 10% solution of Sarkosyl (20 μl) was added on the top layer of the sucrose gradients. Then the reaction mixture (0·2 ml) supplemented with 20 μl of 1 M magnesium acetate after the end of the reaction, was added carefully on the top of the layer. It was stirred gently with a glass rod in order to obtain mild lysis of the cells. The white complex that formed immediately was centrifuged for 20 min at $70{,}000 \times g$ at 4°C. The white magnesium–Sarkosyl–membrane–DNA complex was banded between 40 and 25% sucrose TMK layers. The band formed was collected carefully using a pipette with wide end. A 10% TCA solution was added to this fraction and the precipitates filtered on glass fiber paper (Whatman GF/A), washed with cold 5% TCA, then with ethanol, dried and the radioactivity counted. About 90% of the radioactivity was recovered at a band between 40 and 25%, when all bands between each sucrose layer were examined.

Assays

DNA was measured by diphenylamine method [9]. For the measurement of the radioactivity in 5% TCA, 1 ml of the solution was mixed with 10 ml of scintillation solution (667 ml of toluene, 333 ml of triton X-100, 5·5 *g* of DPO and 100 mg of POPOP). The scintillation solution remained clear after the addition of TCA. The radioactivity was measured with a Packard model 2425 scintillation counter. For the measurement of the radioactivity on glass fiber paper, the paper disc was dipped in the scintillation solution, consisting of 4 g DPO (2,5-diphenyloxazole) and 500 mg of POPOP (1,4-bis-[2-(5-phenyloxazolyl)]-benzene) in 1 l. of toluene. A Beckman model LS 133, liquid

scintillation counter, was used for these experiments.

Chemicals

(6-^{3}H) thymidine was purchased from The Radiochemical Centre, Amersham. N-demethyl rifampicin, 3-(4-benzyl-dimethylpiperazines amino methyl)-(cis)-3-formyl rifamycin SV (AF/ABDP), 3-formyl rifamycin SV O-n-hexyloxime (AF/024) and 3-formyl rifamycin SV O-5-hexenyloxime (AF/027) were kindly donated by Dr. Silvestri (Lepetite SpA). Adriamycin was kindly donated by Dr. Kondo (Farmitalia). Actinomycin D was obtained from Banyu Pharmaceutical Co.

RESULTS

The cells obtained from spleens of DDD mice or DDD-*Fv*-2^r mice were carefully suspended in buffer A. About 2×10^7 nucleated cells were incubated with 2 μCi of ^{3}H-TdR (^{3}H-thymidine) for 1 hr at 30°C to follow the kinetics of the incorporation. Figure 1 shows that the incorporation continued linearly for 1 hr. No difference was observed between the cells from DDD mice and DDD-*Fv*-2^r mice. All incubations in subsequent experiments were made for 1 hr.

Incorporation of ^{3}H-TdR was completely inhibited by all of the inhibitors of DNA polymerase tested, except for N-demethyl rifampicin (DMR) (Table 1). On testing the inhibition by DMR at various concentrations, an increase of the activity was found at low concentration of DMR, followed by the decrease of the activity. This increase was always found by repeated experiments on DDD mice (Fig. 2(A)). When the same experiments were performed with cells from DDD-*Fv*-2^r mice it was found that DMR did not show significant inhibition (Fig. 2(B)). The difference between the results suggested that it might be associated with the phenotypic expression of *Fv*-2 gene. As this assumption could be tested by investigating another line of mice C57BL/6, the sensitivity of the cells from C57BL/6 mice was compared with that from C57BL/6-*Fv*-2^s mice, which was established by 12 successive backcrosses of hybrid mice to C57BL/6.

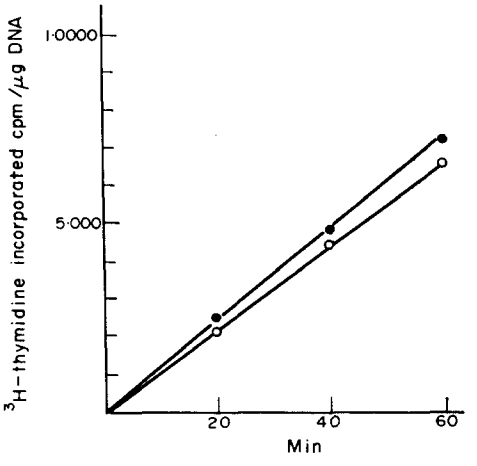


Fig. 1. *Incorporation of ^{3}H-thymidine in spleen cells of mice.* —●— *and* —○— *indicate DDD and DDD-*Fv-2^r *mice respectively.*

Table 1. *Inhibition of incorporation of ^{3}H-thymidine in spleen cells by various antibiotics*

Inhibitors* added	Thymidine incorporated (cpm/μgDNA; mean ± S.D.) DDD	DDD-Fv-2^r	DDD 7 days after FLV† infection
None	7,218 ± 421 (100)‡	7,230 ± 368 (100)	41,802 ± 2,036 (100)
Actinomycin D	202 ± 68 (3)	198 ± 139 (3)	832 ± 121 (2)
Adriamycin	549 ± 52 (8)	417 ± 32 (6)	2,006 ± 158 (5)
AF/ABDP	4 (1)	7 (1)	376 ± 47 (1)
AF/024	6 (1)	——	125 ± 40 (1)
AF/027	4 (1)	——	84 ± 5 (1)
N-demethyl rifampicin	4,966 ± 267 (69)	6,881 ± 224 (94)	41,259 ± 2,456 (99)

*Each inhibitor was added at a concentration of 200μg/ml to the reaction mixture respectively.
†Friend leukemia virus.
‡Values given by the experiments without inhibitors were taken as 100%.

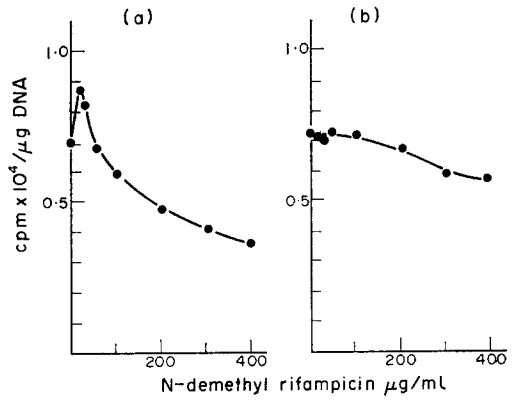


Fig. 2. Effect of N-demethyl rifampicin on the incorporation of ^{3}H-thymidine in spleen cells. (A) A normal DDD mouse with a spleen of 0·1 g. (B) A normal DDD-Fv-2^r mouse with a spleen of 0·15 g.

The cells from C57BL/6-*Fv*-2^s mice were found to be more sensitive to inhibition by DMR than the cells from C57BL/6 mice (Fig. 3(A), (B)), although the curve of the inhibition on the cells from C57BL/6-*Fv*-2^s mice

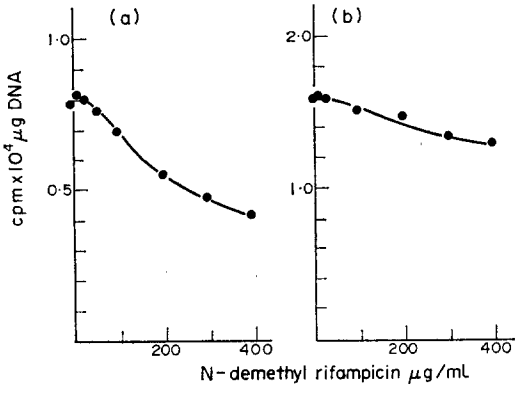


Fig. 3. Effect of N-demethyl rifampicin on the incorporation of ^{3}H-thymidine in spleen cells. (A) A normal C57BL/6-Fv-2^s mouse with a spleen of 0·085 g. (B) A normal C57BL/6 mouse with a spleen of 0·09 g.

was not the same as that on DDD mice. The former did not show the enhancement of the incorporation of thymidine at lower concentration of DMR. The difference might be explained by another gene which was closely linked to *Fv*-2 gene and which could not be replaced in establishing DDD-*Fv*-2^r mice and C57BL/6-*Fv*-2^s mice. DDD-*Fv*-2^r mice were established by the successive cross-intercross system 7 times, while C57BL/6-*Fv*-2^s mice were established by successive backcrosses 12 times and were highly congeneic with C57BL/6 mice.

Since DDD mice and C57BL/6-*Fv*-2^s mice, which possess *Fv*-2^s gene, were both more sensitive with regard to the sensitivity of their spleen cells, these results were in line with the assumption that the product of *Fv*-2^s gene or a gene closely linked to *Fv*-2 gene is related to the sensitivity to DMR as an inhibitor of the incorporation of ^{3}H-TdR.

Since the multiplication of Friend virus complex was observed in DDD-*Fv*-2^r mice [6], the effect of *Fv*-2^s gene seemed to be associated with capability to form specific tumor cells (Friend cells). The DDD mice were infected with the virus and the incorporation of ^{3}H-TdR measured. The rate of the incorporation of ^{3}H-TdR increased twofold on the fifth day and about sixfold from the seventh to fourteenth day, while the spleens were enlarged during the second week (Table 2). Histological studies showed that a large number of large polygonal cells appeared which resembled immature reticulum cells (Fig. 4A). It was considered that the high incorporation of ^{3}H-TdR observed was due to the large numbers of these tumor cells (Friend cells), in the cell suspension (Fig. 4(B)). This high incorporation of ^{3}H-TdR was, however, not inhibited by DMR, even at a concentration of 400 μg/ml (Fig. 5(A)).

Table 2. Weight of spleen and incorporation of ^{3}H-thymidine in spleen cells after infection with Friend leukemia virus

Days after FLV* infection	DDD		DDD-Fv-2^r	
	Weight of spleen (gram)	Thymidine incorporated (cpm/μgDNA; mean±S.D.)	Weight of spleen (gram)	Thymidine incorporated (cpm/μgDNA; mean ± S.D.)
0	0.10	7,218 ± 421	0.15	7,230 ± 368
5	0.15	15,912 ± 2,192	—	—
7	0.35	41,259 ± 5,391	0.20	22,013 ± 1,896
9	0.75	44,212 ± 3,413	—	—
14	1.20	41,115 ± 2,882	0.22	16,297 ± 1,902

* Friend leukemia virus

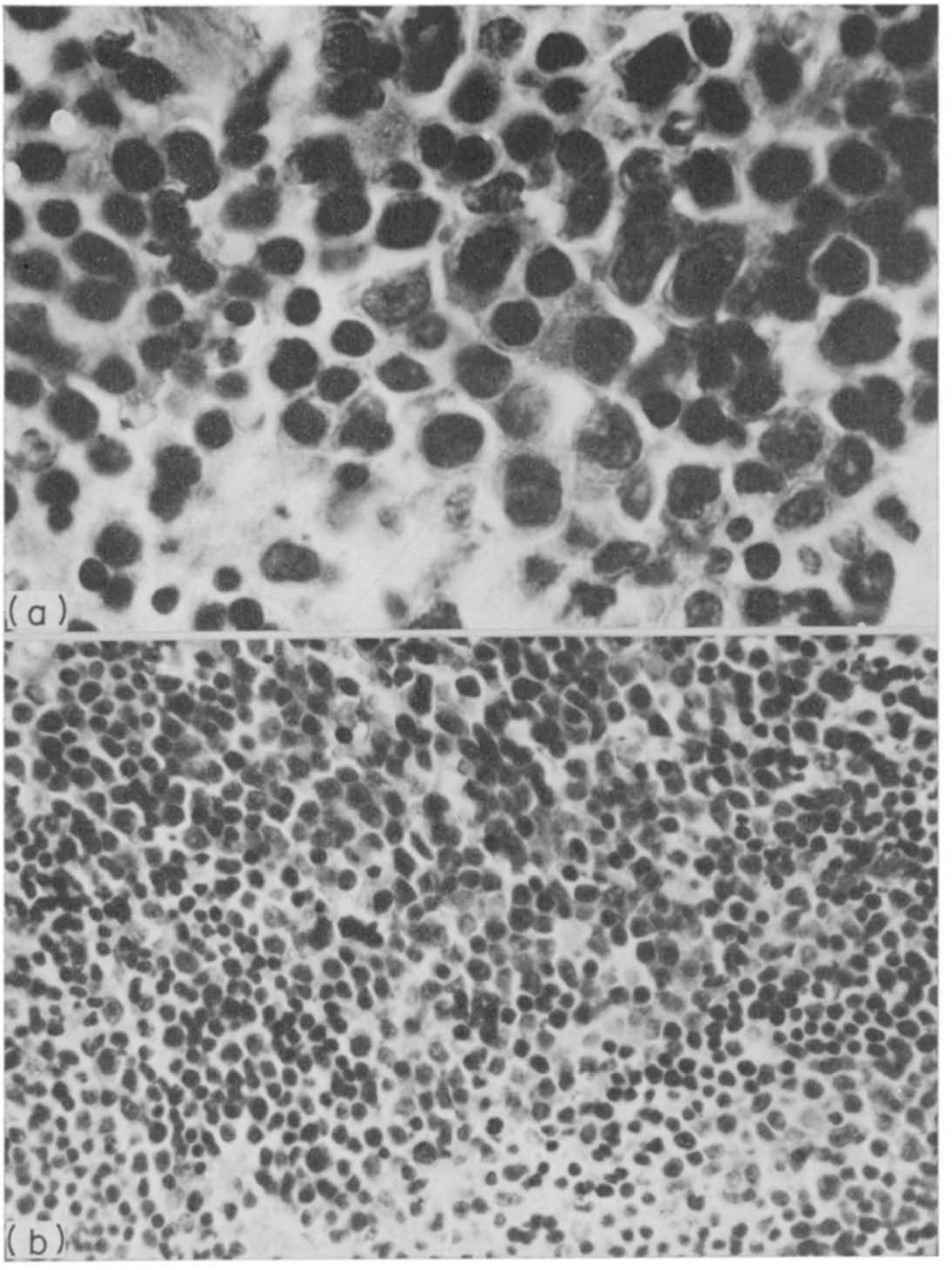


Fig. 4(*a*), (*b*) *Spleen of a DDD mouse developing the rapid splenomegaly after infection of Friend leukemia virus. Marked proliferation of large reticular cells in association with erythroblast-like cells in the red pulp. Hematoxylin and eosin.* (*a*) × 1000. (*b*) × 400.

(to face p. 652)

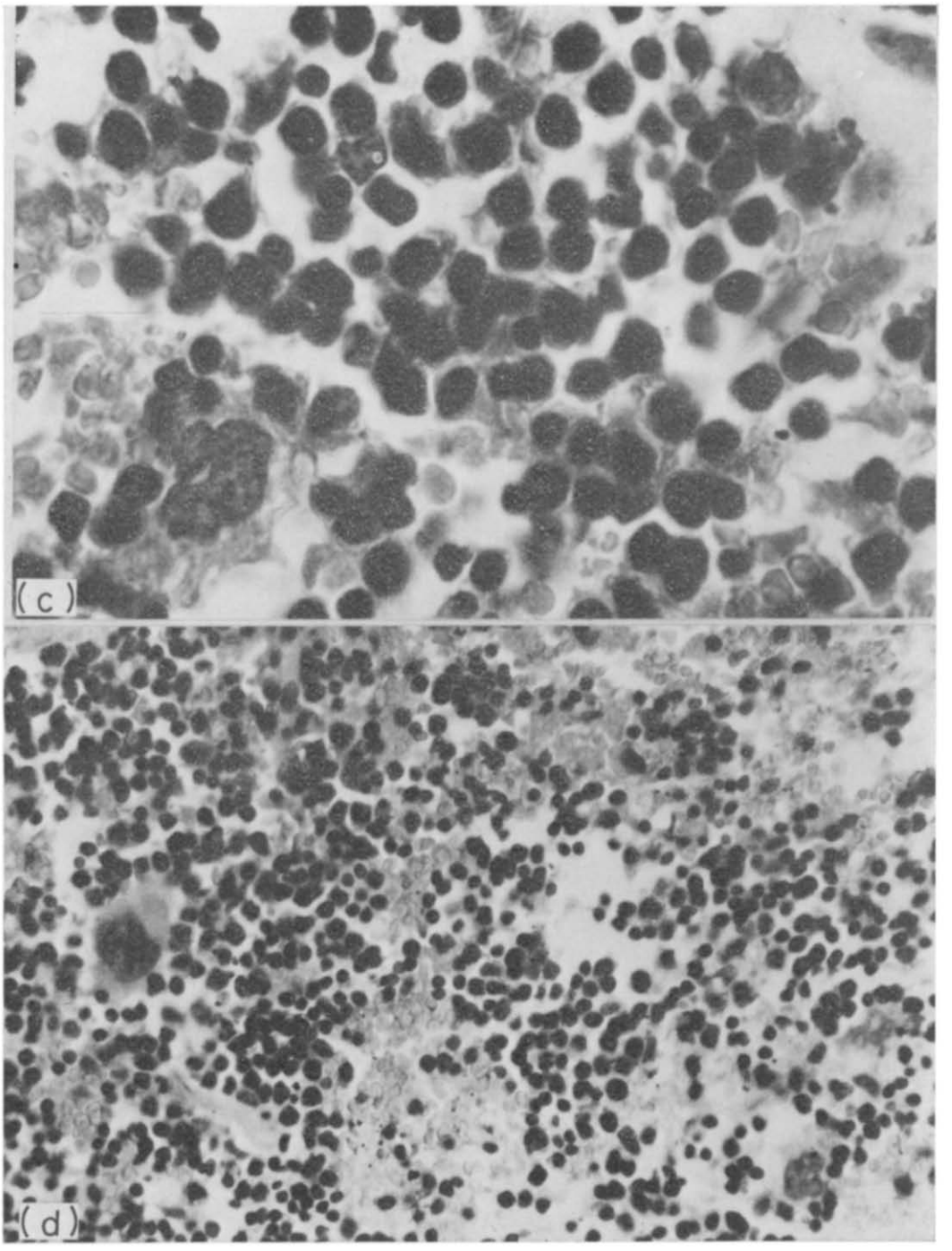


Fig. 4(*c*), (*d*) *Spleen of a DDD mouse showing erythroblastosis in the red pulp after the induction of acetylphenylhydrazine anemia. No existence of large reticular cells, except for megakaryocytes. Hematoxylin and eosin.* (*c*) × 1000 (*d*) × 400.

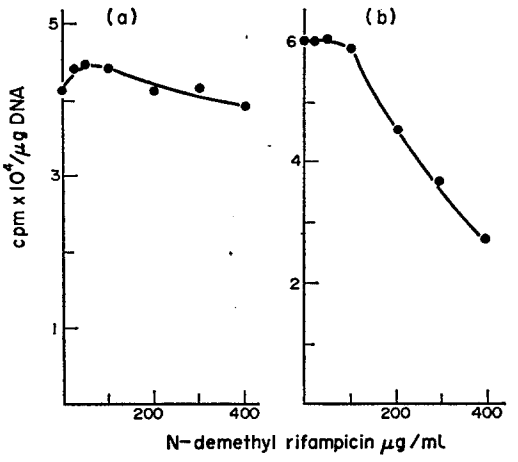


Fig. 5. Effect of N-demethyl rifampicin on the incorporation of ^{3}H-thymidine in spleen cells. (A): A DDD mouse 7 days after infection with Friend leukemia virus with a spleen of 0·35 g. (B): A DDD mouse after the last injection of acetylphenylhydrazine with a spleen of 0·5 g.

The loss of sensitivity to DMR in cells from virus-infected DDD mice was confirmed by assaying DNA–membrane complex. The spleen cells from DDD mice, in which ^{3}H-TdR was incorporated, were lysed with detergent Sarkosyl and the complex associated with DNA together with membrane was centrifuged. Most of the radioactivity was recovered between layers of 25 and 40% of sucrose–TMK solution. The radioactivity in this fraction was decreased when DMR was added to the assay mixture (Fig. 6(A)).

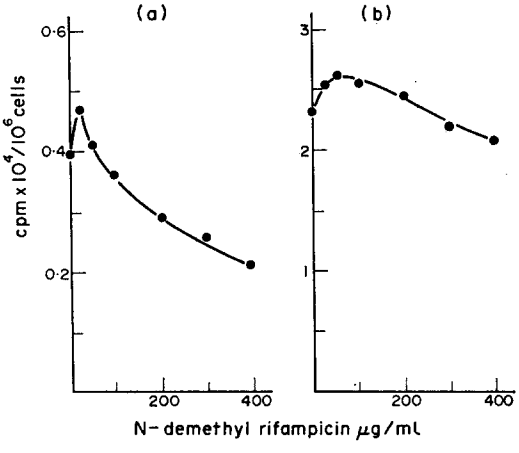


Fig. 6. Effect of N-demethyl rifampicin on the incorporation of ^{3}H-thymidine into DNA-membrane complex of spleen cells. (A) A normal DDD mouse with a spleen of 0·12 g. (B) A DDD mouse 7 days after infection with Friend leukemia virus with a spleen of 0·36 g.

The incorporation of ^{3}H-TdR was increased 6 fold in virus-infected DDD mice as shown in Table 2. The inhibition by DMR was, however, not significantly changed as shown in Fig. 6(B). This result confirmed that the incorporation of thymidine into DNA was changed by the viral infection.

In order to see whether the loss of the sensitivity was specific to the Friend tumor cells, DDD mice were treated with acetylphenylhydrazine, which induced an erythroblastosis. Since Friend disease has been reported to give proliferation of erythroid cells [10, 11], the cells of both erythroid types were compared on mice infected with virus or with an induced erythroblastic anemia. Many proerythroblastic cells were observed in spleens of mice treated with acetylphenylhydrazine (Fig. 4(D)) and the incorporation of ^{3}H-TdR was raised 8 fold. This high incorporation seemed to be associated with these cells. The incorporation of ^{3}H-TdR into these cells was however, sensitive to DMR as shown in Fig. 5(B). From these studies, it was inferred that the insensitivity in Friend cells cannot be simply explained by the presence of large numbers of erythroid precursor cells.

DISCUSSION

Several rifamycin derivatives strongly inhibited the incorporation of thymidine into the spleen cells of normal and Friend virus-infected mice except DMR. The same results were obtained either by the standard assay or by the DNA–membrane complex assay [8]. Thus, the decreased inhibition by DMR was unlikely caused by the impermeability of the cell membrane. DMR probably inhibited polymerase. DMR gave rise to the enhanced incorporation in cells from DDD mice at lower concentration of DMR and the increasing inhibition at higher concentration. Similar effects were reported on derivatives of rifamycin, using purified polymerase from murine leukemia virus [12].

It was reported that rifampicin did not inhibit a purified mitochondrial DNA-dependent RNA polymerase, while it did inhibit the crude preparation of the enzyme [13]. This suggested that the sensitivity to rifamycin can be altered by some components associated with the enzyme. The sensitivity of the cells from C57BL/6-*Fv*-2^s can be explained by such components. If the action of *Fv*-2^s gene is related to such components, the hybrids were expected to be sensitive to DMR, because *Fv*-2^s was dominant over *Fv*-2^r gene [5, 7]. The hybrid (C57BL/6-*Fv*-2^s × C57BL/6) F_1 mice were tested for sensitivity to DMR in the same way. Almost the same inhibition curve was obtained as that from C57BL/6-*Fv*-2^s

mice. These results were in support of the assumption that the product of gene *Fv*-2^s can be a component, which might be involved in the activity of DNA polymerase.

The inhibition was not the same in DDD mice and in C57BL/6-*Fv*-2^s mice. This difference may be due to the fact that C57BL/6-*Fv*-2^s mice were established by 12 repeated backcrosses, whilst DDD-*Fv*-2^r mice were established by 7 crosses. C57BL/6-*Fv*-2^s mice thus possessed smaller number of genes closely linked to *Fv*-2^s gene than the number of genes replaced in DDD-*Fv*-2^r mice. The other genes closely associated with *Fv*-2^s gene might be related to the enhanced incorporation of thymidine at lower concentration of DMR found in DDD mice.

A six fold increase of the incorporation of ^{3}H-TdR was observed in the Friend virus-infected spleen cells of DDD mice. This significant increase seemed to be caused by the Friend cells. DDD-*Fv*-2^r mice infected with the virus showed neither such high incorporation of the thymidine nor so many Friend cells when the spleens were examined microscopically, a small increase of erythroblasts and of megakaryocytes was observed.

The Friend cells stem from the erythroid series as shown by using the transplantation of bone marrow [14] or by assaying the hemoglobin synthesis [15]. The effect of DMR on Friend cells seemed to differ from that on the proerythroblastic cells in acetylphenylhydrazine-treated mice. Since both the tumor cells and the erythroblasts belong to the same line of erythroid cells, the insensitivity to DMR of Friend cells was probably related to the transformation induced by the virus. Transplants into F_1 hybrid hosts from the Friend virus-infected spleen cells of parental lines were reported to give rise to the enhanced were reported to give rise to the enhanced hybrid resistance in the early stage of the infection [16, 17].

This study on the change of the sensitivity to DMR with respect to *Fv*-2 gene would give clue to the biological changes of transformed cells.

Acknowledgements—Grateful acknowledgement is made to Dr. T. Odaka, The Institute of Medical Science, University of Tokyo, for his kindness in providing us mice with different genotypes and also for valuable discussions. The authors are also grateful to Dr. K. Nagashima, Department of Pathology, Faculty of Medicine, University of Tokyo, for carrying out histological studies on spleens of these mice. The authors wish to thank Prof. E. H. Cooper of The University of Leeds for the reading and correcting the manuscript.

REFERENCES

1. C. Friend, Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. *J. exp. Med.* **105,** 307 (1964).
2. P. J. Dawson, W. M. Rose and L. H. Fieldsteel, Lymphatic leukemia in rats and mice inoculated with Friend virus. *Brit. J. Cancer* **20,** 114 (1966).
3. T. Odaka and T. Yamamoto, Inheritance of susceptibility to Friend mouse leukemia virus. *Jap. J. exp. Med.* **32,** 405 (1962).
4. A. Axelrad, Genetic and cellular basis of susceptibility or resistance to Friend leukemia virus infection in mice. *Canad. Cancer Conf.* **8,** 313 (1968).
5. F. Lilly, *Fv*-2: Identification and location of a second gene governing the spleen focus response to Friend leukemia virus in mice. *J. nat. Cancer Inst.* **45,** 163 (1970).
6. T. Odaka, Inheritance of susceptibility to Friend mouse leukemia virus. VII. Establishment of a resistant strain. *Int. J. Cancer* **6,** 18 (1970).
7. T. Odaka, Inheritance of susceptibility to Friend mouse leukemia virus. V. Introduction of a gene responsible for susceptibility in the genetic complement of resistant mice. *J. Virol.* **3,** 543 (1969).
8. G. Y. Trembley, M. J. Daniels and M. Schaechter, Isolation of a cell membrane–DNA–nascent RNA complex from bacteria. *J. molec. Biol.* **40,** 65 (1969).
9. W. C. Schneider, Determination of nucleic acid in tissues by pentose analysis. In *Methods in Enzymology*. Edited by S. P. Colowick and N. O. Kaplan, Vol. III, p. 680. Academic Press, New York (1957).
10. Y. Ikawa, H. Sugano and K. Oota, Spleen focus in Friend's disease: Histological study. *Gann* **58,** 61 (1967).
11. Y. Ikawa, H. Sugano and M. Furusawa, Pathogenesis of Friend virus-induced leukemia in mice. In *Gann Monograph on Cancer Research*. Edited by T. Yamamoto and H. Sugano, Vol. XII, p. 33. University of Tokyo Press, Tokyo (1972).

12. S. S. Yang, F. M. Herrera, R. G. Smith, M. S. Reitz, G. Lancini, R. C. Ting and R. C. Gallo, Rifamycin antibiotics: inhibitors of Rauscher murine leukemia virus reverse transcriptase and purified DNA polymerases from human normal and leukemic lymphoblasts. *J. nat. Cancer Inst.* **49,** 7 (1972).
13. B. D. Reid and P. Parsons, Partial purification of mitochondrial RNA polymerase from rat liver. *Proc. nat. Acad. Sci. (Wash.)* **68,** 2830 (1971).
14. T. Odaka and M. Matsukura, Inheritance of susceptibility to Friend mouse leukemia virus. VI. Reciprocal alteration of innate resistance or susceptibility by bone marrow transplantation between congenic strains. *J. Virol.* **4,** 837 (1969).
15. S. Sassa, F. Nakao, Y. Ikawa and H. Sugano, Heme synthesis in Friend ascites tumor cells. *Proc. Soc. exp. Biol. (N.Y.)* **127,** 527 (1968).
16. G. B. Rossi, G. Gudkowicz and C. Friend, Evidence for transformation of spleen cells one day after infection of mice with Friend leukemia virus. *J. exp. Med.* **131,** 765 (1970).
17. G. B. Rossi, G. Gudkowicz and C. Friend, Transformation of spleen cells three hours after infection *in vivo* with Friend leukemia virus. *J. nat. Cancer Inst.* **50,** 249 (1973).

Europ. J. Cancer Vol. 11, pp. 657–667. Pergamon Press 1975. Printed in Great Britain

Identification of The Inhibitor of Labelled Thymidine Incorporation into HeLa Cell DNA Present in Endometrial Extract

S. CHEVALIER and W. G. VERLY
Department of Biochemistry, University of Montreal, Montreal, Canada

Abstract—*An extract of bovine endometrium was found to decrease ^{3}H-thymidine incorporation into DNA of HeLa cells, but also, although to a lesser extent, of Ehrlich and Novikoff cells. Thymidine in the extract was responsible for about 80% of this inhibition. No inhibitor strictly specific for HeLa cells could be detected.*

An extract of human endometrium decreased, while an extract of human myometrium increased ^{3}H-thymidine incorporation into DNA of HeLa cells. The inhibitory effect of the endometrial extract was also observed on Ehrlich and Novikoff cells. The endometrial extract contained thymidine which was absent from the myometrial extract; thymidine in the endometrial extract was, however, a preparation artefact. Both human endometrial and myometrial extracts possessed an enzymic activity that converted thymidine into thymine; at high doses, the myometrial extract also decreased ^{3}H-thymidine incorporation into DNA of HeLa cells.

Addition of unlabelled thymidine to the incubation medium has not the same inhibitory action in different kinds of cells on ^{3}H-thymidine incorporation into DNA. HeLa cells are very sensitive to this dilution effect. The differences in behavior of various cellular systems can be wrongly interpreted as the result of the action of a tissue-specific inhibitor.

INTRODUCTION

IT HAS been proposed that tissue growth is under the control of "antitemplates" [1] or "chalones" [2]. In principle, a chalone is secreted by cells in the extracellular fluid and exerts an inhibitory effect on the proliferation of cells of the same kind; this tissue-specificity seems to be the most important property of the chalones to enable each tissue to control its own growth by a negative feedback mechanism. On the other hand, the chalones so far described have no species-specificity.

Hinderer, Volm and Wayss [3] found that a crude preparation from human endometrium inhibited the incorporation of ^{3}H-thymidine into DNA of HeLa cells, but not of cells from the amelanotic melanoma of the Syrian hamster or of mouse fibroblasts (L cells) cultured *in vitro*; it had no effect either on Yoshida sarcoma or Fortner III melanoma ascites cells. On the other hand, a crude preparation of human myometrium stimulated the incorporation of ^{3}H-thymidine into DNA of HeLa cells as well as of the other kinds of cells tested; this was attributed to non-specific nutritive influences. Because HeLa cells are cancer cells derived from the cervix epithelium of a human uterus, Hinderer *et al.* [3] concluded that the human endometrium contained

Accepted 20 May 1975.

*The research was supported by grants from the National Cancer Institute and the Medical Research Council of Canada. S. Chevalier was recipient of a MRC studentship.

The authors wish to thank Dr. J. Gagnon from Ste-Justine Hospital, Montreal, who supplied the human endometrium and myometrium and Miss J. Flamand who provided the HeLa cells used in this work.

an endometrial chalone. The aim of this work was to isolate, from the endometrial crude extract prepared by the technique of Hinderer *et al.* [3], the inhibitor of ^{3}H-thymidine incorporation into the DNA of HeLa cells. While this work was under way, Wayss and Volm [4] observed that the crude endometrial extract was also active on human fetal liver and kidney cells; they then revised their previous opinion and concluded that the endometrial inhibitor was not a chalone.

MATERIAL AND METHODS

Solutions

Antibiotic solution. 1·00 g of streptomycin sulfate (General Biochemicals) and 0·63 g of penicillin G potassium salt (Calbiochem) are dissolved in water and the volume brought to 100 ml.

MEM-antibiotic solution. 10·7 g of minimum essential medium (Eagle) powder with Spinner salts and L-glutamine (F-14, Gibco) and 2·2 g $NaHCO_3$ are dissolved in water; 4·0 ml of phenyl red solution (0·05 g/l, 510 Gibco) and 10 ml of antibiotic solution are added; the solution is brought to 1000 ml. The solution is sterilized by passing through a 0·45 μm Millipore filter.

Trypan blue solution. 0·4% trypan blue (525, Gibco) in Hanks' solution.

Hanks' solution. Gibco 406S diluted 10 times and sterilized 15 min at 130°C. To 100 ml of this solution, 2·5 ml of sterile 5% $NaHCO_3$ are added to have a 0·015 M $NaHCO_3$ final concentration. The pH is 7·4 in a 95% air–5% CO_2 atmosphere.

Cells

About 75×10^6 HeLa cells are inoculated into a 1 l. Spinner flask (Bellco Glass Inc.) containing 300 ml of MEM-antibiotic solution; calf serum (Tissue Culture Service Ltd.) is added to obtain a 5% concentration. After a 3-day incubation at 37°C, a 0·5 ml aliquot of the cell suspension is mixed with 0·1 ml of trypan blue solution and, after 5 min, the cells are counted in a hemacytometer. The 300-ml culture usually yields $3·0–3·5 \times 10^8$ HeLa cells; dead cells do not exceed 2% in the population. The culture is transferred into tubes and centrifuged 5 min at 1000 rev/min; the sediments are then put in Hanks' solution to have about $2·5 \times 10^6$ cells/ml.

Ehrlich cells are inoculated i.p. into male Swiss white mice (20–25 g) and Novikoff cells into male Sprague–Dawley rats (150–175 g). The ascites fluid is collected one week later. The fluid is centrifuged and the cells washed 4 times with Hanks' solution. They are finally put in suspension in Hanks' solution to have about $2·5 \times 10^6$ cells per ml.

Preparation of tissue extract

Bovine endometrium is dissected from adult cow uterus taken immediately after the animal has been killed. Human endometrium and myometrium were provided by Dr. J. Gagnon of Ste-Justine Hospital, Montreal. Tissues were dissected after hysterectomy of 20–45 year-old women, and frozen to −20°C until needed. These uterus did not show any major pathology, in particular no cancer. Fresh bovine endometrium or thawed human endometrium and myometrium, kept in crushed ice, are cut into pieces with scissors, minced in an equal volume of cold water, then crushed and sonicated with a Polytron model PT 45/2 (7000 rev/min, 5000 Hz).

From the homogenates, 105,000 ***g*** supernatants are prepared by two different methods:

—Method I (Hinderer *et al.* [3]): the homogenate is lyophilized and the powder extracted with water (20 mg/ml, 4°C, 12 h). The suspension is centrifuged at 100,000 rev/min for 15 min in a RC-2B Sorvall centrifuge; the supernatant is centrifuged at 105,000 ***g*** for 150 min in a Beckman L2-65 centrifuge.

—Method II: the homogenate is immediately centrifuged at 10,000 rev/min for 15 min in a RC-2B Sorvall centrifuge. The 105,000 ***g*** supernatant is obtained after centrifugation for 150 min at 105,000 ***g*** in a Beckman L2-65 centrifuge.

The clear supernatants are kept frozen at −10°C.

Assay of the action on ^{3}H-thymidine incorporation into DNA

The activity of our preparation on ^{3}H-thymidine incorporation into DNA is tested on 10^7 HeLa cells (3·9 ml of the cell suspension in Hanks' solution obtained previously) incubated into 25 ml Erlenmeyer flasks containing a maximum of 1·0 ml of preparation in the assay or an equal volume of water into the control,* and Hanks' solution to adjust the volume at 4·9 ml. Tritiated thymidine labelled in the

*In some cases, larger volumes of preparations are lyophilized to a powder and dissolved in Hanks' solution (maximum 1·0 ml) before the assay; Hanks' solution is then added to the control.

methyl group (4 μCi; 6 Ci/mmol; Bioresearch) in 0·1 ml of Hanks' solution is added bringing the total volume to 5·0 ml. The flasks are incubated for 1 hr at 37°C in an atmosphere of 95% air–5% CO_2 (v:v) with continuous shaking. The incubation is stopped by cooling the flasks in ice.

In experiments where the activities of our preparations on HeLa, Ehrlich and Novikoff cells are compared, the incubation is carried out for 15 min only; other conditions remain unchanged.

For the determination of the DNA specific radioactivity, the flask content is transferred to a 15-ml Corex tube and is centrifuged for 10 min at 6000 rev/min in the refrigerated RC-2B Sorvall centrifuge. The incubation medium is pipetted away, then 3·0 ml of 10% cold trichloroacetic acid (TCA) containing unlabelled thymidine (100 μg/ml) are added; after vigourous stirring with a Vortex mixer, the tubes are centrifuged again. The supernatant is discarded and the manipulation is repeated on the sediment with 2·5 ml of 10% cold TCA. The last sediment is suspended in 5 ml of 95% ethanol, the suspension is centrifuged as above, and the supernatant discarded; this manipulation is repeated twice on the sediment. The final sediment is suspended in 2·5 ml of 5% TCA and the tube heated at 90°C for 15 min with occasional shaking. After cooling at room temperature, the tube is centrifuged for 15 min at 10,000 rev/min and the supernatant collected [5].

A 1 ml aliquot of this supernatant is used for the determination of the radioactivity, while the DNA content is estimated on another 1 ml aliquot by the diphenylamine method, the absorbance being read at 600 nm [6]. The DNA specific radioactivity (DNA sp. rad) is arbitrarily given in disintegrations per min per 1000 A_{600}; the standard deviation for the control is 6%. The inhibited fraction (I.F.) of ^{3}H-thymidine incorporation into DNA is expressed as:

$$\text{I.F.} = \frac{\text{DNA sp. rad. (control)} - \text{DNA sp. rad. (assay)}}{\text{DNA sp. rad. (control)}}$$

As did Verly *et al.* [6] with liver chalone, we found a linear relationship between 1/(1—I.F.) and the amount of bovine endometrium supernatant (method I) added in the assay flask. By analogy with the liver chalone unit, we have defined the HeLa unit as the amount of inhibitor necessary to reduce by 50% the amount of ^{3}H-thymidine incorporated into the DNA of HeLa cells in our experimental conditions (1 hr at 37°C). The number of units (U) is given by the equation: U = I.F./(1—I.F.). To minimize the error in U, we use only I.F. values between 0·30 and 0·70.

Separation of incubation medium, TCA-soluble and TCA-insoluble fractions of HeLa cells [7].

After incubation of HeLa cells in the presence of ^{3}H-thymidine, the suspension, cooled at 4°C, is centrifuged and the supernatant (incubation medium) collected. The cells are washed with 5 ml of Hanks' solution containing 500 μg of unlabelled thymidine, then with 5 ml of Hanks' solution; each washing is followed by a centrifugation. After removing the supernatant from the last centrifugation, 2 ml of cold 5% TCA are added to the cells; the mixture is homogenized in a Potter–Elvehjem apparatus before being centrifuged to separate the TCA-soluble and TCA-insoluble fractions.

Chromatography on Dowex-50 [8]

The sample is placed on top of a 1 × 26 cm column of Dowex AG 50W X2 equilibrated with 0·1 M ammonium formate buffer pH 3·2. Elution is performed with the same buffer at a rate of 0·5 ml/min and fractions of 3·0 ml are collected. The column was calibrated with thymine, thymidine and thymidine phosphates. Table 1 gives the elution position of the different compounds: thymidine is well separated from thymine and the thymidine phosphates; the three thymidine phosphates (dTMP, dTDP and dTTP) are not retained by the ion-exchanger and emerge in the same position.

Dialysis

The supernatant is placed in a cellophane bag (Visking Co) with pores permeable to molecules below 4000 D, and dialyzed against five volumes of water at 4°C with three changes; the whole process lasts 20 hr. The dialysate is lyophilized and the residue is taken in water; a 10 min centrifugation at 10,000 rev/min in a Sorvall RC-2B, rotor SS-34, allows discarding of the insoluble material.

Chromatography on Sephadex G-15

A column of 2·5 cm dia (Pharmacia) is filled in the cold room at 4°C with Sephadex G-15 up to 45 cm and equilibrated with water. The sample dissolved in 2·5 ml of water is placed on top of the Sephadex column and the elution is performed with water at a rate of 5 ml/hr;

fractions of 2·0 ml are collected. V_0 (74 ml) was determined with Pharmacia blue dextran.

Table 1. *Chromatography on Dowex-50 of thymine, thymidine and thymidine phosphates*

Compound	Maximum (fraction number)
Thymine	13–14
Thymidine	11–12
dTMP	4–5
dTDP	4–5
dTTP	4–5

Thymine and thymidine were bought from Sigma; thymidine monophosphate (dTMP), thymidine diphosphate (dTDP) and thymidine triphosphate (dTTP) came from NBC. About 200 μg of the compound in 1 ml of 5% TCA were placed on top of a 1 × 26 cm column of Dowex AG 50W X2. The elution was performed with 0·1 M ammonium formate buffer, pH 3·2; fractions of 3 ml were collected and their absorbance at 260 mm determined. The Table gives the fraction corresponding to the maximum of the elution peak.

Thin layer chromatographies

The lyophilized sample is dissolved in 50 μl of water. Three different systems were used:
—System C: cellulose MN 300 plate, Macherey–Nägel; solvent: n-butanol–acetic acid–water (60:15:25);
—System CM: carboxymethylcellulose MN 300 CM plate, Macherey–Nägel; solvent: n-butanol–acetic acid–water (60:15:25);
—System PEI: polyethyleneiminecellulose MN-polygram cel 300/PEI plate, Macherey–Nägel; solvent: 0·25 M NaCl in water.

After chromatography, the plates are examined under a u.v. lamp and the fluorescent spots are delineated. For the detection of the ninhydrin-positive compounds, the chromatograms are cut in two longitudinally and one half-band is immersed in 0·5% ninhydrin in acetone and dried. The other half-band is divided in 1 cm portions; the powder of each portion is removed and eluted with 1 ml of water. The absorbance at 260 nm is determined in a model 130 Hitachi Perkin–Elmer spectrophotometer; 100 μl aliquots are used for the biological assay.

Ethanol precipitation

To the 105,000 *g* supernatant kept at 4°C, pure ethanol is added dropwise, with continuous stirring, up to a concentration of 60% (v:v); the precipitate (fraction I) is collected by centrifugation at 10,000 rev/min for 15 min in the RC-2B Sorvall centrifuge. To the new supernatant, pure ethanol is further added up to 87% (v:v); the second precipitate (fraction II) is also collected by centrifugation. The fraction soluble in 87% ethanol (fraction III) is lyophilized. Each fraction is dissolved in a minimum volume of water.

Protein determination

The protein content of the various preparations is determined by the method of Lowry *et al.* [9] using bovine serum albumin as standard.

RESULTS

Bovine endometrium

The 105,000 *g* supernatant, prepared from bovine endometrium according to method I, was tested for its action on ^{3}H-thymidine incorporation in the DNA of HeLa, Ehrlich and Novikoff cells. Table 2 shows that a dose containing 0·31 mg of proteins was inhibitory in all cases although the magnitude of the inhibition varied from one cell type to another; the inhibition was greater with HeLa cells.

(1) *Purification of the main inhibitor*

Bovine endometrium supernatant (150 ml) prepared by method I was submitted to dialysis: more than 70% of the recovered inhibitory activity on HeLa cells appeared in the dialysate; the specific activity (number of inhibitory units per mg of proteins) in the dialysate was about 10 times higher than in the supernatant. The 3 l. dialysate was lyophilized and the residue taken in 3 ml of water; the solution was centrifuged to discard the insoluble material.

This clear solution from the dialysate (2·5 ml; 1·8 mg of proteins; 3850 inhibitory units) was chromatographed on Sephadex G-15. The inhibitor was eluted in a single peak (V_e/V_0 = 2·7) (Fig. 1); the pooled fractions corresponding to this peak were called DS–G15. Table 2 shows that DS–G15 was active on HeLa, Ehrlich and Novikoff cells. Thymidine, which was the main low mol. wt. inhibitor found in a bovine liver extract by Lenfant *et al.* [10], emerges with a V_e/V_0 of 2·8 from the same Sephadex G-15 column.

Table 2. Inhibitory activities of bovine and human endometrium preparations on HeLa, Ehrlich and Novikoff cells

Preparation	Proteins (mg)	I.F. HeLa	I.F. Ehrlich	I.F. Novikoff
Bovine endometrium				
105,000 g supernatant	0·31	0·67	0·53	0·39
DS-G15	0·0005	0·41	0·29	0·30
Ethanol precipitation				
Fraction I (0–60%)	0·38	0·54	0·41	
Fraction II (60–87%)	2·47	0·59	0·43	0·47
Fraction III (soluble in 87%)	0·02	0·72	0·57	0·58
Human endometrium				
105,000 g supernatant	4·60	0·49	0·37	0·15

The supernatants were prepared by method I; the other endometrial preparations are described in the text. The same dose (expressed in mg of proteins) was added into the incubation medium containing ^{3}H-thymidine and HeLa, Ehrlich or Novikoff cells. Controls without endometrial preparation were carried out. The incubation lasted 15 min at 37°C before the determination of the DNA specific radioactivity; the inhibited fraction (I.F.) of tritium incorporation into DNA was calculated.

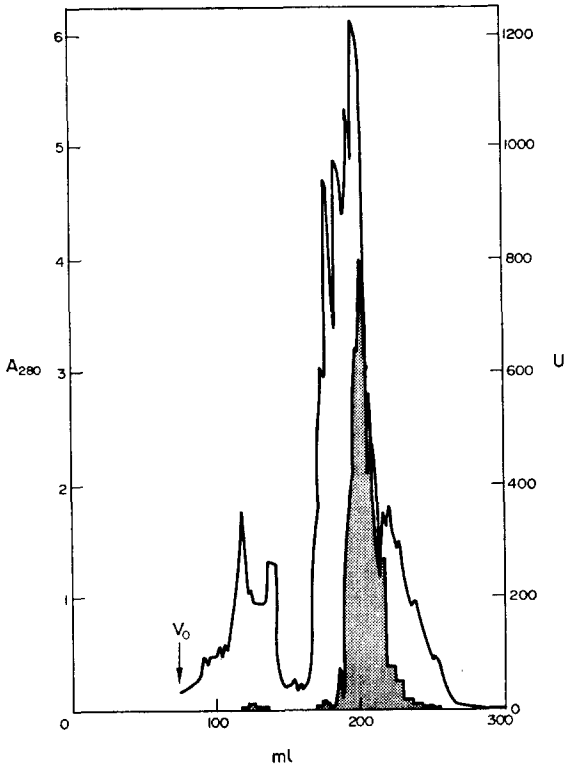


Fig. 1. Chromatography on Sephadex G-15 of the dialysate from the bovine endometrium supernatant

A portion of the concentrated dialysate (1·8 mg of proteins; 3850 inhibitory units) was chromatographed in water on a 2·5 × 45 cm Sephadex G-15 column. Fractions of 2 ml were collected at a rate of 5 ml/hr. The continuous line gives the absorbance at 280 nm, while the graded line limiting the stippled area gives the number (U) of inhibitory units in each fraction. V_o (74 ml) = void volume.

DS–G15, in parallel with thymidine, was submitted to three successive thin layer chromatographies in different systems.

A portion (1 ml) of DS–G15 was lyophilized and the residue taken in 50 µl of water. This solution (200 inhibitory units) and 45 µg of thymidine, also dissolved in 50 µl of water, were first chromatographed on cellulose (system C). Thymidine gave one fluorescent spot of Rf 0·75. By contrast, DS–G15 gave six fluorescent spots and three spots reacting with ninhydrin; analysis of the water eluate from the cellulose powder of 1-cm long portions of the chromatogram, showed a peak of inhibitory activity associated with the fluorescent, ninhydrin-positive, spot of Rf 0·75 (Fig. 2).

The pooled eluates of the Rf 0·75 spots from several C-chromatograms of thymidine or inhibitor were lyophilized; the residues (called THY-C75 or DS-G15-C75) were dissolved in water and 50 µl of each were chromatographed on carboxymethylcellulose (system CM). THY-C75 gave one fluorescent spot of Rf 0·75, while DS-G15-C75 (400 units) gave three ninhydrin-positive spots and one fluorescent spot; the inhibitor was associated with the fluorescent, partly ninhydrin-positive, spot of Rf 0·75 (Fig. 2). After elution from their corresponding spots of two CM-chromatograms, lyophilization and redissolution in a minimum of water, 50 µl of THY-C75-CM75 and 50 µl of DS-G15-C75-CM75 (540 units) were chromatographed on PEI-cellulose (system PEI). In each case, there was only one fluorescent, ninhydrin-negative, spot of Rf 0·82; the inhibitory activity was associated with this spot (Fig. 2). The eluates from the Rf 0·82 spots of the PEI-chromatograms were called respectively THY-

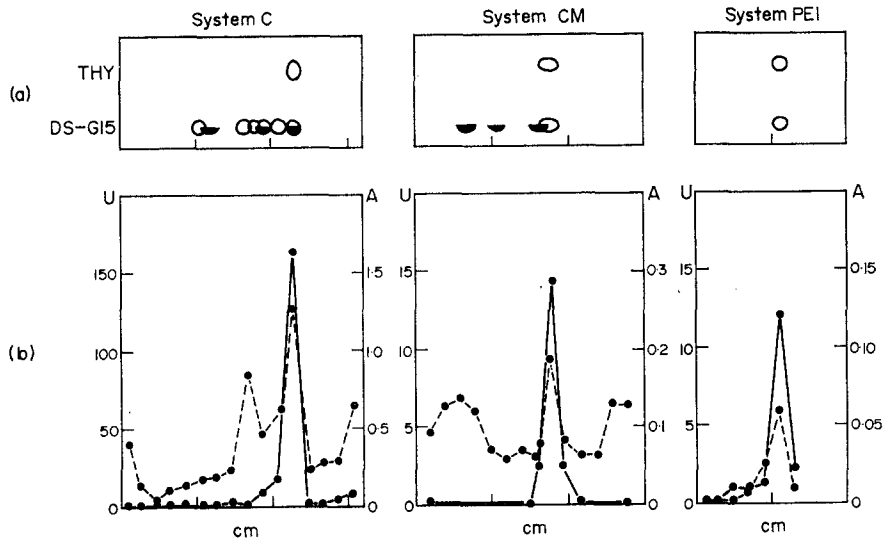


Fig. 2. Purification by thin layer chromatographies of the main bovine endometrium inhibitor. The inhibitor (DS-G15), in parallel with thymidine (THY), was submitted to three successive thin layer chromatographies: DS-G15 and thymidine were applied on C-plates; the eluates from the Rf 0·75 spots of the C-chromatograms were applied on CM-plates; the eluates from the Rf 0·5 spots of the CM-chromatograms were applied on PEI-plates. Cm = migrated distance in cm.

(a) 50 µl of the aqueous solution of the sample was applied on the plate and developed as described above. Fluorescent spots are encircled; ninhydrin-positive regions, revealed on half-bands, are the black half-spots.

(b) absorbance (A) at 260 nm (dotted line) and the number (U) of units of inhibitory activity on HeLa cells (continuous line) were measured on the eluate from the powder.

C75-CM75-PEI82 and DS-G15-C75-CM75-PEI82.

Thymidine and inhibitor were compared at each step of purification. The u.v. spectra of THY-C75-CM75-PEI82 and DS-G15-C75-CM75-PEI82 are identical (Fig. 3). Equivalent amounts (A^*_{260} × volume) of THY-C75-CM75-PEI82 and DS-G15-C75-CM75-PEI82 have the same inhibitory action on ^{3}H-thymidine incorporation into DNA of HeLa cells (Table 3).

Table 3. Comparison of the inhibitory activity of thymidine and the fractions containing the bovine endometrium main inhibitor taken in the course of purification

Preparation	I.F.
DS–G15	0·42
Thymidine	0·64
DS–G15–C75	0·64
THY–C75	0·68
DS–G15–C75–CM75	0·68
THY–C75–CM75	0·73
DS–G15–C75–CM75–PEI82	0·56
THY–C75–CM75–PEI82	0·58

Equivalent amounts ($\mu l \times A_{260}$) of thymidine or inhibitor solutions were added to the incubation medium containing HeLa cells and ^{3}H-thymidine; the control received an equal amount of water. After 1 hr at 37°C, the DNA specific radioactivities were measured and I.F. values calculated.

(2) *Thymidine content of the endometrium supernatant*

Ten millilitres of 105,000 *g* supernatant were lyophilized. The residue, dissolved in 2·5 ml of water, was placed (211 units) on a 2·5 × 45 cm column of Sephadex G-15 and eluted with water. The absorbance at 280 nm of the collected fractions and their biological activity on ^{3}H-thymidine incorporation into DNA of HeLa cells were measured. Stimulatory factors were separated from 357 units of inhibitory factors. The greater part of the inhibitory factors was in a peak having the position of thymidine (Fig. 4).

(3) *Search for an inhibitor specific for HeLa cells*

The 105,000 *g* supernatant was submitted to fractional precipitation with ethanol. Table 2 shows that fraction I (0–60% ethanol), fraction II (60–87% ethanol), and fraction III (soluble in 87% ethanol) contained inhibitors, but that the inhibitory action of none of these fractions was specific for HeLa cells.

(4) *Action of endometrial supernatant on DNA replication*

HeLa cells (10^7) were incubated in the presence of 4 µCi of ^{3}H-thymidine (6 Ci/mmol) with 0·1 ml of endometrial supernatant in a

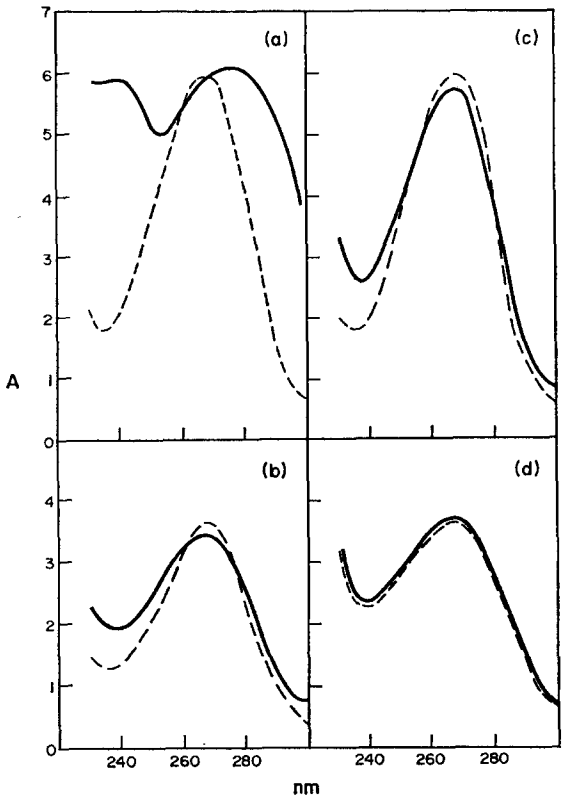


Fig. 3. Comparison of the u.v. spectra of thymidine and of the inhibitory fractions in the course of the purification of the bovine endometrium main inhibitor

The spectra were recorded with the Cary 14R spectrophotometer. The continuous lines give the absorbance (A) of the fractions containing the main inhibitor, while the dotted lines give the absorbance of the thymidine solutions.

(a) *DS-G15 and thymidine*
(b) *DS-G15-C75 and THY-C75*
(c) *DS-G15-C75-CM75 and THY-C75-CM75*
(d) *DS-G15-C75-CM75-PEI82 and THY-C75-CM75-PEI82.*

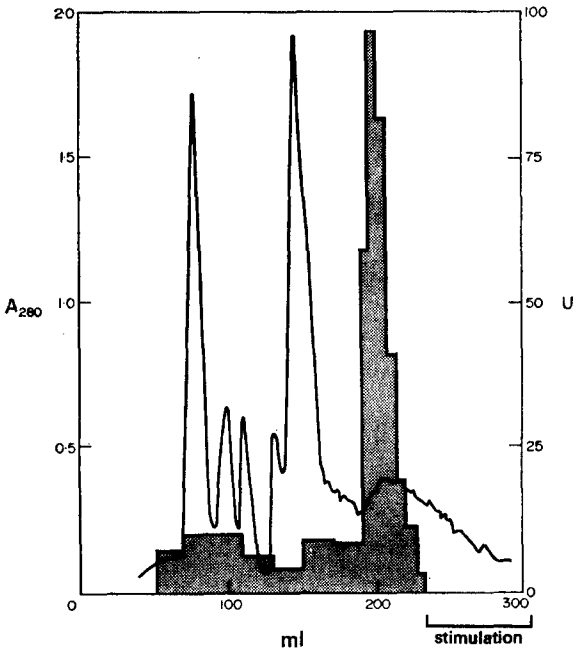


Fig. 4. Chromatography on Sephadex G-15 of the bovin endometrium supernatant

Lyophilized bovine endometrium supernatant (211 units) in 2·5 ml of water was placed on a 2·5 × 45 cm column of Sephadex G-15 and eluted with water. The continuous line gives the absorbance at 280 nm, while the graded line limiting the stippled area gives the number (U) of units of inhibitory activity. There is, after 230 ml, a region containing stimulatory factors. On this column, thymidine is eluted around 200 ml.

total volume of 5 ml. After 1 hr at 37°C, the cells were separated from the incubation medium and treated with TCA.

Analysis on Dowex-50 of the incubation medium showed that the radioactivity was mainly in the position of thymidine; the presence of the endometrial supernatant increased by 25% the amount of ^{3}H-thymidine that was not used by the cells (Fig. 5).

Analysis on Dowex-50 of the TCA-soluble fraction showed that the radioactivity was mainly in the position of the thymidine phosphates. Incubation with the endometrial supernatant strongly decreased this radioactivity (Fig. 5); by analogy with the procedure followed to calculate the inhibition of ^{3}H-thymidine incorporation into DNA, an I.F. of 0·75 can be calculated.

The TCA-insoluble fraction was utilized to estimate the DNA specific radioactivity. The endometrial supernatant was responsible for an I.F. of 0·77.

Human endometrium and myometrium

The 105,000 *g* supernatant from human endometrium was prepared using either method I or method II; a 105,000 *g* supernatant was prepared from human myometrium using only method I. The action of increasing amounts of these supernatants on ^{3}H-thymidine incorporation into DNA of HeLa cells was investigated (Fig. 6). At low doses, containing less than 2 mg of proteins, the endometrial supernatant method I stimulated the incorporation; inhibition appeared only at larger doses. The endometrial supernatant method II was tested only at doses containing more than 2 mg of proteins; its inhibitory effect was similar although lower than that of the endometrial supernatant method I. The myometrial supernatant was mainly stimulatory, although inhibition appeared at doses containing more than 10 mg of proteins.

The same dose (4·6 mg of proteins) of the endometrial supernatant method I was assayed on HeLa, Ehrlich and Novikoff cells. The results are analogous to those obtained with

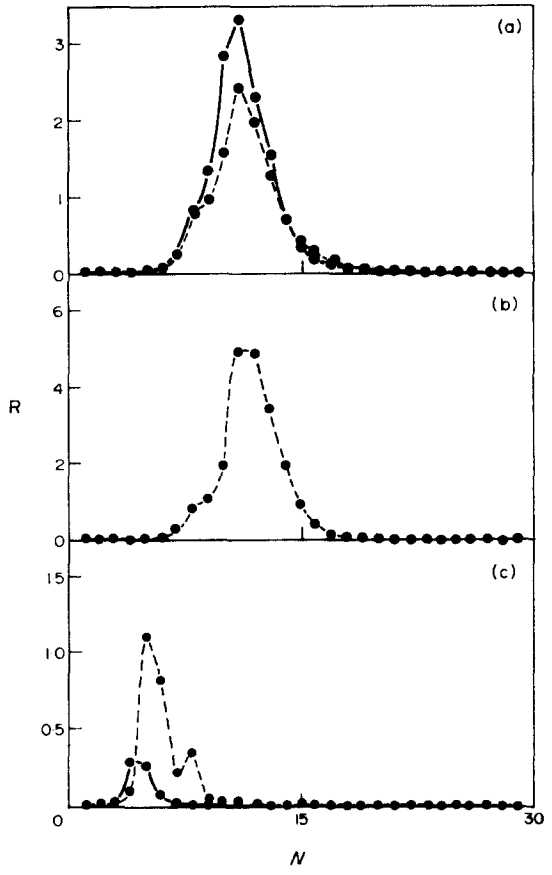


Fig. 5. Action of bovine endometrium supernatant on ^3H-thymidine uptake by HeLa cells and on the radioactivity of the intracellular thymidine phosphates

HeLa cells were incubated in the presence of ^3H-thymidine for 1 hr at 37°C with bovine endometrium supernatant (3·4 units) and a control without supernatant. A 1-ml cliquot of the incubation medium (a) or of the cellular TCA-soluble fraction (c) were chromatographed on Dowex-50 and the radioactivity measured in the eluted fractions. The continuous lines correspond to the incubation with the supernatant and the dotted lines to the control. A chromatography with commercial ^3H-thymidine is presented in (b); note the impurity eluted before the thymidine peak. N = fraction number; R = dis/min × 10^{-5}.

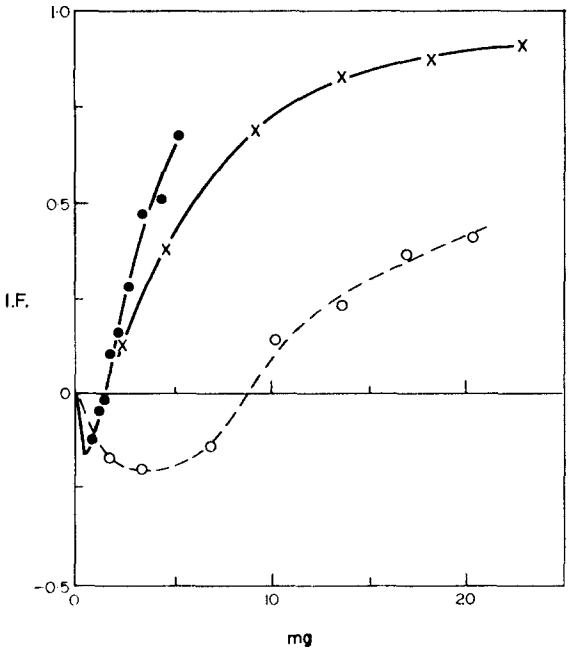


Fig. 6. Action of human endometrium and myometrium supernatants on ^3H-thymidine incorporation into DNA of HeLa cells

I.F. is given as a function of the amount of supernatant (mg of proteins). ●, endometrial supernatant method I; ×, endometrial supernatant method II; ⊙, myometrial supernatant method I.

bovine endometrial supernatant: the inhibition of ^{3}H-thymidine incorporation into DNA was observed with the three kinds of cells, although the effect was more important with HeLa cells (Table 2).

To see whether human endometrial and myometrial supernatants contained thymidine, they were chromatographed on Dowex-50; a trace amount (0·04 μCi) of purified ^{3}H-thymidine* was added to locate the elution peak of this nucleoside. The collected fractions were assayed for radioactivity and for their action on HeLa cells. Figure 7 shows that, when the endometrial supernatant method I was chromatographed, the greater part of the inhibitory activity migrated with thymidine. By contrast, no inhibitory activity could be eluted from the Dowex-50 when the endometrial supernatant method II or the myometrial supernatant method I were chromatographed.

^{3}H-thymidine was incubated for 1 hr at 37°C with the endometrial (method I or II) or myometrial (method I) supernatants, then chromatographed on Dowex-50. In every case, the peak of radioactivity was no longer in the position of thymidine; ^{3}H-thymidine had been converted into ^{3}H-thymine (Fig. 8).

Thymidine

The action of increasing amounts of unlabelled thymidine on the ^{3}H-thymidine incorporation into DNA was studied with HeLa, Ehrlich and Novikoff cells. Figure 9 shows a linear relationship between 1/(1–I.F.) and the amount of unlabelled thymidine added. The slopes are the same for HeLa and Ehrlich cells, but it is 2·3 times smaller with Novikoff cells.

*Commercial ^{3}H-thymidine very often contains an impurity which is eluted from Dowex-50 immediately before the thymidine peak. This impurity can be seen in Fig. 5(b). Purified ^{3}H-thymidine is prepared from commercial ^{3}H-thymidine by taking only the fractions corresponding to thymidine after a chromatography on Dowex-50.

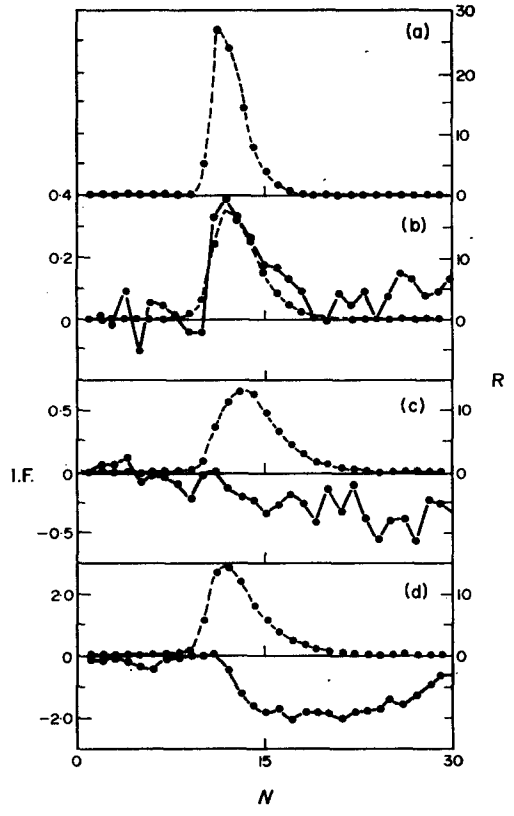


Fig. 7. Dowex-50 chromatographies of human endometrium and myometrium supernatants.

The lyophilized supernatants were taken in 2 ml of water; after removal of the insoluble material by centrifugation, 0·04 μCi of purified ^{3}H-thymidine was added to the clear solution which was placed on a 1 × 26 cm column of Dowex AG 50W X2 at 4°C. The elution was performed with 0·1 M ammonium formate buffer, pH 3·2, and 3 ml fractions were collected; 0·5 ml was used to determine the radioactivity (dotted line), while the remaining 2·5 ml were lyophilized and the residue taken in 1 ml of Hanks' solution for the assay on HeLa cells (I.F.; continuous line). N = fraction number; R = dis/min × 10^{-3}. (a) purified ^{3}H-thymidine alone; (b) endometrium supernatant method I (42 mg of proteins); (c) endometrium supernatant method II (92 mg of proteins); (d) myometrium supernatant method I (46 mg of proteins).

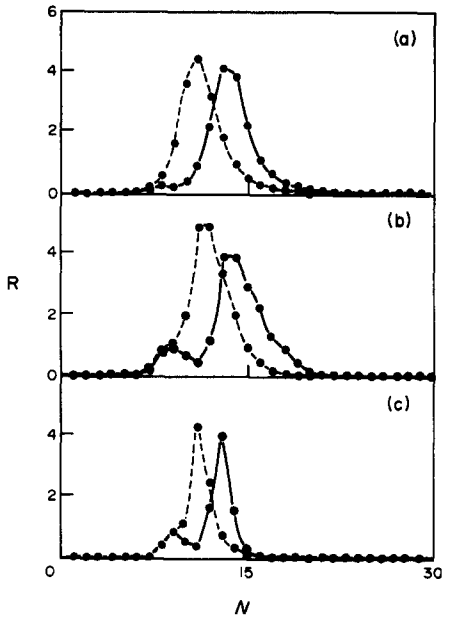


Fig. 8. Action on thymidine of human endometrium and myometrium supernatants.

The supernatant (1 ml) was added to 4 ml of Hanks' solution containing 4 μCi of ^{3}H-thymidine. After 1 hr of incubation at 37°C, the solutions were cooled at 4°C and 1 ml aliquots were chromatographed on Dowex AG 50W X2 (continuous line). Controls of ^{3}H-thymidine incubated without supernatant were run on the same column (dotted line). N = fraction number; R = dis/min × 10^{-5}. (a) endometrial supernatant method I (6·9 mg of proteins); (b) endometrial supernatant method II (13·8 mg of proteins); (c) myometrial supernatant method I (36·0 mg of proteins).

DISCUSSION

To have enough material to isolate the inhibitor, we first used bovine endometrium. A supernatant was prepared following the method of Hinderer, Volm and Wayss [3]: the homogenate was lyophilized, the resulting powder was extracted with water and the extract centrifuged at 105,000 *g* for 150 min. Bovine endometrium supernatant inhibits the incorporation of ^{3}H-thymidine into DNA of HeLa cells, but it is also active on Ehrlich and Novikoff cells.

To purify the inhibitor, the supernatant was first dialyzed: more than 70% of the recovered inhibitory activity was found in the dialysate. When chromatographed on Sephadex G-15,

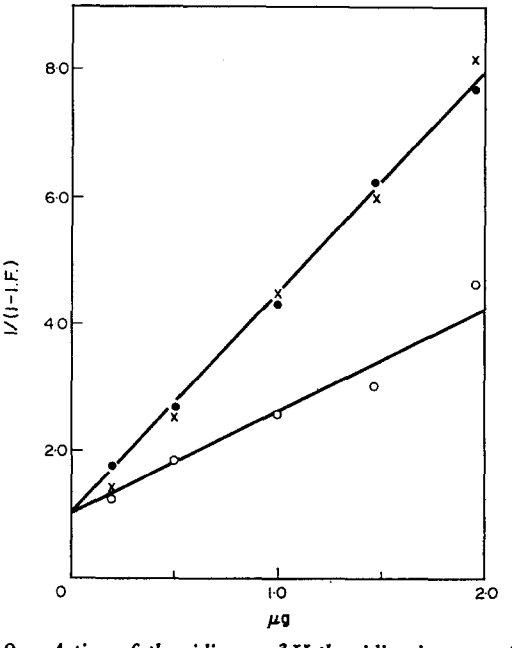


Fig. 9. Action of thymidine on ^{3}H-thymidine incorporation into DNA of HeLa, Ehrlich and Novikoff cells.

Increasing amounts of unlabelled thymidine were added to cells incubated with 4 μCi of ^{3}H-thymidine (6 Ci/mmol) in a total volume of 5 ml. After 15 min at 37°C, the DNA specific radioactivity was determined. The graph gives 1/(1–I.F.) against the amount (μg) of unlabelled thymidine. ●, HeLa cells; ×, Ehrlich cells; ⊙, Novikoff cells.

the dialysable fraction gave a single peak of inhibitory activity with a $V_e/V_0 = 2{\cdot}7$, i.e., in the elution position of thymidine. Further purification of the dialysable inhibitor in three successive thin layer chromatographies showed that it was indeed thymidine: the spectrum of the pure inhibitor was identical to that of thymidine; equivalent amounts ($A_{260} \times$ volume) of inhibitor and thymidine had the same action on ^{3}H-thymidine incorporation into DNA of HeLa cells.

The supernatant of bovine endometrium was directly chromatographed on Sephadex G-15 to determine what part of its inhibitory activity was due to thymidine: 211 inhibitory units were placed on the column; stimulatory factors were separated from 357 units of inhibitory factors of which 78% were in the position of thymidine.

An attempt to separate an inhibitor specific for HeLa cells was made by fractional precipitation of the supernatant with ethanol. Three fractions (0–60%; 60-87%; soluble in 87%) were examined: none was specific for HeLa cells.

Because our results with bovine endometrium seemed to differ from those of Hinderer *et al.* [3] with human endometrium, we decided to repeat the work with human tissues. Supernatants of human endometrium and myometrium were prepared following the procedure of these authors. In agreement with them, we found that, at the doses they used, the endometrial supernatant inhibited while the myometrial supernatant stimulated the incorporation of ^{3}H-thymidine into DNA of HeLa cells. However, our endometrial supernatant was also inhibitory for Ehrlich and Novikoff cells; the relative effects were exactly the same as with the supernatant of bovine endometrium.

Using Dowex-50 chromatography, the human endometrial supernatant was found to contain thymidine which was absent from the human myometrial supernatant. Moreover, the human endometrial supernatant contained an enzyme that hydrolyzed thymidine into thymine. The human myometrial supernatant also possessed this enzymic activity and, indeed, this supernatant was inhibitory at high doses; it is likely that, at high doses, the destruction of ^{3}H-thymidine was more important than the nutritive effect of the extract.

The presence of thymidine in liver extract has been reported by Lenfant *et al.* [10]. The occurrence of thymidine in endometrial but not in myometrial supernatant could be explained by the fact that endometrium possesses more cell nuclei per unit weight than myometrium and that the cell turnover is high in the endometrium so that this tissue might contain necrotic cells. It was, however, still possible that thymidine was a preparation artefact: the beef liver homogenate of Lenfant *et al.* [10], which was heated at 50°C before addition of ethanol, contained thymidine, while the rabbit liver homogenate of Deschamps and Verly [7], which was kept at 4°C during all the manipulations, did not contain this nucleoside. To investigate this point, a human endometrial supernatant was prepared in a more direct way: the homogenate was immediately centrifuged at 105,000 *g*. This supernatant (method II) had a similar but lower effect on ^{3}H-thymidine incorporation into DNA of HeLa cells than the supernatant prepared according to the method of Hinderer *et al.* [3]; both supernatants contained an enzymatic activity that destroyed ^{3}H-thymidine, but thymidine was not found in supernatant method II. Thymidine is thus a preparation artefact of method I.

The salvage pathway, which utilizes exogenous thymidine in these *in vitro* experiments, is a minor route compared to the endogenous synthesis of thymidine phosphates. Deschamps and Verly [7] calculated that, with regenerating liver slices and in their experimental conditions, less than 1/5000 of the thymine in the synthesized DNA derived from the ^{3}H-thymidine of the incubation medium. A decrease of ^{3}H-thymidine incorporation into DNA is not necessarily indicative of an inhibition of DNA synthesis.

Various causes can decrease ^{3}H-thymidine incorporation into cellular DNA: (1) presence of thymidine in the extract which lowers the specific radioactivity of the labelled precursor in the incubation medium [10]; (2) addition of enzymes which convert thymidine into thymine [11–14] that can no longer be used by the cell [15]; (3) decrease of the plasma membrane permeability to thymidine or decrease of the phosphorylation of this deoxynucleoside [8]; (4) variation in the size of the intracellular thymidine phosphate pool; (5) decrease of the conversion of the thymidine phosphates into DNA. Only the latter cause corresponds to an inhibition of DNA synthesis. To demonstrate that it is really the case, it is necessary to measure the specific radioactivity of the deoxynucleoside phosphates: one is then in a position to calculate the amount of DNA synthesized from the radioactivity incorporated into this macromolecule. Using this technique, Deschamps and Verly [7] showed that their purified liver inhibitor decreased DNA

synthesis in regenerating liver slices. A totally different situation was observed with bovine endometrium supernatant and HeLa cells: when incubated with ^{3}H-thymidine in the presence of the supernatant, the radioactivity in the cellular thymidine phosphates was decreased by the same factor as the radioactivity in DNA. It must be concluded that the rate of DNA synthesis remained unchanged: if the endometrial supernatant inhibits ^{3}H-thymidine incorporation into DNA, it has no action whatsoever on DNA synthesis in HeLa cells.

The effect of the addition of unlabelled thymidine to the incubation medium on ^{3}H-thymidine incorporation into DNA was studied with HeLa, Ehrlich and Novikoff cells. The incubation medium contained 0·16 μg of ^{3}H-thymidine; 0·27 μg of unlabelled thymidine had to be added to have a 50% reduction of tritium incorporation into DNA with HeLa and Ehrlich cells, and 0·62 μg with Novikoff cells. The dilution factors are respectively 2·7 and 4·9; Lenfant *et al.* [10] found a factor of 5·8 with regenerating liver slices and one much greater with kidney slices. These differences can be interpreted wrongly as the result of the action of a tissue-specific inhibitor. HeLa cells are, among the biological systems so far studied, one of the most sensitive to the inhibition, by unlabelled thymidine, of ^{3}H-thymidine incorporation into cellular DNA.

Thymidine has the same action on HeLa and Ehrlich cells, while bovine or human endometrial supernatants were more active on HeLa cells than on Ehrlich cells. But we have seen that thymidine is not the only non-specific inhibitor in these supernatants: human endometrium also contains enzymes that destroy the ^{3}H-thymidine of the incubation medium; moreover stimulatory factors are present in those crude preparations.

We are thus unable to confirm the conclusion of Hinderer, Volm and Wayss [3] that the factor in endometrial extract which inhibits thymidine incorporation into HeLa cell DNA is a chalone. Indeed, these authors observed later [4] that their crude endometrial extract had no tissue specificity since it was also active on human fetal liver and kidney cells; Volm and Wayss [16] even came to the conclusion that the chalone concept, developed for the epidermis, could perhaps not be extended to other tissues. This is likely too pessimistic; it might well be that HeLa cells are just not the right cells to use to discover the endometrial chalone. Besides, two kinds of chalones have been described: those interfering with S phase and those inhibiting mitosis; the assay used in the work of Hinderer *et al.* [3] and in our work would only enable to find an inhibitor of DNA replication.

REFERENCES

1. P. Weiss, *In Biological Specificity and Growth*, G. Butler, Princeton University Press, Princeton (1955).
2. W. S. Bullough, C. O. Hewett and E. B. Laurence, *Exp. Cell Res.* **36,** 182 (1964).
3. H. Hinderer, M. Volm and K. Wayss, *Exp. Cell Res.* **59,** 464 (1970).
4. K. Wayss and M. Volm, *Verh. dtsch. Ges. Path.* **54,** 496 (1970).
5. W. C. Schneider, *Methods in Enzymology.* (Edited by S. P. Colowick and N. O. Kaplan) Vol. III, p. 680. Academic Press, New York (1957).
6. W. G. Verly, Y. Deschamps, J. Pushpathadam and M. Desrosiers, *Canad. J. Biochem.* **49,** 1376 (1971).
7. Y. Deschamps and W. G. Verly, *Biomedicine*, In press (1975).
8. G. Nilsson, *Exp. Cell Res.* **59,** 207 (1970).
9. O. H. Lowry, N. J. Rosebrough, A. F. Farr and R. T. Randall, *J. biol. Chem.* **193,** 265 (1951).
10. M. Lenfant, L. Kren-Proschek, W. G. Verly and H. Dugas, *Canad. J. Biochem.* **51,** 654 (1973).
11. M. Friedkin and D. Roberts, *J. biol. Chem.* **207,** 245 (1954).
12. S. Perry and J. C. Marsh, *Proc. Soc. exp. Biol. (N.Y.)* **115,** 51 (1964).
13. W. C. Dewey, R. M. Humphrey and B. A. Dedeta, *Exp. Cell Res.* **80,** 349 (1968).
14. M. Miyamoto and H. Terayama, *Biochim. Biophys. Acta. (Amst.)* **228,** 324 (1971).
15. A. A. Plentl and R. Schoenheimer, *J. biol. Chem.* **153,** 203 (1944).
16. M. Volm and K. Wayss, *Naturwissenschaften* **58,** 458 (1971).

Europ. J. Cancer Vol. 11, pp. 669–673. Pergamon Press 1975. Printed in Great Britain

The Effect of Mouse Embryo Fibroblasts on the Binding of Carcinogenic Polycyclic Hydrocarbons by Neoplastic L Cells

I. M. BUJURINA, L. A. MITTELMAN and M. A. PANOV

Laboratory of Mathematical Methods in Biology, Moscow State University, Moscow, U.S.S.R.

Abstract—*Cocultivation of normal mouse fibroblasts and neoplastic L fibroblasts in a mixed culture leads to accumulation of ^{3}H-benzo(a)pyrene (^{3}H-BP) metabolites in L cells. These metabolites most probably are transferred directly into L cells from normal cells by passing the pool of ^{3}H-BP metabolites in culture medium. Accumulation of firmly-bound ^{3}H-BP metabolites in the mixed culture proceeds more slowly than in a pure culture of normal fibroblasts. This decline of accumulation rate of firmly-bound ^{3}H-BP metabolites in the mixed culture as compared to pure culture may be indicative of an influence of L cells upon normal ones.*

INTRODUCTION

CARCINOGENIC polycyclic hydrocarbons, *e.g.*, 7,12-dimethylbenzo(α)anthracene (DMBA) and benzo(α)pyrene (BP) are known to be toxic for fibroblasts of rodents [1]. The toxic effect depends upon the metabolism of these carcinogens within the cells and is due to formation of toxic metabolites in the process of oxidation of the hydrocarbons [2–4]. When normal fibroblasts are cultured with added carcinogenic hydrocarbons, large quantities of metabolites firmly bound to macromolecules (possibly by covalent bonds) accumulate both in the cells and culture medium [5]. Many lines of neoplastic fibroblasts, including L cells, are resistant toward the toxic action of carcinogenic hydrocarbons; concomitantly these cells metabolize the hydrocarbon much less effectively [6–9]. In all probability, this is due to diminished activity of microsomal oxidases metabolizing polycyclic hydrocarbons in tumour cells [3, 10]. In a previous paper [11] it was shown that DMBA is toxic for L cells growing in direct contact with normal fibroblasts. Similar results have been obtained by other authors [12]. In this connection it was suggested that DMBA metabolites, produced in normal fibroblasts, are transferred to L cells where they exert their toxic action. The present paper shows that L cells, growing in contact with normal fibroblasts, accumulate firmly-bound metabolites of BP at the same time, the amount of these metabolites accumulated by normal fibroblasts diminishes, and the metabolism of BP is qualitatively altered.

Accepted 21 March 1975.

METHODS

Two types of cells were used: normal mouse embryo fibroblasts (1st passage) and neoplastic mouse fibroblasts of L line. The sources of cells and conditions of cultivation have been described earlier [11]. Normal cells were seeded in small (100 ml) glass Pavitski flasks ($4 \cdot 10^6$ cells per flask). Twenty four to 48 hr later, $8 \cdot 10^6$ L cells were seeded in some of these flasks on the surface of a confluent monolayer of normal cells ("mixed culture"). Other flasks with normal cells were used as controls as well as cultures of $8 \cdot 10^6$ per flask of L cells seeded on glass. Later, control cultures will be designated as "pure" so as to distinguish them from

"mixed" ones. When the culture medium was changed 24 hr after the seeding of L cells, ^{3}H-BP at a final concentration of 0·01 μg/ml was added to all flasks (Amersham, spec. act. 25 Ci/mM). When incubation with the carcinogen was accomplished, the culture medium was collected, the cell monolayer rinsed with a cold Hanks solution and incubated for 1 hr in Hanks solution at 4°C to remove extracellular ^{3}H-BP [13]. Cells of the pure cultures of both the normal fibroblasts and the L line were detached from the glass with 0·2% trypsin at 37°C. In the mixed cultures L cells and normal fibroblasts were harvested separately. The cell monolayer was rinsed with a cold trypsin solution; 60–90 sec later a cold Hanks' solution with 10% bovine serum was added and the L cells were detached from the surface of the normal monolayer by gentle agitation. Detachment of L cells was checked microscopically. A possible admixture of normal fibroblasts in the L cells in the suspension thus obtained was measured by counting normal and L cells separately in a cell counting chamber no longer than 1–2 min after preparation of the suspension. In a preliminary experiment it was found that this period is sufficient for 90% of the L cells detached from the glass to become rounded, whereas not more than 10% of normal fibroblasts were rounded within this interval. Counts accomplished on blind preparations of the L cell suspensions detached from the surface of normal fibroblasts showed that the admixture of the latter does not exceed 10%. The normal fibroblasts remaining on the glass surface after the L cells had been detached were harvested after trypsin treatment at 37°C.

The content of BP metabolites in different fractions of both cells and culture medium was determined as described earlier [5]. Cells were collected from Hanks' solution by centrifugation at 2000 ***g***. Cell pellets and aliquots of the medium were extracted with 3 volumes of ethanol and the precipitates formed were washed twice with methanol: chloroform (1:2) for complete extraction of unbound BP metabolites. The washed precipitates containing firmly bound BP metabolites were dissolved in Hyamine 10X and the radioactivity was measured in a Mark II liquid scintillation spectrometer. Some aliquots of the medium were precipitated by 5% trichloroacetic acid and the radioactivity of the precipitate was determined after it was dissolved in a mixture of Hyamine 10X and Triton X100. Accumulation of water soluble metabolites in the culture medium was assessed as a difference in the radioactivity of acid-insoluble fractions of the fresh medium and the conditioned one [13]. The amount of non-metabolized BP in the culture medium was measured as radioactivity of the n-octane extract of the medium [14].

RESULTS

The accumulation of strongly-bound BP metabolites 24 hr after addition of ^{3}H-BP in the pure culture of L cells appeared to be 25–70 times lower than in the pure culture of normal fibroblasts. The results of one experiment are shown in Table 1. The data obtained demonstrate differences in the accumulation of firmly-bound BP metabolites between the cultures of normal and transformed cells. This is in agreement with the data of other authors [5]. The amount of firmly-bound metabolites accumulated by L cells in the mixed cultures is 6–10-fold higher as compared with the L cells in the pure cultures (Table 1). Accumulation of these metabolites by the L cells of the mixed culture is only 2–4 times lower than by normal fibroblasts of the same culture. It should be noted that normal fibroblasts in the mixed culture bind firmly 1·5–3 times less BP metabolites than in pure culture. The differences in accumulation of firmly-bound metabolites between the cells of pure and mixed cultures became apparent as early as 1 hr after the addition of ^{3}H-BP.

The amount of metabolites of carcinogenic hydrocarbons leaving the cells for the culture medium is known to exceed markedly their amount in the cell monolayer [13]. Therefore, the data on accumulation of metabolites of carcinogens in the medium can be used for evaluating the ability of the cells to metabolize carcinogens. In Table 1 data on the accumulation of ^{3}H-BP metabolites in the culture medium of pure and mixed cultures of both normal fibroblasts and L cells are presented. Proteins in the medium of pure culture of L cells bind 5–20 times less metabolites firmly than proteins in the medium of pure culture of normal fibroblasts. In the medium of mixed cultures, a lower amount of firmly-bound BP metabolites' accumulates than in the medium of pure culture of normal fibroblasts. The clearance rate of non-metabolized BP as well as the rate of accumulation of water-soluble BP metabolites in mixed and pure cultures did not differ.

Table 1. *^{3}H-BP metabolism in pure and mixed cultures of normal fibroblasts and L cells*

	Amount of firmly bound metabolites in cells (dis/min × 10^{-3} per 10^6 cells)		Amount of BP metabolites in medium				Amount of non-metabolized BP in medium (dis/min × 10^{-6}/flask)	
			Firmly bound dis/min × 10^{-6}/flask		Water soluble dis/min × 10^{-6}/flask			
	1 hr	24 hrs	1 hr	24 hrs	1 hr	24 hrs	1 hr	24 hrs
Pure cultures of normal fibroblasts	27 ± 4*	71 ± 3	0	1·56 ± 0·02	0	5·2 ± 0·3	31·9 ± 0·6	8·0 ± 0·6
Pure cultures of L cells	1·0 ± 0·2	1·0 ± 0·2	0	0·18 ± 0·03	0	1·9 ± 0·5	34·0 ± 0·2	31·3 ± 0·5
Normal fibroblasts of mixed cultures	18 ± 2	45 ± 4	0	1·26 ± 0·08	0	5·1 ± 0·5	31·5 ± 0·8	7·7 ± 0·3
L cells of mixed cultures	5·3 ± 0·7	10 ± 2						

*Mean of triplicate determination ± S.D.

Table 2. *Time course of ^{3}H-BP metabolism in pure and mixed cultures of normal fibroblasts and L cells*

	Amount of firmly bound metabolites in cells (dis/min × 10^{-3}/flask)			Amount of BP metabolites in medium						Amount of non-metabolized BP in medium (dis/min × 10^{-6}/flask)		
				Firmly bound (dis/min × 10^{-3}/flask)			Water soluble (dis/min × 10^{-6}/flask					
Time of incubation with BP (hr)	1	8	24	1	8	24	1	8	24	1	8	24
Pure cultures of L cells	6·6* (0·2)	7·7 (0·1)	11·9 (0·9)	0	0	85 (10)	0	1·8 (0·8)	1·6 (0·6)	35·3 (0·4)	34·0 (0·5)	33·4 (0·7)
Pure cultures of normal fibroblasts	50 (14)	175 (16)	207 (7)	100 (34)	804 (12)	1895 (9)	0	3·0 (0·7)	5·8 (0·4)	33·8 (1·2)	20·6 (0·7)	8·8 (0·8)
Mixed cultures	68·4 (0·4)	180 (33)	163 (7)	57 (31)	614 (60)	1290 (260)	0	2·8 (0·6)	5·4 (0·6)	34·1 (0·5)	18·5 (0·4)	7·7 (2·1)

*Mean of triplicate determination ± S.D. (in parentheses).

Accumulation of firmly-bound BP metabolites decreased as early as several hours after the beginning of cultivation with the carcinogen. An experiment illustrated in Table 2 was designed to investigate the time course of ^{3}H-BP metabolism in mixed and pure cultures of both normal fibroblasts and L cells. In this experiment all cells of mixed culture were harvested together; therefore the amount of firmly-bound metabolites per cell was not calculated. During 8 hr of cultivation with BP the same amount of firmly-bound metabolites accumulated in the cell layer of both mixed culture and pure culture of normal fibroblasts. However, the amount of firmly-bound BP metabolites in the medium of mixed culture is 25% lower than in the pure culture of normal fibroblasts, whereas the amount of non-metabolized carcinogen in the medium of a mixed culture is the same as (or less than) that in the medium of a pure culture of normal fibroblasts. During 24 hr of cultivation with BP the cell layer of a mixed culture accumulates significantly less firmly-bound metabolites than normal fibroblasts in pure culture of normal cells. This difference reflects a decrease of the accumulation of firmly bound BP metabolites in normal fibroblasts of the mixed culture described above. It is to be noted that the number of normal fibroblasts was equal in pure and mixed cultures.

In order to decide whether the ability of L cells to accumulate firmly-bound BP metabolites persists after their separation from normal fibroblasts, the following experiments were performed. A mixed culture of normal and L cells was grown for 72 hr, L cells were then detached from their normal counterparts by the method described above. The suspension of L cells thus obtained was seeded in culture flasks after addition of ^{3}H-BP. As a control, L cells without previous contact with BP were

seeded similarly. After 24 hr of cultivation all cells were harvested and the quantity of firmly bound BP metabolites accumulated by the cells was measured. The L cells incubated with BP after 72 hr of cultivation together with normal fibroblasts contained the same quantity of firmly-bound BP metabolites as the L cells without previous contact with normal cells. In both variants of cultivation, the L cells did not metabolize BP, nor did they accumulate its firmly-bound metabolites in the culture medium.

DISCUSSION

The results of these experiments have shown that, unlike the situation is pure culture, L cells grown in a mixed culture with normal fibroblasts are able to accumulate significant amounts of firmly-bound BP metabolites. Transformed cells are known to be incapable of metabolizing aromatic hydrocarbons. It is quite probable that accumulation of these metabolites in L cells reflects their transfer from normal to transformed cells. Apparently, this transfer is not realized through the culture medium, as the concentration of firmly-bound metabolites in the medium is much less than that in the cellular layer. In all probability, the BP metabolites able to form stable complexes with macromolecules are transferred directly from the normal fibroblasts, where they were produced, to the L cells. It is a probable mechanism of toxic action of carcinogenic hydrocarbons upon L cells in mixed cultures [11]. The presence of highly permeable contacts between normal and L cells in mixed cultures [15] suggests the existence of a continuous hydrophobic phase between these cells. Along these intercellular hydrophobic bridges, metabolites of carcinogenic hydrocarbons, insoluble in water, may be transferred from cell to cell. After separation from normal fibroblasts, L cells lose their ability to accumulate firmly-bound BP metabolites.

Consideration of the metabolism of carcinogenic hydrocarbons in mixed culture shows that, not only do the normal cells influence the transformed cells but the latter also influence the normal cells. This is evident mainly from decreased accumulation of firmly bound BP metabolites by normal fibroblasts in mixed cultures. This decrease is due possibly to the efflux of metabolites from the site of their formation in normal cells into L cells. This explanation is, however, not sufficient since the total amount of firmly-bound metabolites in the cell layer of a mixed culture is significantly lower than that in the layer of normal fibroblasts in a pure culture (Table 2). Decreased accumulation of firmly-bound BP metabolites in normal fibroblasts due to contact with L cells is apparently a consequence of altered BP metabolism in mixed cultures. In fact, the rate of accumulation of firmly-bound BP metabolites in the mixed culture was lower than that in the pure culture of normal fibroblasts (Tables 1, 2), whereas the number of normal cells in both cultures was the same. The total rate of BP metabolism assessed as clearance rate of radioactivity from octane extracts in mixed culture and in pure culture of normal cells is equal. The rate of accumulation of water soluble BP metabolites in these two types of cultures is also equal. Thus the change of accumulation of firmly-bound BP metabolites in mixed culture may reflect qualitative alterations of BP metabolism by normal fibroblasts growing in contact with L cells. Specifically, these alterations may be due to changes in the character of BP epoxides formed in normal cells as immediate precursors of firmly-bound metabolites [16].

The nature of the effect of L cells upon normal ones is not clear. It cannot be excluded that BP metabolism in normal fibroblast is altered due to its contact with numerous L cells, i.e. a process similar to contact inhibition takes place. On the other hand, one can suggest that some substances capable of changing the character of BP metabolism flow through contacts from L cells into normal fibroblasts. To choose between these and other possibilities, it is necessary to investigate other aspects of metabolism both in normal and L cells in mixed culture.

REFERENCES

1. L. A. Andrianov, G. A. Belitsky, Yu. M. Vasiliev, L. V. Domnina, O. Yu. Ivanova, L. V. Olshevskaya, M. A. Panov, I. L. Slavnaya and A. Ya. Khesina, The action of carcinogenic hydrocarbons on cells. In *Medicine II*, **69**. (Edited by L. M. Shabad) Moscow, Moscow (1971).
2. L. N. Andrianov, G. A. Belitsky, O. Ju. Ivanova, A. Ya. Khesina, S. S. Khitrovo, L. M. Shabad and Ju. M. Vasiliev, Metabolic degradation of 3,4-benzopyrene in the cultures of normal and neoplastic fibroblasts. *Brit. J. Cancer* **21**, 566 (1967).

3. L. DIAMOND, C. SARDET and G. H. ROTHBLAT, The metabolism of 7,12-dimethylbenz(a)anthracene in cell cultures. *Int. J. Cancer* **3,** 838 (1968).
4. H. V. GELBOIN, E. HUBERMAN and L. SACHS, Enzymatic hydroxylation of benzopyrene and its relationship to cytotoxicyty. *Proc. nat. Acad. Sci. (Wash.)* **64,** 1188 (1969).
5. M. A. PANOV and O. YU. SAKHAROVA, Metabolism of ^{3}H-benzo(a)pyrene in murine embryo cell cultures. *Biol. nauki.* **2** (1971).
6. V. B. STARIKOVA and J. M. VASILIEV, Action of 7,12-dimethylbenz(alpha)-anthracene on the mitotic activity of normal and malignant rat fibroblasts *in vitro*. *Nature (Lond.)* **195,** 42 (1962).
7. J. BERWALD and L. SACHS, *In vitro* cell transformation with chemical carcinogens. *Nature (Lond.)* **200,** 1182 (1963).
8. L. J. ALFRED, A. GLOBERSON, J. BERWALD and R. T. PREHN, Differential toxicity response of normal and neoplastic cells *in vitro* to 3,4-benzopyrene and 3-methylcholantrene. *Brit. J. Cancer* **18,** 159 (1964).
9. L. DIAMOND, Effect of carcinogenic hydrocarbons on rodent and primate cells *in vitro*. *J. cell comp. Physiol.* **66,** 183 (1965).
10. D. W. NEBERT and H. V. GELBOIN, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. II. Cellular response during enzyme induction. *J. biol. Chem.* **243,** 6250 (1968).
11. L. A. MITTELMAN, JU. JU. SHAROVSKAYA and JU. M. VASILIEV, Toxic effect of 7,12-dimethylbenz(a)anthracene on neoplastic cells grown in mixed cultures with normal fibroblasts. *Int. J. Cancer* **10,** 667 (1972).
12. H. MARQUARDT and C. HEIDELBERGER. Influence of feeder cells and inducers and inhibitors of microsomal mixed-function oxidases on hydrocarbon-induced malignant transformation of cells derived from mouse prostate. *Cancer. Res.* **32,** 721 (1972).
13. I. M. BUZHURINA, YU. M. VASILIEV, M. A. PANOV and O. YU. SAKHAROVA, Binding of H^3-benz(a)pyrene with proteins and nucleic acids in murine embryo cell cultures. *Cytologia* **13,** 1256 (1971).
14. G. A. BELITSKY and A. YU. KHESINA, Metabolism of some polycyclic hydrocarbons in cultures of embryo and malignant cells of different species of mammals. In *Tissue Culture in Oncology*. (Edited by N. N. BLOCHIN) 211. Proceedings of the Institute of Experimental and Clinical Oncology of the Academy of Medical Sciences of the U.S.S.R., Moscow, in Russian (1968).
15. L. JU. BOITSOVA, S. A. KOVALEV, L. A. MITTELMAN, T. V. POTAPOVA, E. N. RIVKIN, L. M. CHAILAKHYAN and JU. JU. SHAROVSKAYA. Comparative study of permeability of plasmatic and contact membranes of normal and transformed fibroblasts in cell culture. In *Biophysics in Membranes* (Edited by Z. A. JANUSHKEVICHUS) 175. Proceedings of the Symposium on Membrane Biophysics, Materials of the symposium, Kaunas, in Russian (1971).
16. N. KINOSHITA, B. SHEARS and H. V. GELBOIN, K-region and non-K-region metabolism of benzo(a)pyrene by rat liver microsomes. *Cancer Res.* **33,** 1933 (1973).

Europ. J. Cancer Vol. 11, pp. 675–676. Pergamon Press 1975. Printed in Great Britain

Letter to the Editor

Preliminary Pharmacological Studies with 1,6-dimesyl-2,3-4,5-dianhydro-L-iditol in Patients with Cancer

J. CSETÉNYI, V. BIHARI, SZ. I. SZÁMEL, I. P. HORVÁTH and S. ECKHARDT
National Institute of Oncology, XII.Ráth György-u 7–9, 1525 *Budapest, Hungary*

DMDAI (1,6-dimesyl-2,3-4,5-dianhydro-L-iditol) is a new alkylating agent with a relatively low toxicity [1]. It has been shown to exert a pronounced inhibitory action on a broad spectrum of transplantable tumors [2]. Its effect on bone marrow stem cells is moderate [3]. DMDAI has already entered the first phase of clinical testing, and in connection with such investigations the purpose of our study was to provide data on absorption, excretion and metabolism of the drug in patients.

Seven hospitalized subjects with advanced cancer were selected for our trial. DMDAI-1-^{3}H (specific activity: 1 mCi/mM) triturated with non-radioactive DMDAI was administered orally in a single therapeutic dosage of 3·7 mg/kg to each patient. All medications were stopped at least one week prior to study.

Heparinized venous blood samples and excreted urine were collected at 0, 0·5, 1, 2, 4, 8, 12, 24, 48 and 72 hr after dosing. Duplicate samples from whole blood and its components were assayed in liquid scintillation spectrometer (LKB–Wallac 18000). Urinary metabolites extracted with ethylacetate were separated by thin layer chromatography (Silica Gel G; Solvent system: ethylacetate saturated with water), then subjected to radioassay. Alkylating compounds were located by 4-/p-nitrobenzyl/-pyridine (NBP) reaction.

Our results indicated that radioactivity was detectable in whole blood within 1 hr after the oral treatment in all cases. Peak levels at about 4 hr were followed by slow decline (Fig. 1). The terminal increase of the curves, ensuing from 48 to 72 hr, might be due to the accumulation of ^{3}H compounds in blood cells. This suggestion is supported by the distribution patterns of radioactivity between RBC and plasma (Fig. 2). Plasma level curves of radioactivity showed that the mean half-life of absorption was 0·9 hr. Peak values were obtained at about 4 hr after dosing, then the curves declined in 2 distinct phases with half-life ranges of 3·1 to 9 hr and 20 to 27·4 hr, respectively.

In urine, 63 to 72% of the administered ^{3}H dose was recovered within 72 hr and DMDAI as well as 5 metabolites could be separated. Four of these metabolic products showed alkylating abilities (Table 1). Their distribution in time (Fig. 3) demonstrated that only a minimal amount of unchanged drug was removed by renal route. The major metabolite M-1 showed no alkylating ability.

In conclusion, the orally administered DMDAI was readily absorbed and it was distributed initially in about equal proportions between RBC and plasma. Subsequent increase of RBC/plasma ratio might only be related to metabolite(s) incorporation. The rapid biotransformation of DMDAI into 4 alkylating agents may be of therapeutic significance.

Acknowledgements—The authors are indebted to Research Institute for Pharmaceutical Chemistry, Hungary, for their generous supply of DMDAI-1-^{3}H. The excellent assistance of Mrs. K. Máté is greatly appreciated.

Accepted 15 May 1975.

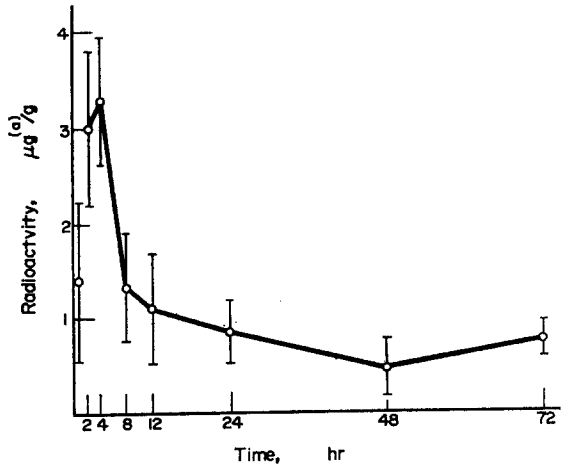


Fig. 1. *Mean* (±*S.E.*) *levels of radioactivity in whole blood after a single oral dose of DMDAI-*1-^{3}*H in* 7 *patients.* (*a*) *Values are computed as equivalent DMDAI.*

Table 1. *Thin layer chromatography of urine after DMDAI-*1-^{3}*H administration*

Product	R_f values of radioactive zones	NBP detection
M–1	0·00–0·10	–
M–2	0·15–0·25	+
M–3	0·30–0·40	+
M–4	0·42–0·48	+
M–5	0·50–0·75	+
DMDAI	0·65–0·75	+

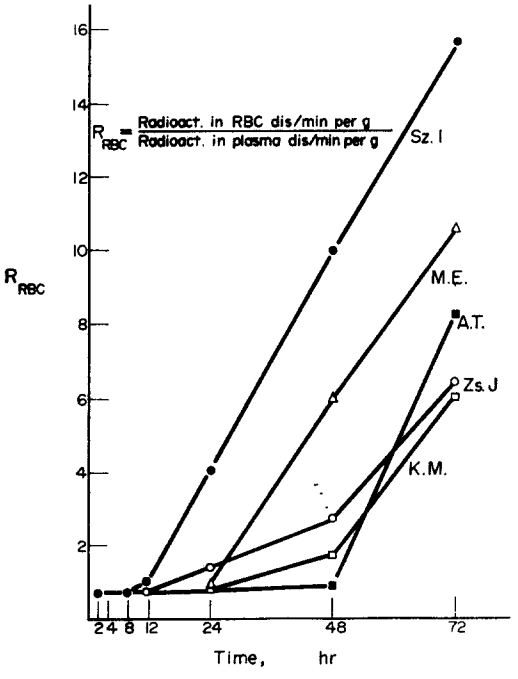


Fig. 2. *Relative concentration of radioactivity in RBC compared with the plasma level after a single oral dose of DMDAI-*1-^{3}*H.*

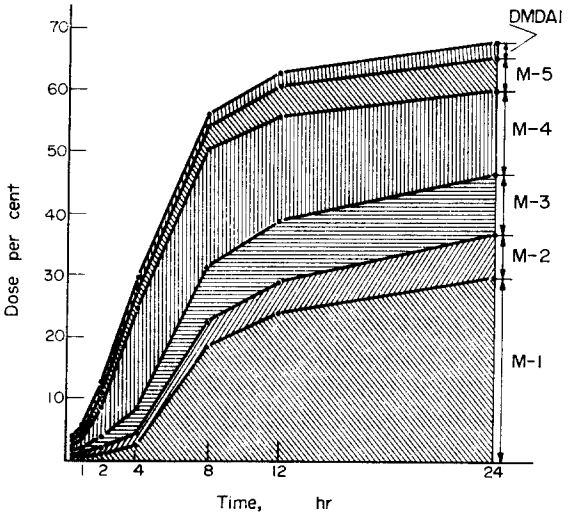


Fig. 3. *Mean cumulative urinary excretion of DMDAI and its* 5 *metabolites in* 7 *patients.*

REFERENCES

1. T. Horváth and L. Varga, *Neue Zuckerderivate mit zytostatischer Wirksamkeit. V. Ung. Krebstagung, Budapest*, 1961. Publishing House of the Hungarian Academy of Sciences, Budapest (1962).
2. E. Csányi and M. Halász, 1,6-Di-0-methansulphonyl-2,3-4,5-dianhydro-L-iditol, a new potential anti-tumour agent. *Arzneimittel-Forsch.* **23,** 861 (1973).
3. M. Füzy, P. Lelieveld and L. M. van Putten, The effect of cytostatic hexitol derivatives on haematopoietic stem cells, the L1210 leukaemia and C22LR osteosarcoma. *Europ. J. Cancer*, **11,** 169 (1975).

Europ. J. Cancer Vol. 11, pp. 677–678. Pergamon Press 1975. Printed in Great Britain

Announcement

International Conference on Environmental Mutagens

The Second International Conference on Environmental Mutagens will be held in Edinburgh from 11th to 15th July 1977 under the patronage of H.R.H. The Duke of Edinburgh, K.G., K.T. Full details will be circulated to members of the American, European, Japanese and Indian Environmental Mutagen Societies. Other interested parties may obtain further information from:

The Secretary-General, Dr. P. Brookes,
Institute of Cancer Research,
Pollards Wood Research Station,
Nightingales Lane,
Chalfont St. Giles,
Bucks, HP8 4SP,
England.

Recent Journal Contents (1975)

International Journal of Cancer *September, 1975*

N. Gabelman, S. Waxman, W. Smith and S. D. Douglas: Appearance of C-type virus-like particles after co-cultivation of a human tumor cell line with rat (XC) cells.

M. Suurküla and B. Boeryd: Tumour metastasis in mice with reduced immune reactivity. III. Studies with three weakly antigenic tumours in thymectomized and/or sub-lethally irradiated mice.

F. Bairstow and C. Heidelberger: Increased thymidine uptake by methylcholanthrene-treated C3H/10T1/2 cells.

A. M. Prince, W. Szmuness, J. Michon, J. Demaille, G. Diebolt, J. Linhard, C. Quenum and M. Sankale: A case/control study of the association between primary liver cancer and hepatitis B infection in Senegal.

M. Glaser, H. Kirchner and R. B. Herberman: Inhibition of *in vitro* lymphoproliferative responses to tumor associated antigens by suppressor cells from rats bearing progressively growing gross leukemia virus-induced tumors.

Y. S. Okada and A. Hakura: Temperature-dependent properties of cells transformed by a thermosensitive mutant (TS-121) of polyoma virus. II. Characterization of 121-6 cells.

C. O'Toole, K. Helmstein, P. Perlmann and G. Moberger: Cellular immunity to transitional cell carcinoma of the urinary bladder. III. Effects of hydrostatic pressure therapy.

A. Colombatti, D. Collavo, G. Biasi and L. Chieco-Bianchi: Genetic control of oncogenesis by murine sarcoma virus Moloney pseudotype. I. Genetic of resistance in AKR mice.

A. Colombatti, D. Collavo, G. Biasi and L. Chieco-Bianchi: Genetic control of oncogenesis by murine sarcoma virus Moloney pseudotype. II. A dominant epistatic susceptibility gene.

S. Kumar and G. Taylor: Non-organ-specific and tumour-specific antibodies in children with Wilms' tumour.

A. Bekierkunst: Immunotherapy and vaccination against cancer with nonliving BCG and cord factor (trehalose-6,6′-dimycolate).

J. H. Dean, J. L. McCoy, D. Lewis, E. Appella and L. W. Law: Studies of lymphocyte stimulation by intact tumor cell and solubilized tumor antigen.

J. Tamerius, I. Hellström and K. E. Hellström: Evidence that blocking factors in the sera of multiparous mice are associated with immunoglobulins.

Y. Langelier, R. Mandeville and R. Simard: Detection of virus-specific DNA sequences by quantitative *in situ* hybridization during infection with SV40.

M. O. Reynier, C. Lafarge-Frayssinet and C. Frayssinet: Ultrastructural alterations and modifications of nuclear RNA of rat liver by the combined action of thioacetamide and aflatoxin.

L. M. J. Harwood and P. H. Gallimore: A study of the oncogenicity of adenovirus type 2 transformed rat embryo cells.

British Journal of Cancer *September, 1975*

D. J. R. Laurence, C. Turberville, S. G. Anderson and A. M. Neville: First British Standard for carcinoembryonic antigen (CEA).

R. D. Watson, A. G. Smith and J. G. Levy: The detection by immunodiffusion of tumour associated antigenic components in extracts of human bronchogenic carcinoma.

K. James, G. J. A. Cluni, M. E. A. Woodruff, W. H. McBride, W. H. Stimson, R. Drew and D. Catty: The effect of *Corynebacterium parvum* therapy of immunoglobulin class and IgG subclass levels in cancer patients.

V. K. Lui, J. Karpuchas, P. B. Dent, P. B. McCulloch and M. A. Blajchman: Cellular immunocompetence in melanoma: effect of extent of disease and immunotherapy.

D. Collavo, G. Biasi, A. Colombatti and L. Chieco-Bianchi: *In vitro* and *in vivo* evaluation of T and B lymphocyte functions in AKR mice.

B. M. Jones and A. R. Turnbull: Horizontal studies of cell-mediated immune reactions to autologous tumour antigens in patients with operable mammary carcinoma.

D. G. Hopper, M. V. Pimm and R. W. Baldwin: Levamisole treatment of local and metastatic growth of transplanted rat tumours.

R. D. Rubens, S. Vaughan-Smith and R. Dulbecco: Augmentation of cytotoxic drug action and X-irradiation by antibodies.

L. J. Peters: A study of the influence of various diagnostic and therapeutic procedures applied to a murine squamous carcinoma on its metastatic behaviour.

L. J. Peters: The natural history of metastasis of a syngeneic murine squamous carcinoma and the prognostic implications of primary tumour size and duration of growth.

M. Ellison, D. Woodhouse, C. Hillyard, M. Dowsett, R. C. Coombes, E. D. Gilby, P. B. Greenberg and A. M. Neville: Immunoreactive calcitonin production by human lung carcinoma cells in culture.

G. D. Zanelli, P. B. Lucas and J. F. Fowler: The effect of anaesthetics and vasodilators on blood perfusion in transplanted mouse tumours.

M. J. Peckham, H. T. Ford, T. J. McElwain, C. L. Harmer, K. Atkinson and D. E. Austin: The results of radiotherapy for Hodgkin's disease.

Short Communication

P. Thomas and J. H. Westwood: Carboxyl esterase activity of carcinoembryonic antigen.

Letter to the Editor

R. Schoental: Chromium carcinogenesis, glycidal formation and tanning.

Europ. J. Cancer Vol. 11, pp. 677–678. Pergamon Press 1975. Printed in Great Britain

Announcement

International Conference on Environmental Mutagens

The Second International Conference on Environmental Mutagens will be held in Edinburgh from 11th to 15th July 1977 under the patronage of H.R.H. The Duke of Edinburgh, K.G., K.T. Full details will be circulated to members of the American, European, Japanese and Indian Environmental Mutagen Societies. Other interested parties may obtain further information from:

The Secretary-General, Dr. P. Brookes,
Institute of Cancer Research,
Pollards Wood Research Station,
Nightingales Lane,
Chalfont St. Giles,
Bucks, HP8 4SP,
England.

Recent Journal Contents (1975)

International Journal of Cancer *September, 1975*

N. Gabelman, S. Waxman, W. Smith and S. D. Douglas: Appearance of C-type virus-like particles after co-cultivation of a human tumor cell line with rat (XC) cells.

M. Suurküla and B. Boeryd: Tumour metastasis in mice with reduced immune reactivity. III. Studies with three weakly antigenic tumours in thymectomized and/or sub-lethally irradiated mice.

F. Bairstow and C. Heidelberger: Increased thymidine uptake by methylcholanthrene-treated C3H/10T1/2 cells.

A. M. Prince, W. Szmuness, J. Michon, J. Demaille, G. Diebolt, J. Linhard, C. Quenum and M. Sankale: A case/control study of the association between primary liver cancer and hepatitis B infection in Senegal.

M. Glaser, H. Kirchner and R. B. Herberman: Inhibition of *in vitro* lymphoproliferative responses to tumor associated antigens by suppressor cells from rats bearing progressively growing gross leukemia virus-induced tumors.

Y. S. Okada and A. Hakura: Temperature-dependent properties of cells transformed by a thermosensitive mutant (TS-121) of polyoma virus. II. Characterization of 121-6 cells.

C. O'Toole, K. Helmstein, P. Perlmann and G. Moberger: Cellular immunity to transitional cell carcinoma of the urinary bladder. III. Effects of hydrostatic pressure therapy.

Europ. J. Cancer Vol. 11, pp. 679–688. Pergamon Press 1975. Printed in Great Britain

Chemotherapy as a Primary Treatment of Hodgkin's Disease (204 New Cases, 1 to 8 Years of Follow-up)*

Cl. JACQUILLAT†, M. WEIL, G. AUCLERC, J. P. DESPREZ-CURELY, C. CHASTANG‡, C. GARÇON‡, J. P. CHANTALOU‡, M. F. GEMON-AUCLERC, J. DELOBEL, M. DANA, J. TANZER, F. TEILLET, V. IZRAEL, N. CHELLOUL, C. WEISGERBER, M. DE HEAULME‡, M. BOIRON, J. BERNARD

Unité de Chimiothérapie, Institut de Recherches sur les Leucémies et les Maladies du Sang, Université Paris VII, Hôpital Saint-Louis, 2 Place du Docteur A. Fournier, 75475 Paris Cedex 10, France.

Abstract—*Two hundred and four untreated patients with Hodgkin's disease were given 6 monthly courses of combined therapy with nitrogen mustard, vincristine, procarbazine, and prednisone (MOPP), followed by monthly vinblastine (protocol H2 65). Fifty of these patients were non-randomly selected to receive complementary or prophylactic irradiation to large fields, either supradiaphragmatic (28 cases), subdiaphragmatic (7 cases) or both of these (15 cases). Similarly, 50 other patients were chosen to receive reinductions with MOPP every three months during the first year, then every 6 months. All treatment was stopped after four years. Splenectomy was not performed routinely but 19 patients underwent splenectomy to define precisely the stage of their disease. Review of lymphography led to reclassification of 37 patients to that of stage II; 91% of whom entered remission, 48% being complete. Of the 109 stage III patients, 52% had a complete remission and 38% a partial remission (P.R.). Of stage IV patients, 40% obtained a C.R. and 26% a P.R.*

Remission curves reached a plateau at the 42nd month: 74 ± 6% for those who entered into C.R. and 56 ± 8% for those with a P.R. ($P < 0{\cdot}03$). These patients have been at risk for more than 42 months and 14 have been followed for periods between 5 and 8 years.

Other factors may have influenced these results: increased age (more than 50 years) greatly decreased the rate of remission ($P < 0{\cdot}001$); the more advanced the anatomical stage the worse the prognosis: but paradoxically, histological lymphocytic depletion (L.D.) had a better prognosis than other types ($P < 0{\cdot}02$). The clinical signs (A or B) did not influence the duration of the remission, but abnormalities in the parameters of biological activity (b) worsened the prognosis ($P < 0{\cdot}01$). Patients who were given complementary radiotherapy had a longer remission ($P < 0{\cdot}001$).

A relapse occurred in 38 patients, but never after the 42nd month. In 21 cases, the relapse occurred in the lymphatic areas which had been involved initially, and in the other 17, there was visceral involvement. In addition, there was a correlation between histological cell type and mode of recurrence: local recurrences occurring predominantly in lymphocytic predominance (L.P.), nodular sclerosis (N.S.) and widespread relapses occurring in mixed cellularity (M.C.) and (L.D.).

The overall survival reached a plateau at the 48th month of 72 ± 10%, this being influenced by the same factors as the remission. However, the beneficial effects of complementary irradiation ($P < 0{\cdot}05$) and reinductions ($P < 0{\cdot}05$) were certain.

Thus the value of polychemotherapy is confirmed. Prognosis in Hodgkin's disease may be related directly to the administration of polychemotherapy before the radiotherapy. These two modalities must be used in flexible association dependent on the staging which must include all the parameters.

Accepted 27 March 1975.

For footnotes see over page

INTRODUCTION

PETERS [1] and others determined that localized forms may be cured by appropriate irradiation [2]. The study of the sites of relapses led to the concept of the predominance of lymphatic spread of the disease. Most authors have adopted these methods, illustrated by Kaplan's research, of prophylactic irradiation of the lymphatic drainage areas. They have admitted that cures may be hoped for in localized forms and stage III A with dissemination on both sides of the diaphragm, but that stage III B and, more particularly stage IV, frequently, can only benefit from palliative therapy [3].

Progress in chemotherapy has destroyed this principle: good combination chemotherapy, when indicated, may be as curative as radiotherapy [4–6]. Both methods are complementary and their combination should increase significantly the rate of cures in the near future [7].

MATERIAL AND METHODS

This study comprises 204 consecutive, previously untreated patients [8] admitted to the Hôpital Saint-Louis from June 1965 to February 1973 with diagnoses of disseminated forms of Hodgkin's disease (stages III and IV). Within the same period, 490 other patients with stages I or II were treated by various combinations of irradiation and chemotherapy and some results have already been published [9, 10].

The protocol H2 65 [11] includes induction and maintenance phases.

The induction phase, similar to MOPP [12] consisted of 6 monthly courses of the following combination: vincristine (VCR) (1, 4 mg/m2) and nitrogen mustard (HN2) (6 mg/m2 i.v.) on the 1st and 8th days; procarbazine (MIH) (100 mg/m2 p.o.) from 1st to 14th day, prednisone (PDN) (40 mg/m2) from 1st to 14th day of every alternate course. The maintenance phase included one monthly dose of vinblastine (VLB) (0·2 mg/kg i.v.) for 4 years; some patients received additional treatment in a non-random way. In 50 patients VLB was replaced by a course of MOPP at 3 and 6 months and then every 6 months. In 29 patients mantle field irradiation or inversed Y field was carried out after the onset of C.R. ("prophylactic" irradiation). In 21 other patients large field irradiation (4000 rads in 4 weeks with 60 Co) was undertaken because of the persistence of radiological abnormalities ("complementary" irradiation). This chronology is shown on Fig. 1.

All histological examinations were revised in our department and classified according to the WHO classification [13].

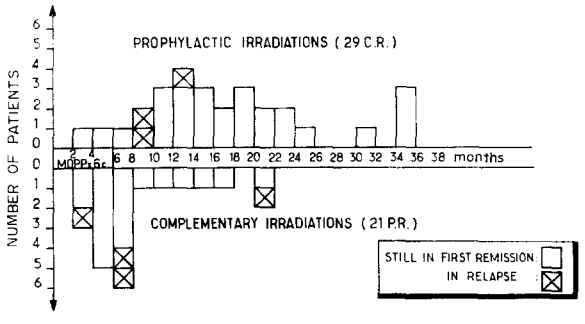


Fig. 1. Interval between chemotherapy and large field irradiations.

The examinations required in the evaluation of our patients included:

—systemic symptoms (night sweats; fever; weight lost);

—physical examination (lymph nodes; Waldeyer's ring; liver and spleen)

—laboratory tests (full blood count, bone marrow aspiration and bone marrow biopsy, serum iron and iron binding capacity). Sedimentation rate and plasma fibrinogen level, hepatic function tests [14];

—radiological and isotopic exploration; chest X-ray and mediastinal tomography; bilateral iliolumbar lymphography; splenic and hepatic scans; skeletal survey;

—a bone marrow biopsy was done in 167 of 204 patients, all patients except those of anatomical stages I, IE, II and II E.

We used the Ann Arbor classification and considered as stage IV only non-contiguous localisation. For visceral involvement arising by contiguity we staged the patient according to the lymph node involvement followed by the letter E to indicate the presence of an extranodal disease [13]. In addition to clinical stages

*Supported by a grant from Institut de la Santé et de la Recherche Médicale (Professeur L. Burg, Commission 13, by USPHS Research Grant CA 13239–01 from the National Cancer Institute and by Régionale d'Assurance Maladie de Paris.

†To whom request for reprints should be addressed.
‡Département de Biomathématique, Université Paris VI, Faculté de Médecine Pitié-Salpétriere (Professeur F. Gremy).

A and B [15] we considered also the biological activity. Our patients were considered "a" if none or one, "b" if two or more of the following parameters were present: sedimentation rate ≧ 40 mm (1st hour), plasma fibrinogen ≧ 5 g‰; serum albumin ≦ 35 g‰; α_2 globulin ≧ 10 g‰; γ globulin ≧ 22 g‰ serum iron ≦ 70 γ‰.

In 37 patients, revision of the lymphogram caused us to modify the initial staging: lymphography which was considered initially as suspect was finally regarded as normal, which explains why some stage II patients received a protocol designed for disseminated disease [16]. The principal data for each patient were entered into files under 59 items and treated by the computer according to the "A.I.D.E." system [17]. Histograms, survival curves, X_2 tests were obtained. Two hundred and sixty-

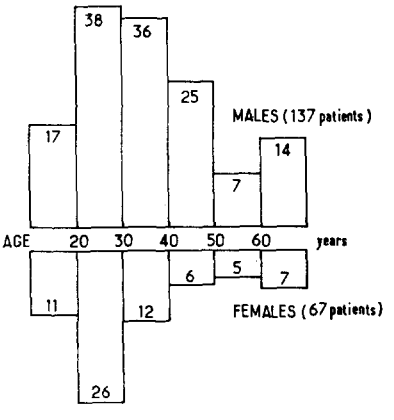


Fig. 2. Repartition of the patients by age and sex.

six actuarial curves were analyzed and 172 comparative series curves with calculations of the s.d. at a 3-monthly interval were made.

In addition, 19 patients had a laparotomy to provide histological material because of splenomegaly or an ambiguous lymphogram (among 10 with clinical splenomegaly, 8 were found to have histological splenic involvement).

The criteria for hepatic involvement is based on the combination of the following signs:

—clinical hepatomegaly confirmed by radioisotopic scans;

—bromsulphalein retention test;

—serum alkaline phosphatase level;

—serum transaminase level.

Nineteen patients had a liver biopsy after splenectomy: in the 4 cases where the first three signs were present, the liver biopsy was positive.

The distribution of patients by age, sex and anatomical stages according to the histological type and the clinico-biological type are shown on Fig. 2 and Tables 1 and 2. The frequency of histological M.C. and L.D. in stage III is confirmed and the lack of correlation between clinico-biological types and anatomical stages may be noted. C.R. is defined as the complete disappearance of all objective and subjective disease manifestations. P.R. is defined as a 50% or greater reduction of all tumoral masses. The distinction between C.R. and P.R. may be difficult to define if only minor radiological abnormalities persist such as discrete mediastinal enlargement or minor structural defects on lymphography. Moreover,

Table 1. Relation between anatomical stages and histological types

Anatomical stages	Histological types L.P.	N.S.	M.C.	L.D.	Unknown	Total
I_E	—	1	—	—	—	1
II	1	11	10	3	2	27
II_E	1	4	2	—	2	9
III	20	20	52	7	4	103
III_E	1	1	3	1	—	6
IV	3	7	19	5	4	38
IV BM	—	3	13	4	—	20
All stages	26	47	99	20	12	204

Table 2. Relation between anatomical stages and clinical and biological types

Stages	Aa	Ab	Ba	Bb	Total
I_E	—	1	—	—	1
II	4	1	2	20	27
II_E	1	4	—	4	9
III	14	30	7	52	103
III_E	1	3	—	2	6
IV	—	3	3	32	38
IV BM	2	3	2	13	20
All stages	22	45	14	123	204

these abnormalities may be present at the end of the six courses and disappear thereafter, increasing the ambiguity of the classification. Patients were examined monthly as outpatients for the first 6 months; every 2 months for the second 6 months, then every 3 months during the second and the third year; and thereafter every 6 months. Physical examination, laboratory and radiological tests were performed at each visit. Relapses are defined by the reappearance of tumoral involvement and/or of major clinical or biological abnormalities.

RESULTS

Induction results

Overall results. One hundred and fifty-two patients received the six scheduled courses and 10 patients died from disease before the 6th course. In 12 patients the protocol was changed because of resistance before the 6th course. In 30 patients changes were made despite initial response to treatment. In 11 patients (6 P.R., 5 C.R.) large field irradiations were delivered. In 12 patients maintenance treatment with VLB was undertaken. In 7 patients another combination chemotherapy was given. The overall percentage of good results was 84% with 47% C.R. and 37% P.R. but if we limit our analysis to consider the patients who received 6 courses the rate of remission was 93%.

Table 3. *Results according to age*

Results	< 20 yr	20–35 yr	35–50 yr	> 50 yr	Total
C.R.	12	49	30	5	96
P.R.	13	28	22	13	76
Failures	3	6	3	9	21
Inductions deaths	—	2	3	6	11
	28	85	58	33	204

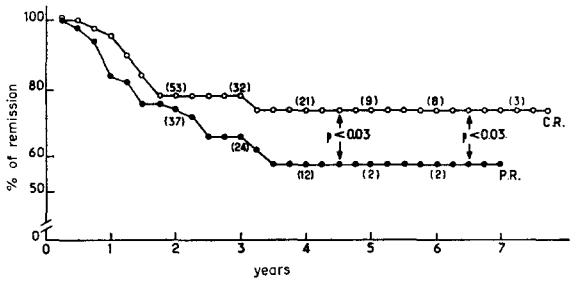


Fig. 3. *Comparison between duration of C.R.* (96 *patients*) *and P.R.* (76 *patients*).

Eleven patients (5·5%) died during the induction phase. In only 3 cases was there a direct relationship between treatment and death. In the other patients deaths were related to the disease or to the poor condition of the patient on admission. The predominance of males and of relatively elderly patients was noteworthy ($P < 0{\cdot}001$).

(2) *Analysis of induction results.* There was no difference in the overall results between males and females (29 C.R., 31 P.R. of 67 females; 67 C.R., 45 P.R. of 137 males).

—As shown in Table 3, age was an important parameter; the frequence of remission was reduced significantly in those over 50 years of age ($P < 0{\cdot}001$).

—Anatomical stage played a part as well (Table 4). The frequency of remissions was less in stage IV than in other stages ($P < 0{\cdot}02$) but in patients with bone marrow involvement the rate of remission was 80% (30% C.R.). All parenchymatous involvement regressed with the same frequency: lung (21 C.R. in 27 patients), liver (18 C.R. in 23 patients), marrow (6 C.R. in 20 patients).

Neither histological cell type nor clinico-biological variety played a role in the frequency of remission attainment.

Duration of remissions

For greater uniformity, we took as a basis for the duration of remissions, the onset of treatment. Some patients had not received 6 courses of treatment: 30 patients had received between 3 and 5 courses; 10 who had achieved a rapid remission had a prophylactic or complementary irradiation; 20 patients began the maintenance treatment before the 6th month because of poor tolerance of chemotherapy. Figure 3 shows the duration of C.R. and P.R.; for C.R. there was a levelling off of the curve to $74 \pm 6\%$ from the 39th month. For P.R. there was a levelling off to $56 \pm 8\%$ from the 42nd month. As already stated, P.R.'s are a heterogeneous group. In some patients, initial P.R.'s converted into C.R.'s by a complementary treatment, irradiation or chemotherapy. Remission

Table 4. *Results according to stages*

Induction results	I_E	II	II_E	III	III_E	IV	IV BM	Total
C.R.	1	14	2	54	4	15	6	96
P.R.	—	11	5	38	1	11	10	76
Failures	—	2	2	6	1	6	4	21
Induct. deaths	—	—	—	5	—	6	—	11
	1	27	9	103	6	38	20	204

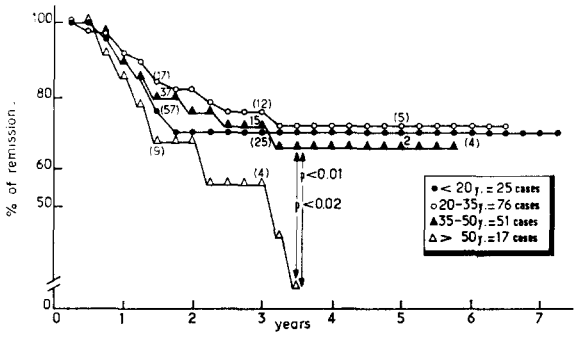


Fig. 4. *Effect of age on duration of remission* (*C.R.* + *P.R.*).

duration was influenced by host factors as well as by treatment.

Age. As shown on Fig. 4, duration of remission was significantly reduced for those over 50 years of age (for patients under 50 years, the "plateau" was reached at 70 ± 23% from the 39th month and for patients over 50 years, at 28 ± 31% at the 42nd month). Sex did not influence significantly the duration of C.R.

Disease stage. Disease stage did not influence the duration of C.R. and it is noteworthy that no relapse occurred after 15 months in stage IV in spite of the fact that most patients had been

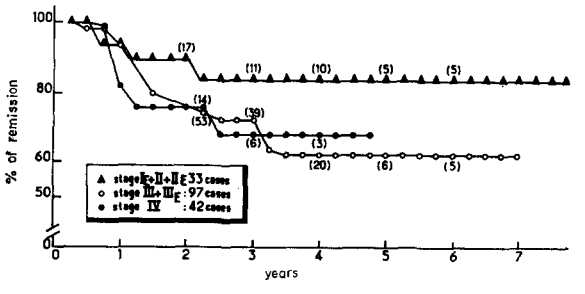


Fig. 5. *Effect of anatomical stages on duration of remission (C.R. + P.R.).*

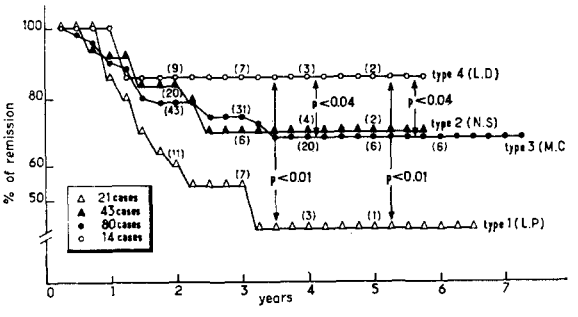


Fig. 6. *Duration of C.R. + P.R. according to histological variety.*

in C.R. for more than 4 years. In contrast, the duration of C.R. + P.R. was significantly better in stages I E, II, II E than in stages III and III E or than in stage IV (Fig. 5).

In stage IV, duration C.R. + P.R. was significantly better if no bone marrow involvement was detected ($P \leqq 0{\cdot}01$) with a respective levelling off of the curves at 86 ± 14% and 51 ± 32% at 15 months.

Histological type. Whereas there was no difference in the duration of C.R. according to histopathological classification, we observed a paradoxical influence on C.R. + P.R.; as shown on Fig. 6, L.D. demonstrated significantly better prognosis than L.P. ($P < 0{\cdot}01$) or M.C. ($P < 0{\cdot}04$).

Clinico-biological type. We noted no difference in the duration of C.R. according to initial clinical activity (A or B). On the contrary, in stage III there was a significant difference according to biological activity (a or b) ($P < 0{\cdot}01$). We observed no relapse in the "Ba" patients and 1 relapse in the Aa group but a levelling off of the curve to 58 ± 11% at 39 months for the "b" patients. Biological activity was thus a more significant prognostic parameter than clinical activity (Fig. 7).

Therapeutic factors. Because of the unequal distribution of irradiation treatment according to stage, we restricted our analysis to stages III and III E. In Table 5 the classification of 204 patients is shown as a function of their treatment after the induction period. The

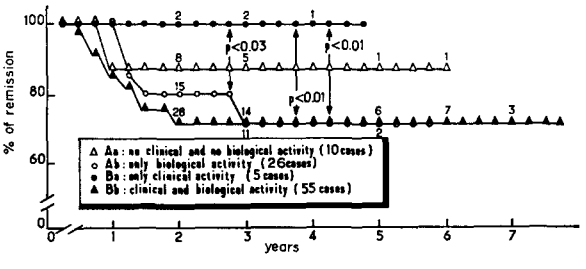


Fig. 7 *Duration of C.R. according to the clinical and to biological activity.*

various prognostic features studied in these three groups were sex, age, histological type, anatomical and clinico-biological stages.

In spite of the fact that these groups were not randomized, comparison shows an homogeneous distribution between these groups except for anatomical stages, there being a significantly greater number of localized forms (stages I E, II and II E) in the group with irradiation than in the other two groups. As a result of this difference, only stages III and III E were analysed.

There were 19 prophylactic and 4 complementary irradiations and only 23 irradiations in stages III and III E. Whereas the prophylactic irradiation was generally given 6 months after the completion of the 6 courses of MOPP, the complementary therapy was given one or two months after the end of the combination chemotherapy.

As shown on Fig. 8, duration of C.R. + P.R. was better in patients who were irradiated

Table 5. Group comparison

			Patients in C.R. or P.R.			
		All patients	VLB alone	VLB+ irradiation	VLB+ reinductions	Total C.R.+P.R.
Sex	M/F	137/67	48/25	29/21	35/14	112/60
Age	<20 yr	28	12	8	5	25
	20–35	85	32	26	19	77
	35–50	58	19	14	19	52
	>50	33	10	2	6	18
Anatomical stages	I E	1	0	1	0	1
	II	27	7	16	2	25
	II E	9	1	4	2	7
	III	103	43	23	23	92
	III E	6	5	0	0	5
	IV	38	7	5	14	26
	IV BM	20	10	1	5	16
Clinical and biological	Aa	22	9	8	3	20
	Ab	45	19	13	9	41
	Ba	14	5	2	2	9
	Bb	123	40	27	35	102
Histological	LP	26	11	5	5	21
	NS	47	17	12	14	43
	MC	99	38	26	18	82
	LD	20	2	6	7	15
	Unknown	12	5	1	5	11
Total		204	73	50	49	172

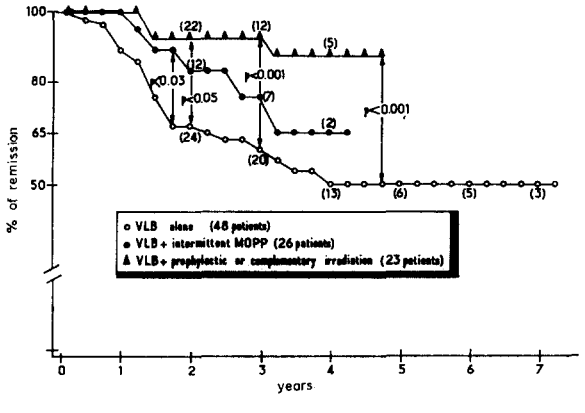


Fig. 8. Stages III+IIIE: effect of irradiation and reinduction on duration of remission (C.R.+P.R.).

($P < 0{\cdot}001$). There was no difference between prophylactic and complementary irradiation.

Toxicity

The overall toxicity reflected the individual toxicity of each drug: MIH produced vomiting; VCR produced pareses and polyneuritis but never led to major impotence. N_2H and MIH were myelosuppressive. Minor toxicity was rated 1 (54 patients); serious toxicity leading to postponement or to decrease of the drugs was rated 2 (7 patients) and lethal toxicity was rated 3 (2 patients). Toxicity was greater in females ($P < 0{\cdot}01$) and in patients over 50 years of age. There was also a relationship between toxicity and duration of treatment since most of these drugs, particularly VCR demonstrated cumulative toxicity. However,the 2 deaths from treatment were observed during the induction phase of treatment.

Irradiation after 3 (6 patients), 4 (2 patients), 5 (3 patients) and 6 (39 patients) MOPP courses was tolerated without significant hematological toxicity. In the 19 patients who had a splenectomy prior to treatment we noted no difference in tolerance.

Relapse pattern and survival

Table 6 compares the stage of disease at the onset of treatment and at the first relapse. In 21 patients, there was a correlation between the site of the largest masses before treatment

and the site of the initial recurrence, confirming Frei's study [18]. In 17 patients relapse involved one or several organs and reflected dissemination of the disease. It is noteworthy that initial distribution of histological cell type was significantly different. In patients with local recurrences, 14 out of 18 patients had histologic L.P. or N.S.; in disseminated relapses 15 out of 18 patients had histologic M.C. or L.D. These findings suggested a possible correlation between histological cell type and the tendency for lymphatic or hematogenous spread [19].

Survival of C.R.'s, P.R.'s and of failures are shown in Fig. 9. Eighty-eight per cent of patients who attained C.R. are still alive at 46 months. The figures for P.R. are $70 \pm 13\%$ at 48 months. These results may be further improved. Complementary irradiation in stage III improved our

Table 6. *Correlation between initial stage and stage at relapse*

Initial stages	Stage at relapse I	II	III	IV	IV BM	Total
II	2	2	—	—	—	4
III	4	5	6	8	2	25
IV	1	—	—	2	—	3
IV BM	—	1	—	4	1	6
All stages	7	8	6	14	3	38

results significantly with a levelling off of the survival to $94 \pm 5\%$ at 36 months which is consistent with our observation that many relapses occur at the site of major initial involvement. Intermittent courses of MOPP alternating with VLB maintenance also improved our results in stages III and IV with a levelling off of the survival curve of C.R. + P.R. to $90 \pm 6\%$ ($P < 0{\cdot}05$): a similar result is obtained with complementary irradiation (Fig. 10).

The poor survival of treatment failures (median < 1 year, 13/21 patients dead at 30 months) shows that in these patients no other combination was active. The difference between survival and duration of complete and partial remission is indicative mainly of the results of the relapse treatments. Of 38 relapses treated by radiotherapy, another combination chemotherapy, or both treatments, good results were obtained in 18 cases (7 C.R.; 11 P.R.): 16 patients died (14 from disease, 2 from accidents); 4 remained alive with active

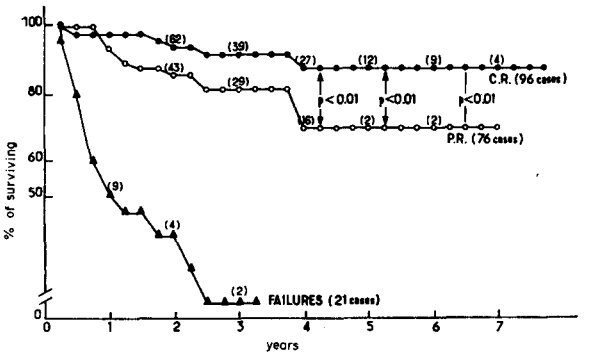


Fig. 9. *Comparison between survival of C.R., of P.R., of failures.*

disease. In disseminated relapses (IV, IV BM) only 3 partial remissions were achieved.

DISCUSSION

This study confirms the striking effectiveness of the H2-65 program in Hodgkin's disease and suggests that cures may be hoped for even in stages III and IV [18, 20, 21].

The results compare favorably with those obtained in stage III by irradiation alone. Peters showed, for stages III and IV, 10% survival at 5 years [1]. For Hodgkin's disease staged with the help of laparotomy and splenectomy (which enables to detect early stage III), Kaplan quoted, for stage III, a survival of 62% at 6 years and for stage IV, of 40% at 3 years with intensive megavoltage radiotherapy. The results of the H2 protocol may be also compared to other combination chemotherapy results. Using the same attack treatment (MOPP) without maintenance, Canellos [12] and De Vita [4] obtained, for stages III and IV, 67% survival at 5 years and 59% at 6 years. Frei demonstrated, in stages III and IV, the necessity of maintenance therapy after 6 induction courses of MOPP: remission rate was 75% in patients who were given 1 course of MOPP every 2 months for 18 months during remission, and 35% in patients without

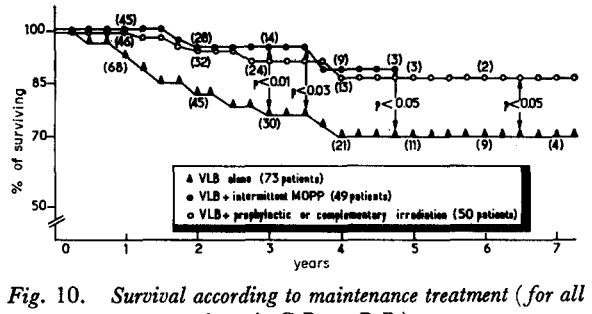


Fig. 10. *Survival according to maintenance treatment (for all patients in C.R. or P.R.).*

maintenance therapy [22]. Other combinations were tried with less favorable results [23, 24], such as alternating courses of VCR and MIH with VLB; chlorambucil and PDN; or a three drug combination [25]. Replacement of N2H by BCNU (1-3-Bis (2-Chloroethyl)-1-nitrosourea) did not seem to alter the results (Nissen [26]).

Among promising new approaches are the incorporation of bleomycin, a non-myelotoxic agent, into the MOPP combination and possibly the clinical exploitation of the striking synergism that has been observed in experimental systems between cyclophosphamide and the nitrosoureas [20].

More effective combinations with new drugs which could reduce the number of unresponsive patients and of disseminated relapses are obviously highly desirable.

The results of chemotherapy and radiotherapy in stage III were reported by some investigators. With a two-drug combination followed by radiotherapy to involved areas or by total nodal radiotherapy, or with total nodal therapy preceding two-drug chemotherapy, or with total nodal radiotherapy alone, Hoogstraten [27] reported a median duration of remission of approximately two years. The same median duration is reported by Tubiana [28] for stages III and IV with a four-drug combination (MIH, VLB, triethylene-melamine and PDN), complementary local radiotherapy and supplementary long term chemotherapy with VLB [29]. In our study the combination of MOPP followed by large field irradiation therapy for three years achieved distinctly better results in 50 patients (94% survival at 3 years).

A program of total nodal irradiation therapy followed by MOPP is in progress in Stanford and, although still preliminary, the results seem to demostrate the superiority of irradiation + chemotherapy over radiotherapy alone [3].

This study also allows the interpretation of prognostic features in disseminated Hodgkin's disease. Some of these parameters are well known and common to all Hodgkin's disease. Age over 50 years worsens the prognosis and remissions are less frequent in stage IV than in other stages. Nevertheless, it should be noted that if a complete remission is obtained, its duration is equal in stages III and IV. Moreover, whereas in Hodgkin's disease treated by irradiation therapy. histological L.P. or N.S. are deemed to be more favorable than M.C. or L.D.; we observed L.D. to be significantly better than any other one [30]. The study of the three therapeutic methods with the histological types by the χ_2 test showed no significant difference in the distribution.

Chemotherapy is thus strongly indicated in such histological types. Whereas no prognostic difference was noted with the presence or absence of clinical symptoms (A and B), the biological activity (b), which is not taken into consideration in the Ann Arbor classification, worsens the prognosis significantly. By a factor analysis approach we proved that pruritus is particularly correlated with alteration of the biological activity [31].

A sophisticated prognostic index [8, 32] would require multifactorial analysis but a pragmatic score taking into account anatomical stage, clinical and biological activity, and histological type should be a guide to the duration of combination chemotherapy prior to radiotherapy. For example: a feeble score, an average, a high and a very high score could be treated respectively with 3, 6, 9 and 12 months of chemotherapy prior to local or extended field irradiation. For a low score, no maintenance chemotherapy would be required. For an average score, 2 years of chemotherapy; and for high and very high scores, 4 years of maintenance chemotherapy with regular reinductions may be required.*

In our opinion, chemotherapy should be combined with local radiotherapy in most cases of local disease since after irradiation most relapses occur at a distance from the initial sites. After mantle or inverted Y irradiation plus a minor chemotherapy program, remission durations were no better for stages I and II than for stages III treated by the protocol H2 65 and were worse for stages II Bb (45% C.R. at 5 years) [10].

Perhaps complementary or prophylactic irradiation will add little to the survival of stage III and IV patients treated with MOPP, but MOPP improves the results which can be obtained by radiotherapy in stages I and II. In a recent controlled study dealing with stages I and II, relapses were significantly reduced in 99 patients receiving 3 courses of MOPP prior to irradiation (9 relapses) when compared to 102 patients only irradiated (21 relapses) [10]. This may be due to staging errors since no systematic splenectomy was

*Score I (1), II (2), III (3), IV (5), IV BM (7), E (1), (spleen 1), tumor mass (1), Ba (1), Ab (1), Bb (3), > 50y. (1), In stages I and II M.C. or L.D. (1). In stages III and IV L.P., N.S. (1), vascular invasion (1). Score points are indicated in parenthesis. Score additions: low: 1 to 2; average: 3 to 5; high: 6 to 9; very high $\geqslant$ 10.

performed. However, Peters mentions the possibility of mesenteric involvement after apparently negative laparotomy [1]. Another advantage of chemotherapy when given prior to irradiation is to induce regression of large tumoral masses, thus allowing the use of a more limited X-ray field. On the other hand, the frequency of local relapses in stages III and even IV suggests the use of irradiation to major areas of initial involvement even in disseminated stages.

The results of initial multiple drug therapy suggest the possibility of using localized radiotherapy as a substitute for extended field radiation to clinically benign lymph node areas [24], given that local recurrences are generally in regions where the biggest tumour masses were originally.

It is thus confirmed that in Hodgkin's disease, "chemotherapy and radiotherapy are partners and not competitors" [33]. This should be confirmed in the near future for most cases in both localized and disseminated stages.

Acknowledgements—We would like to thank Professor Gremy (Département de Biomathématique C.H.U. Pitié-Salpétrière, 91, Boulevard de l'Hôpital 75013 Paris, France for help in studies, Dr. D. Salmon for the multifactorial analysis and E. Morgan for the translation.

REFERENCES

1. M. V. Peters, The evolution of the radiotherapeutic concepts in Hodgkin's disease. *Ser. Haemat.* **6,** 117 (1973).
2. H. S. Kaplan, The radiotherapy of regionally localized Hodgkin's disease. *Radiology,* **78,** 553 (1962).
3. H. S. Kaplan, *Hodgkin's Disease.* Harvard University Press, Cambridge, Massachusetts, 462 pp. (1972).
4. V. T. De Vita, A. A. Serpick and P. P. Carbone, Combination chemotherapy in the treatment of advanced Hodgkin's disease. *Ann. int. Med.* **73,** 881 (1970).
5. V. T. De Vita and P. P. Carbone, Chemotherapeutic implications of staging in Hodgkin's disease. *Cancer Res.* **31,** 1838 (1971).
6. Cl. Jacquillat, M. Weil, A. Goguel, G. Schaison, J. Delobel, M. F. Gemon, R. Brunel Kotschi, F. Teillet, M. Boiron et J. Bernard, Chimiothérapie de la maladie de Hodgkin par des associations médicamenteuses. *Presse méd.* **79,** 513 (1971).
7. S. A. Rosenberg and H. S. Kaplan, Hodgkin's disease and other malignant lymphomas. *Calif. Med.* **113,** 23 (1970).
8. G. Auclerc, Polychimiothérapie dans les formes diffuses de la maladie de Hodgkin. *Thèse Fac. Méd. Paris* **VII,** 61 p., 1974.
9. J. Bernard, M. Boiron, F. Teillet, M. Dana, Cl. Jacquillat et M. Weil, Combined Radiotherapy and Chemotherapy in Human Lymphoma (abstract). Congress XIII Intern. Haemat., Munich, 1970: 89. Edited by J. F. Lehmanns.
10. F. Teillet, Ch. Weisgerber, M. Dana, N. Feingold, N. Chelloul, F. Thiebaud, Cl. Jacquillat, M. Boiron, Jean Bernard, Traitement des formes localisées I et II de la maladie de Hodgkin par association de radiothérapie et chimiothérapie. *Press. méd.* (in press).
11. J. Bernard, M. Boiron, A. Goguel, Cl. Jacquillat, J. Tanzer et M. Weil, Traitement de la maladie de Hodgkin par une polychimiothérapie associant moutarde à l'azote, vincristine, methylhydrazine et prednisone. *Presse Med.* **75,** 2647 (1967).
12. G. P. Canellos, R. C. Young, C. Berard and V. T. De Vita, Combination chemotherapy and survival in advanced Hodgkin's disease. *Arch. intern. Med.* **131,** 388, mars (1973).
13. R. J. Lukes and J. J. Butler, The pathology and nomenclature of Hodgkin's disease. *Cancer Res.* **26,** 1063 (1966).
14. F. Teillet, M. Boiron and J. Bernard, A reappraisal of clinical and biological signs in staging of Hodgkin's disease. *Cancer Res.* **31,** 1723 (1971).
15. P. P. Carbone, H. S. Kaplan, K. Musshoff, D. W. Smithers and M. Tubiana, Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res.* **31,** 1860 (1971).
16. J. P. Desprez-Curély, V. Bismuth et P. Petitbon, Valeur des Aspects "Limites" en Lymphographie. Etude du Devenir de ces Aspects Lymphographiques dans la Maladie de Hodgkin. *Actual. Hémat.* 5e Série, 33 (1971).

17. M. De Heaulme, J. P. Chantalou et C. Garçon, La Puissance d'interrogation du systeme Aide. *Colloque IRIA Informatique Médicale*, Tome III, 125–133, Toulouse, mars 1973. Edited by IRIA (Institut de Recherche, d'Informatique et d'Automatique).
18. E. Frei, III, Combination cancer therapy, Presidential address. *Cancer Res.* 2593 (1972).
19. G. Mathé et M. Tubiana, The development of ideas on the natural history and the therapy of Hodgkin's disease. *Ser. Haemat.* **6,** 5 (1973).
20. E. Frei, III, Status and perspectives in chemotherapy of Hodgkin's disease. *Arch. Intern. Med.* **131,** 439 (1973).
21. E. Frei, III, V. T. De Vita, J. H. Moxley, III and P. P. Carbone, Approaches to improving the chemotherapy of Hodgkin's disease. *Cancer Res.* **26,** 1284 (1966).
22. A. C. Aisenberg and J. M. Goldman, Prolongation of survival of Hodgkin's disease. *Cancer (Philad.)* **27,** 802 (1971).
23. L. Stutzman, Combined radiotherapy and chemotherapy of lymphomas and other cancers. *Cancer Res.* **31,** 1845 (1971).
24. L. Stutzman and O. Glidewell, Multiple chemotherapeutic agents for Hodgkin's disease. Comparison of three routines, a cooperative study by acute leukemia Group B. *J. Amer. med. Ass.* **225,** 1202 (1973).
25. J. K. Luce, J. F. Gamble, H. E. Wilson, R. W. Monto, B. L. Isaacs, R. L. Palmer, C. A. Coltman, J. S. Hewlett, E. A. Gehan and E. Frei, III, Combined cyclophosphamide, vincristine and prednisone therapy of malignant lymphoma. *Cancer (Philad.)* **28,** 306 (1971).
26. N. I. Nissen, L. Stuzman, J. F. Holland and O. Glidewell, Chemotherapy of Hodgkin's disease in studies by acute leukemia group B. *Arch. intern. Med.* **131,** 396 (1973).
27. B. Hoogstraten, J. F. Holland, S. Kramer and O. Glidewell, Combination chemotherapy–radiotherapy for stage III Hodgkin's disease. An acute leukemia group B study. *Arch. intern. Med.* **131,** 424 (1973).
28. M. Tubiana and G. Mathé, Combined radiotherapy and chemotherapy in the treatment of Hodgkin's disease. *Ser. Haemat.* **6,** 202 (1973).
29. R. C. Young, G. P. Canellos, B. A. Chabner, P. S. Schein and V. T. De Vita, Maintenance chemotherapy for advanced Hodgkin's disease in remission. *Lancet* **1,** 1339 (1973).
30. A. Rappaport, P. Cole and J. Masson, Correlates of survival after initiation of chemotherapy in 142 cases of Hodgkin's disease. *Cancer (Philad.)* **24,** 377 (1969).
31. I. Brodwski, C. Chastang, G. Auclerc, D. Salmon and Cl. Jacquillat, Prognosis of Hodgkin's disease: a factor analysis analyse factorielle des correspondances) *Approach. Med. Info., Stockholm* **8,** 74 (in press).
32. P. Armitage and E. A. Gehan, Statistical methods for the identification and use of prognostic factors. *Int. J. Cancer* **13,** 16 (1974).
33. J. H. Burchenal, Chemotherapy and radiotherapy: competitors or partners? *Cancer* **22,** 790 (1968).

Europ. J. Cancer Vol. 11, pp. 689–696. Pergamon Press 1975. Printed in Great Britain

Kinetics of Induction and Growth of Precancerous Liver-Cell Foci, and Liver Tumour Formation by Diethylnitrosamine in the Rat*

E. SCHERER and P. EMMELOT

Department of Biochemistry, Antoni van Leeuwenhoek-Laboratory, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

Abstract—*Induction of foci of atypical liver cells (ATPase-deficient islands, which previously have been shown to serve as tumour cell precursor) by a single dose of diethylnitrosamine in partially hepatectomized rats showed direct proportionality between number of islands and dose up to 30 mg DENA/kg, pointing to a one-hit mechanism of induction. The curve relating number of islands to dose levelled off at ≧ 50 mg/kg, and this was interpreted as resulting mainly from the toxic action of the carcinogen. Island size (the number of cells per island) was independent of dose up to 30 mg DENA/kg, but progressively increased in the higher dose range. Increased proliferation of island cells was attributed to the regenerative stimulus stemming from the toxic effect of the carcinogen at the higher doses. Analogous findings resulted from the measurement of island induction in the livers of intact rats receiving 0·9—14 mg DENA/kg daily for 4 weeks.*

A single dose of ≧ 50 mg DENA/kg produced hepatocellular tumours in the partially hepatectomized rat. Liver tumour formation in this system is discussed and the significance of cell proliferation (DNA synthesis) for the various phases of the hepatocarcinogenic process is stressed.

INTRODUCTION

CARCINOGENESIS is a process in which tumour cells are derived from normal cells, apparently by a number of stepwise alterations [1–4].

A cell type that may qualify as the first morphologically detectable cell stage causally involved in chemical carcinogenesis in the rat liver has recently been identified [5]. These cells are persistently changed in morphological, enzyme histochemical and growth properties from the normal parenchymal cells. ATPase deficiency is the main phenotypic marker which we use for the routine identification and quantitation of foci of these cells. Most probably [6], the foci—designated as islands—are clonally derived from liver cells acted upon by carcinogen, in our experiments mostly DENA; thus the sequence: liver cell → island-forming cell → island = population of island cells, identified as an ATPase-deficient focus surrounded by normal, ATPase-positive liver cells, is obtained. The island cells are endowed with the capacity to proliferate for a considerable period of time in the absence of further treatment in an otherwise normal and resting liver. The number of islands induced is markedly enhanced by partial hepatectomy performed 24 hr before the application of 10–20 mg DENA/kg [5]. Islands thus induced do not attain the malignant state and only seldom

Accepted 22 May 1973.

*Part of this investigation has been communicated to the Eleventh International Cancer Congress, Florence, 22–26 October 1974 (Abstracts, Part 3, p. 354).

reach macroscopic dimensions within the life span of the rats [7].

Recent quantitative experiments [7] support the view that these island cells are causally related to tumour formation, serving as tumour cell precursors by being substrate for further carcinogenic action. Accordingly, induction of tumour cell precursors (island cells) and conversion of the latter to tumour cells can be studied separately and quantitatively, allowing dose–response relations to be established for the separate processes.

The present paper reports on the dose–response relationship of island induction by DENA in rat liver (partially hepatectomized and single application; intact and multiple application), quantitating (a) the number of islands formed, (b) the number of cells per island (from which the growth rate of the island cells may be derived), and (c) the total number of island cells present after a fixed period of time as a function of the dose of DENA. On the basis of the finding that a single dose of $\geqq$ 50 mg/kg administered to partially hepatectomized rats leads to liver tumours, and taking morphological data into account, liver carcinogenesis in this system is discussed as a process manifesting itself in at least two stages, *i.e.* island cell induction and focal progression of some island cells to tumour cells.

MATERIALS AND METHODS

Female rats of the Sprague–Dawley strain (specific pathogen-free, Zentralinstitut für Versuchstierzucht, Hannover, Germany) were fed a standard diet (Hope Farms, Woerden, The Netherlands) and given water *ad libitum*. Body weight amounted to 200–300 g with a deviation of $\pm$ 25 g at the start of an experiment.

Two-thirds hepatectomy was performed according to Higgins and Anderson [8] between 10 a.m. and 3 p.m., 20–24 hr prior to DENA application. DENA (Merck-Schuchardt, München, Germany) in tap water, was applied by stomach tube in the concentrations indicated below. The daily doses were administered 6 days per week in an amount of 7/6 the dose indicated in the text.

For quantitation of the islands, rats were killed 8 weeks after induction following a 16 hr fasting period. Livers were removed, weighed, and representative portions of the anterior and posterior right, and the two caudate lobes (in the case of hepatectomized rats) and of total liver (in the case of intact rats) were frozen on solid CO_2. Cryostat sections (10μm) were stained for ATPase, glucose-6-phosphatase and PAS reactions and by hematoxylin and eosin as described [5]. The quantitation of island number and size has been reported in detail previously [5]. Depending on island number, from 3 to 15 cm^2 of ATPase stained sections were evaluated per animal.

DNA content of the liver was measured by the Schmidt–Thannhauser method [9] and the indole reaction [10] 72 hr after partial hepatectomy corresponding to 48 hr after a single application of DENA using 4 rats per dose level.

For the study of liver tumour formation rats were treated as mentioned below and were kept under standard conditions until they became moribund and were killed. Livers were weighed and pieces from tumours and each lobe were frozen on solid CO_2 for histological examination of sections stained as indicated above.

RESULTS

Single dose application—regenerating liver

Single doses of DENA ranging from 0·3 to 140 mg/kg were administered orally to rats 24 hr after two-thirds hepatectomy, 4–9 animals being used for each dose level in 3 experiments comprising 0·3–30, 0·6–10, and 50–140 mg DENA/kg. The highest dose was lethal but provision of 10% glucose in the drinking water kept half of these rats alive. After 8 weeks ATPase-deficient islands of a similar character as described previously [5], were present in the livers of all rats. In rats which had received a dose of up to 70 mg DENA/kg, liver tissue surrounding the islands was normal in respect to structure, cell morphology, ATPase and glucose-6-phosphatase staining. Higher doses may lead to bile-filled cysts and cirrhosis.

Effect of DENA on liver regeneration

The number of islands induced by a given dose of DENA is considerably enhanced by prior two-thirds hepatectomy [5]. Most probably, DNA synthesis occurring in the regenerative response, enhances the sensitivity of the liver cells to the action of the carcinogen [11–13]. Thus, for dose–response studies in partially hepatectomized rats it is essential to know if liver regeneration is affected in the dose range used. Liver weight and DNA content are recorded in Fig. 1 as a function of DENA dose, 48 hr after the latter's application which corresponds to 72 hr after partial hepatectomy. No effects were seen up to 30 mg DENA/kg, but a dose of 50 mg/kg significantly retarded liver regeneration as shown by the decrease in liver weight and DNA content.

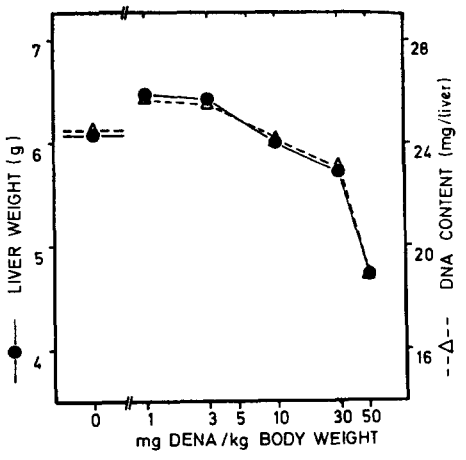


Fig. 1. Weight and DNA content of rat liver 72 hr after partial hepatectomy, as function of the dose of a single application of DENA given 24 hr after the operation. The mean liver weight and DNA content before partial hepatectomy amounted to 9 g and 29 mg respectively. Seventy per cent of the livers were removed by the operation.

Number of islands versus dose

The dose–response curve for island induction is illustrated in Fig. 2 in double logarithmic plot. The curve consists of an ascending limb, a plateau and a descending limb. The ascending limb has a slope of +1 and therefore direct proportionality is obtained between number of islands induced and dose in the range of 0·3 to 30 mg DENA/kg. For doses of ≧ 50 mg DENA/kg no further increase but rather a slight decrease in island number at the highest doses was obtained. Such a diminuation of the response may be the result of toxic manifestations (see previous section) and/or changes in the metabolism of DENA at high dose level.

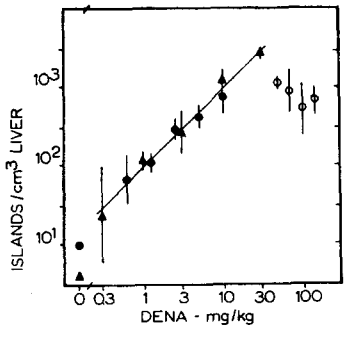


Fig. 2. Number of ATPase-deficient islands as function of the dose of a single application of DENA to partially hepatectomized rats. Results of three experiments. Vertical bars indicate the s.d., the slope of the straight line is +1.

Island size vs. dose

As illustrated in Fig. 3 the size distribution of the islands 8 weeks after induction was about equal for islands induced by doses up to 30 mg DENA/kg. At higher doses (50–140 mg/kg) the size of the islands increased progressively with dose.

Since the mean size of the cells in the various islands was similar and independent of dose, the number of cells per average island (calculated from the mean island volume) was similar up to 30 mg DENA/kg, but increased from 50 to 140 mg/kg (Table 1).

Since islands are most probably derived from single liver cells altered by DENA action [6], it follows that island cells induced by the higher DENA doses had proliferated faster than had those induced by the lower doses. Calculation (from Table 1) of the mean population doubling time of the island cells, assuming all cells to be in cycle and no cell loss, showed

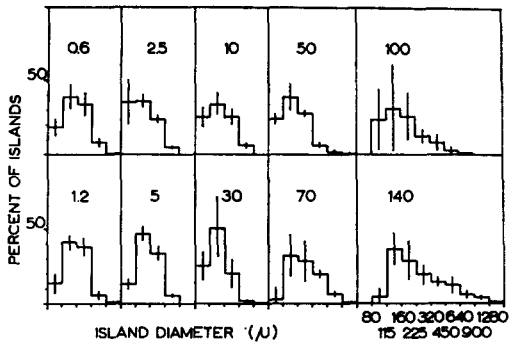


Fig. 3. Size distribution of ATPase deficient islands as a function of the dose of a single application of DENA to partially hepatectomized rats. The mean error is indicated by vertical bars; the dose by figures (mg/kg).

that the cells induced by the highest dose (140 mg DENA/kg) proliferated about two times faster than did island cells induced by 30 mg DENA/kg and lower doses.

Increased island cell proliferation appears to come into operation at a dose level of about 50 mg DENA/kg and thus coincides with the beginning of the plateau in the dose–response curve of island induction (Fig. 2) and the manifestation of toxicity (Fig. 1). Thus, rather than being intrinsically capable of faster growth, the island cells induced by the higher DENA doses may proliferate more rapidly as a result of the toxic effect of this dose level on the liver (see "Discussion").

Total number of island cells per liver versus dose

From the number and size of the islands and the number of cells/cm³ of island tissue [5], the volumetric fraction occupied by island cells and the total number of these cells in the livers 8 weeks after induction by the various single doses of DENA, can be calculated. Results

Table 1. *Mean number of parenchymal cells per ATPase-deficient island* 57 *to* 61 *days after DENA application to* 2/3 *hepatectomized rats*

One cm^3 of island tissue contains $7{\cdot}8 \times 10^7$ cells [5].

Dose of DENA (mg/kg)	0·6	1·2	2·5	5	10	30	50	70	100	140
Cells per island	144	125	100	119	125	90	190	365	1830	3700

illustrated in Fig. 4 show that the curve relating the volumetric fraction and the total number of the island cells to the dose has two main portions:

1. A linear increase in the low dose range due to the direct proportionality between dose and number of islands of equal size and cell content.

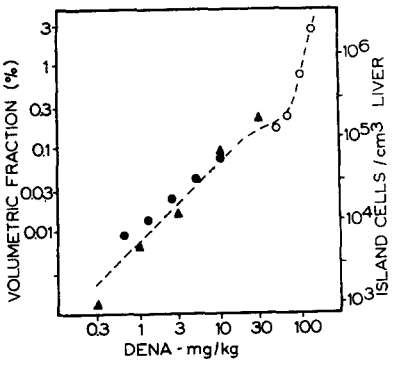


Fig. 4. *Volumetric fraction of liver occupied by ATPase-deficient islands and calculated number of island cells as function of the dose of a single application of DENA to partially hepatectomized rats. Results from three experiments.*

2. A sudden increase in the high dose range where the decrease in island number is far outweighed by the increase in number of cells per island due to their faster proliferation.

Other phenotypic markers

It has been demonstrated previously [5] that among islands a distinction can be made according to whether they demonstrate ATPase deficiency alone or glucose-6-phosphatase deficiency and/or glycogen retention after fasting in addition. The correlation between ATPase deficiency and the other two phenotypic characters was studied quantitatively on parallel sections for the dose range 0·6–10 mg DENA/kg. The relative frequencies of the three staining characters appeared to be independent of dose in this range. Thus for all doses examined it was found that of the ATPase-deficient islands, about 17% contained glycogen after fasting and that half of the latter islands was also deficient in glucose-6-phosphatase staining. Given the direct proportionality between number of ATPase-deficient islands and dose, the latter result implies that a similar relation exists between dose and induction of islands with the two additional characters.

Multiple dose application—intact liver

A similar type of experiment was carried out with intact rats (no partial hepatectomy, 4 rats per dose level) which received 0·9–14 mg DENA/kg daily for 4 weeks, measurements being made after another 6 weeks. The liver surrounding the ATPase-deficient islands was

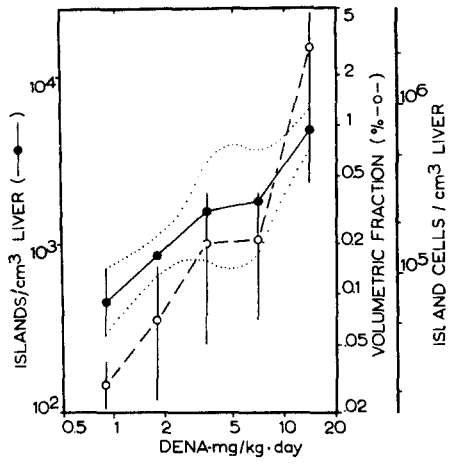


Fig. 5. *Number of ATPase-deficient islands, volumetric fraction of liver occupied by islands, and calculated number of island cells as function of the daily dose of DENA applied for* 4 *weeks to intact rats. The vertical bars and dotted lines indicate the s.d.*

normal for doses up to 7 mg/kg day; at 14 mg/kg day, biliary cysts were present. The dose–response curve in terms of number of islands induced is illustrated in Fig. 5. For the lower doses at least, a slope of +1 is obtained, and, although a straight line could be constructed for the whole dose range, it appears that irregularities occur (see dotted lines for s.d.) above 3·5 mg/kg day, a dose which previously has been shown to exert a toxic effect in rat liver [7].

The volumetric fraction of liver occupied by island cells and the total number of these cells as a function of DENA dose shows a marked increase between 7 and 14 mg DENA/kg day

(Fig. 5), reflecting the increase in the size of the islands (growth rate of the island cells) induced by the latter as compared with the former dose level (Fig. 6). At ≦ 7 mg DENA/kg day the size distribution of the islands was

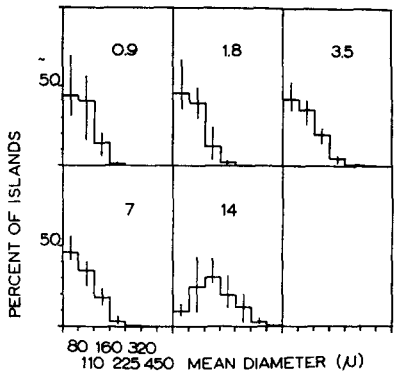


Fig. 6. Size distribution of ATPase-deficient islands as function of the daily dose of DENA applied for 4 weeks to intact rats. Vertical bars indicate the deviation between the 4 animals within a group. Figures the daily dose (mg/kg).

independent of dose, and similar to that obtained after single low doses and partial hepatectomy (Fig. 3).

Parenthetically, glucose-6-phosphatase deficient islands were present in small number, amounting to 5–10% of the number of ATPase-deficient islands. Some 90% of the glucose-6-phosphatase deficient islands were also ATPase deficient. The islands which were both ATPase and glucose-6-phosphatase deficient were generally somewhat larger than ATPase-deficient islands positive in glucose-6-phosphatase. The very few glucose-6-phosphatase deficient islands normal in ATPase staining were small with a diameter of up to 110 μm.

Liver tumour formation by a single application of 50 mg DENA/kg, or higher, to partially hepatectomized rats

Two to three month-old female Sprague Dawley rats used throughout this investigation, received one dose of 20, 50, 70, 140 or 200 mg DENA/kg 24 hr after two-thirds hepatectomy. Apart from acute death, all animals had died by (sacrificed when moribund, e.g. from mammary tumours) or were sacrificed after 24 months. Confirming previous results [7], a dose of 20 mg/kg did not produce carcinomas; only many microscopically large and a few macroscopical islands were observed. However, various types of liver tumours, adequately described by other authors [14, 15], were obtained by the higher DENA doses (Table 2). The hepatocellular tumours were mainly trabecular, well differentiated, and contained glycogen. Glycogen-free carcinomas, lacking trabecular organization and cytoplasmic differentiation of basophilic material were also present next to more anaplastic tumours. The latter tumours, which were the main cause of death by leading to abdominal hemorrhage were indistinguishable from similar tumours found after repeated DENA application; it has previously been argued [7] that such tumours may be of hepatocellular origin. At ≧ 100 mg DENA/kg, no such malignant tumours were encountered, only well differentiated glycogen-containing hepatomas being present; the reproducibility of this result should, however, be tested in a larger experiment. In 4 of 7 animals of these groups, the livers were completely altered by very large biliary cysts.

In the 50 and 70 mg/kg groups the tumours were present in livers which contained well-outlined islands; disorganization of the liver structure hampered clear recognition of islands

Table 2. Formation of hepatocellular carcinomas after a single dose of DENA given to partially hepatectomized rats

Dose mg DENA/kg	Number of animals Total	Acute death	Survivors	Time of death (months)	Hepatocellular carcinomas among survivors
20	9	0	9	24*	0
50	6	0	6	14–22	3/6
70	7	1	6	15–22	3/6
100	5	2	3	13–18	2/3
140†	6	3	3	14–24	1/3
200†	20	19	1	21	0/1

*Sacrificed

†10% glucose in drinking water supplied during 1 week after DENA application.

above 70 mg DENA/kg. Most interestingly, in a number of former cases, a clear topological association between small tumours and (remnants of) island tissue was observed. Figures 7 and 8 are typical illustrations of the presence of a cluster of glycogen-free tumour cells closely associated over a large part of its periphery with an island cell population deficient in ATPase but containing glycogen. These associations were found in the liver of rats which were moribund from mammary tumour—the common cause of death of Sprague Dawley females—and in which liver tumours had not yet reached full or lethal size.

DISCUSSION

Mechanism of island cell formation

Foci of liver cells (islands) characteristically and permanently changed in phenotypic markers and growth behaviour are induced by a single application of a hepatocarcinogen such as DENA [5]. Island formation may represent a sensitive system for studying and assaying a stable precancerous alteration and the process of liver carcinogenesis. Although in general "precancerous" is a rather loose term, it is applied here to a well-defined cell type, the island cell, which according to previous results may serve as a precursor in the process of liver tumour formation [7].

In the present experiments direct proportionality between number of islands induced and DENA in the dose range of 0·3–30 mg/kg for single application and regenerating liver, and 0·9–3·6 mg/kg day for multiple application and intact liver, was obtained. In these dose ranges no toxic effects on the liver were observed. The formation of N-7-ethylguanine in the DNA of the regenerating liver system also showed direct proportionality to the doses studied, i.e. 1–10 mg DENA/kg [16]; a similar result has been reported for dimethylnitrosamine [17]. N-7-ethylguanine, and/or other ethylated products of DNA—which may be expected to exhibit a similar dose dependency [18]—, might be instrumental in the action of DENA at the genetic level in carcinogenesis [19, 20].

From these kinetics the conclusion is drawn that the conversion or "transformation" of a liver cell into an island-forming cell, which then grows out into a microscopical island, most probably occurs by a one-hit process, i.e. by one specific alteration produced by DENA at a critical site of its target cell. The persistently changed phenotype, the very low probability of the induction event (after partial hepatectomy one liver cell per 10^6 being transformed per mg DENA/kg) and the underlying one-hit mechanism suggest that the island cells are permanently altered at the genetic level. Accordingly, the island cell type could result from one specific alteration produced by DENA in the DNA of a liver cell. Partial hepatectomy profoundly increases the number of islands produced by DENA [5]. According to the present view, DNA synthesis triggered by the operation would serve to fix the one-hit structural alteration in DNA, responsible for island cell formation, as a heriditary property prior to its repair [21, 22].

The one-hit kinetics pertain not only to the induction of ATPase-deficient islands but also to those islands which exhibit additional glucose-6-phosphatase deficiency and/or glycogen retention after fasting. This would indicate that the hit may be scored in a DNA segment that, either directly or indirectly, informs or controls various cellular properties.

The relative and absolute decrease in the number of islands induced by a single application of $\geqq$ 50 mg DENA/kg may have various reasons. The diminuation of the response might result from toxic, viz. lethal or sterilising, hits accumulating in cells which have received the "transformation" hit. Secondly, the metabolic capacity of the two-thirds hepatectomized liver to convert DENA into its reactive and biologically active product ($C_2H_5^+$) may be a limiting factor in the case of these higher DENA concentrations. Under the latter conditions, the activity of the microsomal N-deethylating enzymes (which convert DENA to reactive metabolite) may even be further restrained by "self-poisoning" so that a disproportion of the DENA is excreted or metabolized elsewhere in the body [23]. Thirdly, toxic signs in the regenerating liver begin to appear at 50 mg DENA/kg (Fig. 1) and since regeneration enhances island formation [5], impairment of regeneration (DNA synthesis) will decrease island formation.

The island-forming cell and its progeny, the island cells, have apparently acquired the capacity to proliferate, at least in partial independence of the growth control operating in liver [5]. The "growth autonomy" of the island cells is not absolute since island cells induced in intact liver react to partial hepatectomy with increased proliferation [24]. Thus, the regenerative response stemming from the toxic effect of DENA may also speed up island cell growth, as in the case of the 14 mg/kg day dose administered to intact rats (Fig. 5). The situation in the regenerating liver of rats receiving a single toxic application of 70 mg

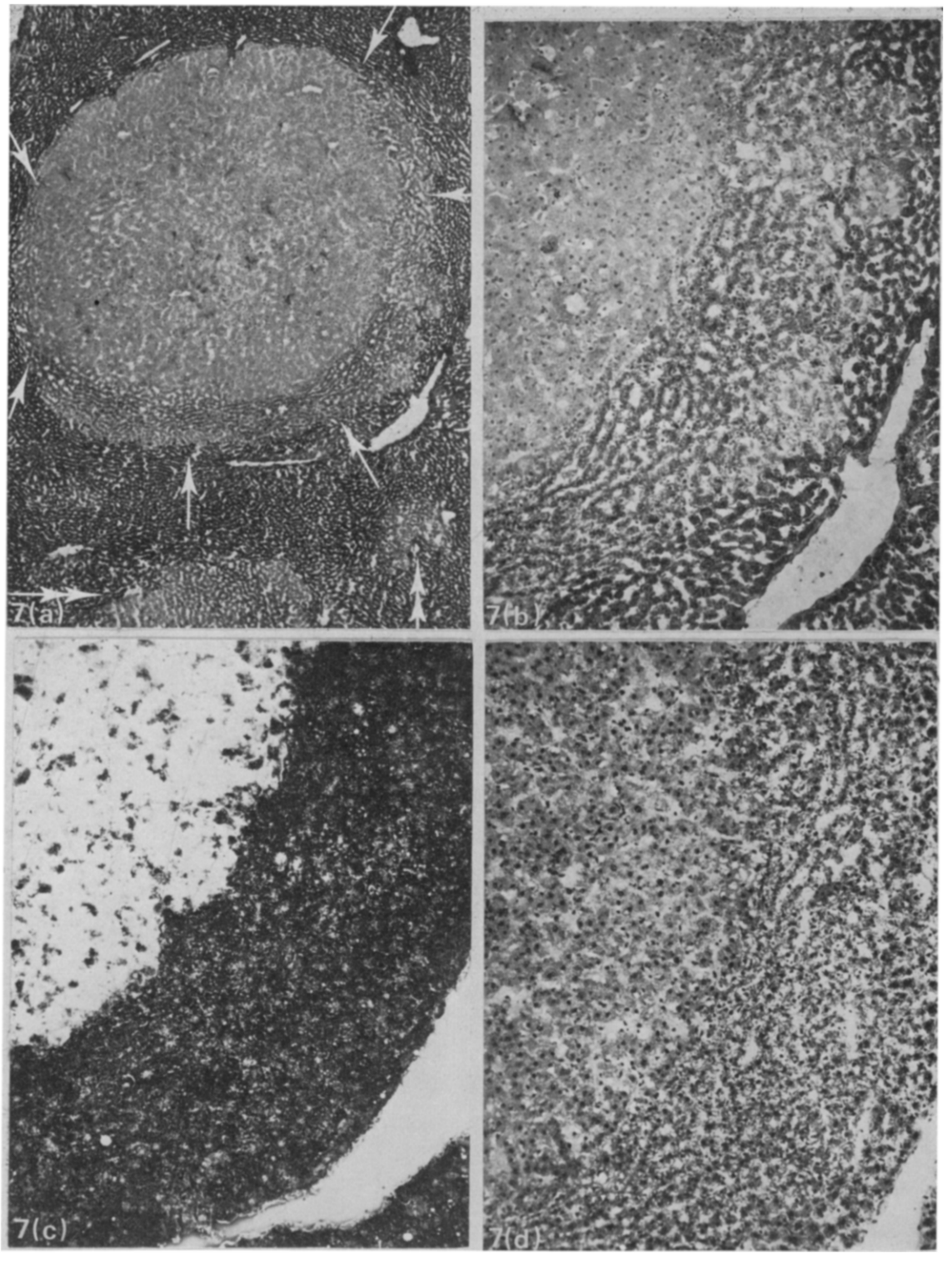


Fig. 7. Close tumour-island association detected in the liver of a non-fasted rat by ATPase staining (a, b). The tumour (trabecular pattern, glycogen deficient (c), basophilic staining (cresylviolet) evenly distributed over the cytoplasm (d)) is in close contact with island cells (less deficient in ATPase staining than the tumour cells (a, b), PAS positive (c), normal basophilic staining of the cytoplasm (d)) over a large part (a, arrows ◀) of its periphery. Two other islands in (a) are indicated by arrows ◀◀ (a, × 30; b–d, × 120).

(to face p. 694)

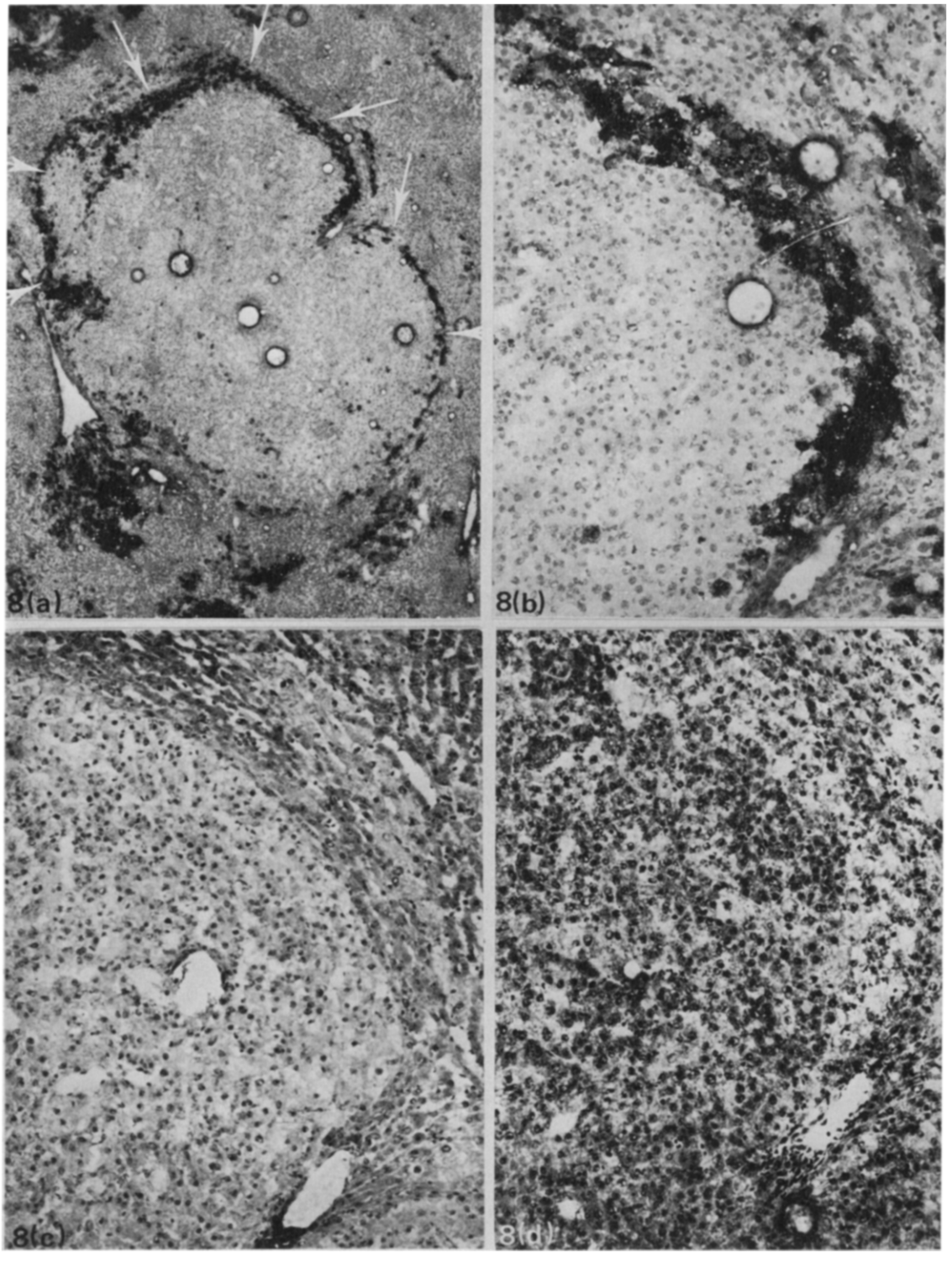


Fig. 8. *Close tumour-island association detected in the liver of a fasted rat by PAS staining* (*a*, *b*). *The PAS negative tumour shows close contact with island cells* (*PAS positive* (*b*), *ATPase deficient* (*c*), *less basophilic than normal liver cells* (*d*)) *over a large part of its periphery* (*a*, *arrows*). *The surrounding liver is compressed by the tumour* (*a*, × 30; *b*, × 120).

DENA/kg or higher may be basically similar but more complicated. Regeneration in these livers is impaired but since the liver weight is fully restored in the course of time, the process should be more protracted as compared with livers subjected to lower (relatively) non-toxic doses of DENA. This more protracted regenerative response may afford an extra growth stimulus to the island cells leading to the observed increase in their numbers (Fig. 4). These effects are likely to be involved in the carcinogenic process since the rate of liver tumour formation has previously been shown to be a function of the total number of island cells present in the liver [7]. Accordingly, toxic effects of carcinogens below the cytolethal level may play an important role in carcinogenesis by providing a growth stimulus for precancerous cells.

Mechanism of liver tumour formation

Liver tumours are induced by a single application of ≧ 50 mg DENA/kg to partially hepatectomized rats [21]. In these livers and in livers of rats receiving one subcarcinogenic dose island cells of similar morphological and enzyme histochemical phenotypes arise and proliferate, although at different rates. The island cells induced by a subcarcinogenic dose result from a one-hit process and, as demonstrated previously [7], such cells may serve as substrate for further carcinogenic action—apparently by receiving additional, i.e. sequential hits—to yield liver tumours. Combining these observations may lead one to suppose that one way in which tumour cells could be formed by a single application of ≧ 50 mg DENA/kg is from liver cells which have received at least 2 concomitant hits, one conferring island-cell character and one allowing such cells to progress in time to malignancy without the need for further carcinogen application. Some morphological support for this view is offered by Fig. 7 and 8, in which tumour cells seem to arise focally in close association with island tissue, although the islands *per se* most probably are of clonal origin [6]. The focal progression in the absence of carcinogen, i.e. the quasi-spontaneous development of heterogeneity in a seemingly homogeneous island cell population [25], could be caused by a small decrease in the fidelity of DNA replication [26, 27]. This decrease may result from a mutation affecting the DNA polymerase [26, 27], induced by a second hit as discussed above. However, decreased fidelity of DNA replication might also be part of the complex of changes (glucose-6-phosphatase deficiency, glycogen retention, ATPase deficiency, etc. [5]) observed in islands induced by only one specific hit. In this case, as compared with the situation of a subcarcinogenic dose, only the greater number of proliferating island cells would decide the malignant outcome, since it would favour the establishment of the critical error required for progression. A third possibility to explain progression is that the normal background ("noise") infidelity of DNA replication might suffice for progression of island cells, again provided that enough proliferating cell at risk and time would be available.

For its operation each of these mechanisms requires DNA synthesis not only for the production of a large number of cells at risk but also for making an error in a critical gene segment and perpetuating that error by another round of DNA synthesis in the face of DNA repair. In addition, the randomness of the genetic changes resulting from infidel DNA replication may explain the general finding [4] that divergent tumour types do arise in liver and that similar types may be antigenically different [28].

Finally, it may be concluded that the well-known enhancing effect of cell proliferation (DNA synthesis [11, 12]) on carcinogenesis is exerted at and beyond the induction stage proper, increasing the number of both the precancerous cells induced and their progeny which constitutes the population at risk to progress to malignancy, and favouring the latter transition.

Acknowledgement—We wish to thank Miss B. Mülder and Mrs. M. van der Steen-Kamerbeek for their competent and careful technical assistance.

REFERENCES

1. W. Nakahara, Critique of carcinogenic mechanism. *Progr. exp. Tumor Res.* **2,** 158 (1961).
2. R. K. Boutwell, Some aspects of skin carcinogenesis. *Progr. exp. Tumor Res.* **4,** 207 (1964).
3. P. Armitage and R. Doll, Stochastic models for carcinogenesis. *Proc. IV. Berkeley Symposium Math. Stat. Probab.* **4,** 19, Univ. California Press, Los Angeles (1961).

4. E. Farber, Carcinogenesis-cellular evolution as a unifying thread: Presidential address. *Cancer Res.* **33,** 2537 (1973).
5. E. Scherer, M. Hoffmann, P. Emmelot and H. Friedrich-Freska, Quantitative study on foci of altered liver cells induced in the rat by a single dose of diethylnitrosamine and partial hepatectomy. *J. natl. Cancer Inst.* **49,** 93 (1972).
6. E. Scherer and M. Hoffmann, Probable clonal genesis of cellular islands induced in rat liver by diethylnitrosamine. *Europ. J. Cancer* **7,** 369 (1971).
7. E. Scherer and P. Emmelot, Foci of altered liver cells induced by a single dose of diethylnitrosamine and partial hepatectomy: Their contribution to hepatocarcinogenesis in the rat. *Europ. J. Cancer* **11,** 145 (1975).
8. G. M. Higgins and R. M. Anderson, Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch. Path.* **12,** 186 (1931).
9. H. N. Munro and A. Fleck, The determination of nucleic acids, *Meth. biochem. Anal.* **14,** 159 (1966).
10. G. Ceriotti, A microchemical determination of desoxyribonucleic acid. *J. biol. Biochem.* **198,** 297 (1952).
11. G. P. Warwick, Effect of the cell cycle on carcinogenesis. *Fed. Proc.* **30,** 1760 (1971).
12. B. A. Kihlman, *Action of Chemicals on Dividing Cells,* Prentice Hall, London (1966).
13. V. M. Craddock, Liver carcinomas induced in rats by single administration of dimethylnitrosamine after partial hepatectomy, *J. natl. Cancer Inst.* **47,** 889 (1971).
14. H. L. Stewart and K. C. Snell, The histopathology of experimental tumors of the liver of the rat. *Acta Un. int. Cancer* **13,** 770 (1957).
15. W. H. Butler, Pathology of liver cancer in experimental animals. *IARC scientific publ.* **1,** 30 IARC, Lyon (1971).
16. E. Scherer and P. Emmelot, unpublished results.
17. V. M. Craddock, Stability of deoxyribonucleic acid methylated in the intact animal by administration of dimethylnitrosamine. *Biochem. J.* **111,** 497 (1969).
18. M. Horikawa, M. Fukuhara, and F. Suzuki, O. Nikaido and T. Suguhara, Comparative studies on induction and rejoining on DNA single-strand breaks by radiation and chemical carcinogen in mammalian cells *in vitro*. *Exp. cell Res.* **70,** 349 (1972).
19. P. D. Lawley, Some aspects of the cellular response to chemical modifications of nucleic acid purines. In *The Purines, Theory and Experiment* Edited by E. D. Bergmann and P. Pullman p. 579, Israel Academy of Sciences and Humanities, Jerusalem (1972).
20. P. Bannon and W. Verly, Alkylation of phosphates and stability of phosphate triesters in DNA. *Europ. J. Biochem.* **31,** 103 (1972).
21. V. M. Craddock, Induction of liver tumours in rats by a single treatment with nitroso compounds given after partial hepatectomy. *Nature (Lond.)* **245,** 386 (1973).
22. R. Goth and M. J. Rajewsky, Persistence of 06-ethylguanine in rat brain: correlation with nervous system-specific carcinogenesis by ethylnitrosourea. *Proc. nat. Acad. Sci. (Wash.)* **71,** 639 (1974).
23. L. Den Engelse and P. Emmelot, Effects of feeding the carcinogen dimethylnitrosamine on its metabolism and methylation of DNA in the mouse. *Chem.-biol. Inter.* **4,** 321 (1971/72).
24. H. Rabes, R. Hartenstein and P. Scholze, Specific stages of cellular response to homeostatic control during diethylnitrosamine-induced liver carcinogenesis. *Experientia* **26,** 1356 (1970).
25. L. Foulds, *Neoplastic Development* Vol. 1, Academic Press, London and New York (1969).
26. R. L. Nelson and H. S. Mason, An explicit hypothesis for chemical carcinogenesis. *J. theor. Biol.* **37,** 197 (1972).
27. L. A. Loeb, C. F. Springgate and N. Battula, Errors in DNA replication as a basis of malignant changes, *Cancer Res.* **34,** 2311 (1974).
28. R. W. Baldwin, Immunological Aspects of Chemical Carcinogenesis. *Advanc. Cancer Res.* **18,** 1 (1973).

Europ. J. Cancer Vol. 11, pp. 697–707. Pergamon Press 1975. Printed in Great Britain

Comparison of the Human Pharmacokinetics of VM-26 and VP-16, Two Antineoplastic Epipodophyllotoxin Glucopyranoside Derivatives

LARRY M. ALLEN and PATRICK J. CREAVEN

Oncologic Pharmacology Section, National Cancer Institute-Veterans Administration, Medical Oncology Branch, Veterans Administration Hospital, Washington, D.C. 20422, *U.S.A.*

Abstract—*The pharmacokinetics of the epipodophyllotoxin glucoside derivatives VM-26 and VP-16 have been evaluated in patients who have received* 67-90 *mg/m² of VM-26 and* 130-290 *mg/m² of VP-16 by short intravenous infusion.*

Whereas VM-26 plasma decay kinetics follow a triexponential function and correspond to a three-compartment open model (mammillary type), VP-16 plasma decay kinetics follow a biexponential decay and correspond to a two-compartment open model.

The 3-times-larger excretion rate (0·112 hr^{-1}) *and correspondingly larger renal clearance* (13·6 *ml/min*) *of VP-16 over VM-26 is correlated with the 3-times-larger equitoxic dose* (290 *mg/m²*).

Although VM-26 appears to be slightly more metabolized (86% *vs.* 66%) *than VP-16, the much larger tolerated dose of VP-16 can compensate for this difference. The discrepancy between the fraction of drug metabolized based on pharmacokinetic analysis and fraction of cumulative metabolite in the urine is explained on the grounds of extensive tissue storage of the parent compounds or their metabolites. The extent of tissue depot was evaluated by reference to body water and indicates it to be more extensive than some long-acting barbiturates. The integral coefficient for VM-26 and VP-16 in the central* (1·14 *vs* 2·26 *hr*) *and peripheral* (3.74 *vs* 3·93 *hr*) *compartment* 2 *suggest that the bioavailability of VM-26 to be only slightly less than VP-16 in those compartments. On the other hand, the bioavailability is greater for VM-26 in peripheral compartment* 3 *compared to peripheral compartment* 2 *of VP-16.*

INTRODUCTION

THE CYTOSTATIC activity of the root of Podophyllum peltatum (May Apple, Mandrake) was first reported by Bentley in 1861 [1] and it was used in the treatment of condyloma accuminatum and other benign proliferative skin lesions [2]. The principal constituents of podophyllin extract (podophyllotoxin) were shown to be metaphase blockers similar to colchicine and the Vinca alkaloids [3, 4]. Numerous attempts to modify the chemical structure of podophyllotoxin led initially to two derivatives which underwent clinical evaluation, both of which proved to be too toxic for clinical usefulness, podophyllic acid ethyl hydrazide and a mixture of purified benzylidene glucosides of Podophyllum emodi [5–7]. A systematic search for more potent and less toxic semi-synthetic podophyllotoxin derivatives [8] led to the introduction into clinical trial in Europe [9–11] and the United States [12–14] of two promising novel epipodophyllotoxin glucopyranosyl derivatives: VM-26, 4′-demethyl-epipodophyllotoxin-9-(4,6-0-thenylidene-β-D-glucopyranoside) and

Accepted 30 May 1975.

VP-16-213, the 4,6-0-ethylidene congener (Fig. 1). These derivatives were found to be active against Sarcoma 180, Ehrlich ascites tumor, and Walker 256 carcinosarcoma, and to be unusual for plant products in that they showed a high degree of oncolytic activity in mice bearing intraperitoneal and intracerebral L1210 leukemia [8, 15–17].

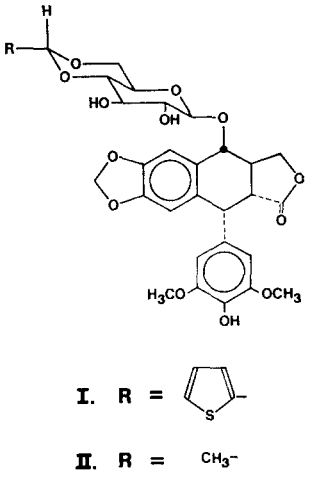


Fig. 1. *Chemical structures of VM-26 (I) and VP-16 (II). The (●) is the position of the tritium label.*

In contrast to other podophyllotoxin analogs, and to colchicine and vinblastine, these new demethylepipodophyllotoxin glucosides do not lead to an increase of cells in metaphase but to a decrease in their mitotic index [15]. The inhibition of DNA, RNA, and protein synthesis by VM-26 and VP-16 in P815 mastocytoma and more recently VM-26 in human lymphoblasts was determined to be secondary to inhibition of cells in the G_2-phase of the cell cycle [18–21]. In addition to this new mechanism of action, VM-26 has been reported to inhibit respiration by Ehrlich ascites tumor cells and electron transport at NADH-dehydrogenase level of mitochondrial and submitochondrial particles from rat liver [22].

Although VM-26 and VP-16 have been evaluated in relatively few tumor types in man, they have shown activity in Hodgkin's disease, non-Hodgkin's lymphoma, and bladder cancer [9-11, 23], and VM-26 has in addition been reported to have activity in brain tumors [13].

Whereas the maximum tolerated dose (MTD) of VM-26 given once weekly in man was found by Muggia *et al.* [12] to be 67 mg/m^2, the MTD for VP-16 given in the same way was recently reported to be 290 mg/m^2 [14]. Furthermore, we have reported recently on some interesting differences in the clinical pharmacology of these compounds [24, 25]. We have now analyzed in some detail the human pharmacokinetics of VM-26 and VP-16 in order to attempt to explain the difference in the equitoxic doses of these two closely related compounds and to account for the different patterns in their plasma decay kinetics [26, 27].

MATERIAL AND METHODS

The pharmacokinetics of VM-26 was evaluated in 5 patients who received 67 mg/m^2 and in one patient who received 90 mg/m^2. The pharmacokinetics of VP-16 was evaluated in 11 patients who received 130-290 mg/m^2 in the course of the Phase-1 clinical trials and for whom a complete set of plasma and urine data was available [26].

VM-26 and VP-16 were obtained from Drug Research and Development (DRD), National Cancer Institute (NCI), Bethesda, Maryland, U.S.A. Tritium-labelled drugs were obtained from Dr. H. Stähelin of Sandoz, Ltd., Basel through DRD, NCI (Fig. 1). VM-26 has a specific activity of 64·419 mCi/mmole and VP-16 has a specific activity of 14·112 mCi/mmole. Both drugs were given as constant i.v. infusions over periods of one-half to one hour. All patients gave written informed consent to entry into the study which was approved by the Human Investigations Subcommittee of this Hospital. Other criteria for entry into the study and patient information are reported elsewhere [24, 25].

Unchanged drug was determined in plasma and urine by $CHCl_3$ extraction and radioisotope counting as described previously [24, 25]. Protein binding determinations were carried out essentially as described before using 5×10^{-4} crystalline human serum albumin [28]. The fraction of binding of radioactive drug was determined from samples taken from inside and outside the dialysis bag after 48 hr at 37°. The data was plotted according to Scatchard and evaluated by least squares regression with an electronic calculator or digital computer [29]. Binding to calf thymus DNA was determined by methods previously described [30–32].

The assumptions usually made in an approach to the analysis of the distribution properties of a drug in a two- or three-compartment open model using common pharmacokinetic expressions [33–35] are: (a) the drug instantaneously equilibrates between the different components in the central compartment volume and, (b) the dose is sufficiently low that metabolic capacity of the body is independent of concentration, and (c) the binding capacity of proteins is independent of concentration. That is, the

concentration is sufficiently low at all times to give a linear relationship between bound and unbound drug.

The best-fit numerical values of the constants in the nonlinear equations describing the time course of the plasma concentration according to the two- and three-compartment open models for VP-16 and VM-26 respectively, were determined by the modified Gauss–Newton procedure [36] in the computer program *NONLIN* [37] as executed on an IBM-370/165 digital computer. The reciprocal of the square of the plasma concentrations were used as weighting factors in the weighted least-squares fitting [38]. Goodness of fit and appropriateness of model were judged from the computer output of the 95% confidence intervals (S-plane and uniplanar), statistical tests on signed residuals [39], correlation matrix, and eigenvalue-eigenvector matrix [40–42].

Renal clearances were evaluated with the aid of the computer program package *MLAB* [43, 44] on a DEC system-10 digital computer. Other calculations were conveniently done by programs we have written for the Wang programmable calculator which are available upon request.

RESULTS

The plasma concentration versus time curve for VM-26 showed a triexponential decay (Fig. 2) where the time dependent variation of the plasma concentration (C_p) could best be described by the equation for a three-compartment open model (mammillary type) as shown in Fig. 3B. In contrast, the plasma concentration versus time curve for VP-16 shows a biexponential decay (Fig. 2) where the time dependent variation of the plasma concentration (C_p) could best be described in Fig. 3A. The post-infusion parameters of these two equations for VM-26 and VP-16 are shown in Table 1. The concentration dependent parameters in Table 1 obtained from the post-infusion evaluation of the plasma concentration versus time plots were corrected for periods of constant rate infusion as described by Loo and Riegelman [45] which enabled assessment of the pharmacokinetic constants and calculation of microscopic transfer constants in Fig. 3A and 3B for the pharmacokinetic models of VP-16 and VM-26 respectively [45, 46].

The distribution volumes for VM-26 and VP-16 were evaluated as described previously [35, 46] and are tabulated in Table 2 where $(Vd)_\beta$ or $_\gamma$ describes the volume of distribution during the β- or γ-phase, respectively, and represents the relative proportion of drug in the body during the β-(γ) or post-distributive phase [46]. V_{t1} and V_{t2} represent the volumes of the peripheral tissue compartment in Fig. 3A and 3B.

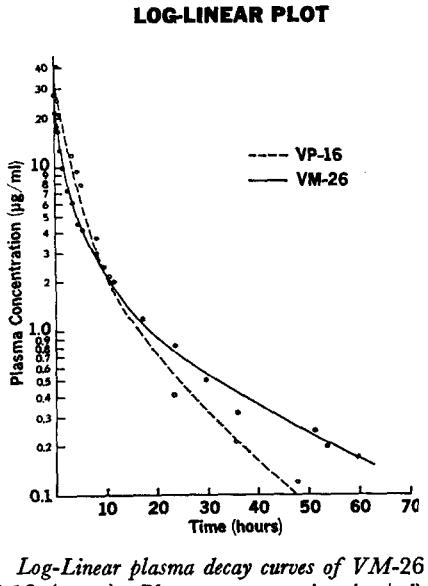


Fig. 2. Log-Linear plasma decay curves of VM-26 (——) and VP-16 (– – –). Plasma concentration (µg/ml) against time (hour). Patient FJ for VM-26 and HR for VP-16.

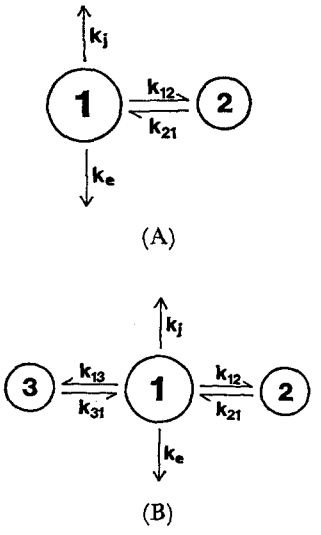


Fig. 3. (A) Two-compartment open pharmacokinetic model scheme. k_{12}, k_{21} are linear transfer constants between central (1) and peripheral compartment (2). k_e and k_j represent excretion rate to urine and all other elimination processes respectively. k_{el}, the elimination rate constant, equals k_e+k_j. Vc in Table 2 is central compartment (1) volume and V_{t1} is peripheral or tissue compartment (2) volume. (B) Three-compartment open pharmacokinetic model (mammillary type) scheme. k_{12}, k_{21}, k_e, and k_j are as in Fig. 3A. k_{13} and k_{31} are linear transfer constants between central (1) and an additional peripheral compartment (3). Vc in Table 2 is central compartment (1) and V_{t1} and V_t2 are peripheral or tissue compartments (2 and 3) volumes.

Table 1. *Data obtained from single intravenous infusion of VM-26 and VP-16 in humans*

For VM-26, $Cp = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$ and VP-16, $Cp = Ae^{-\alpha t} + Be^{-\beta t}$

Subject (mg/m²)	Dose (mg)	A, μg/ml (±S.D.)	α, hr⁻¹ (±S.D.)	B, μg/ml (±S.D.)	β, hr⁻¹ (±S.D.)	C*, μg/ml (±S.D.)	γ*, hr⁻¹ (±S.D.)
VM-26							
DS (67)	100	14·37 (0·41)	1·292 (0·062)	6·34 (0.46)	0·198 (0·009)	0·96 (0·06)	0·018 (0·001)
JCS (67)	88	12·63 (0·47)	1·175 (0·103)	6·47 (0·72)	0·168 (0·008)	2·48 (0·28)	0·048 (0·003)
EM (67)	130	10·97 (0·47)	1·511 (0·147)	9·43 (0·59)	0·181 (0·007)	1·81 (0·14)	0·034 (0·002)
JC (67)	95	7·22 (1·12)	3·978 (1·538)	11·19 (1·37)	0·365 (0·041)	2·55 (0·50)	0·040 (0·007)
CD (67)	123	23·16 (0·95)	1·082 (0·100)	7·65 (1·23)	0·130 (0·013)	1·97 (0·30)	0·027 (0·004)
FJ (90)	121	16·17 (0·72)	1·726 (0·144)	9·63 (0·83)	0·121 (0·013)	1·65 (0·16)	0·038 (0·004)
VP-16							
CD (130)	230	20·54 (2·42)	2·445 (0·449)	12·52 (0·97)	0·161 (0·015)		
NG (130)	205	18·72 (1·00)	1·281 (0·291)	8·12 (0·98)	0·099 (·0007)		
KR (170)	255	10·67 (1·39)	1·740 (0·124)	20·68 (1·33)	0·236 (0·016)		
AA (170)	350	16·07 (1·05)	3·630 (0·549)	17·15 (0·95)	0·134 (0·012)		
TS (170)	230	15·86 (2·62)	1·416 (0·139)	11·14 (0·96)	0·103 (0·012)		
JB (220)	415	28·88 (1·27)	0·354 (0·071)	2·13 (0·91)	0·057 (0·032)		
RJ (220)	363	28·61 (1·21)	0·670 (0·084)	5·02 (0·57)	0·073 (0·019)		
PR (220)	415	23·29 (0·83)	0·377 (0·043)	4·32 (0·57)	0·061 (0·012)		
HR (290)	400	31·81 (2·91)	0·392 (0·043)	4·01 (0·62)	0·083 (0·014)		
KR (290)†	425	34·82 (2·33)	0·298 (0·035)	3·62 (1·44)	0·046 (0·013)		
LBJ (290)	430	21·00 (4·95)	0·364 (0·084)	7·28 (4·95)	0·092 (0·024)		

*Not applicable to VP-16.
†Same patient another course.

Table 2. *Distribution volume of VM-26 and VP-16 following intravenous infusion with reference to plasma concentrations*

Subject	Vc (l.)	V_{t1} (l.)	V_{t2}* (l.)	Vd_{ss}† (l.)	Vd_{ss}/B.W. (%)	Vd_β‡ (l.)	Vd_γ*†‡ (l.)
VM-26							
D.S.	3·69	3·70	22·79	30·18	53·9	4·92	54·12
J.CS	3·39	3·15	3·83	10·37	23·0	4·90	17·16
E.M.	4·76	3·86	8·84	17·46	21·0	6·05	32·30
J.C.	3·02	2·42	10·08	15·52	30·4	2·55	23·25
C.D.	3·08	4·22	7·77	15·07	10·2	5·83	28·06
F.J.	3·37	3·57	8·09	15·03	31·5	5·40	30·15
Mean ±S.D.	3·55 (0·64)	3·48 (0·63)	10·17 (6·39)	17·27 (6·74)	28·45 (14·7)	4·94 (1·26)	30·8 (12·6)
VP-16							
C.D.	5·02	8·16		13·18	17·81	15·18	
N.G.	5·70	10·21		15·91	25·66	19·58	
K.R.	5·94	3·13		9·07	16·20	19·99	
A.A.	5·07	10·84		15·91	17·88	17·48	
T.S.	5·96	8·70		14·66	30·54	16·90	
J.B.	11·37	10·12		21·49	32·65	53·66	
R.J.	8·34	15·00		23·36	37·68	38·73	
P.R.	12·81	13·90		26·71	32·98	46·20	
H.R.	9·66	6·40		16·06	33·46	33·17	
K.R.	9·67	10·47		20·14	35·96	42·67	
L.B.J.	5·29	9·60		26·02	36·65	12·65	
Mean ±S.D.	7·71 (2·79)	9·68 (3·25)		18·41 (5·57)	28·86 (8·11)	27·84 (15·41)	

*Not applicable to VP-16 pharmacokinetic model;
†Vd_{ss}, volume at steady state; $Vd_{ss} = Vc + \Sigma V_{tn}$ *l* B.W. = body weight, Kg.
‡Vd_β, volume of distribution during β-phase; or γ-phase; $Vd_\beta = (k_{e_1}.Vc)/\beta$; $Vd_\gamma = (k_{e_1}.Vc)/\gamma$, ref 46.

The elimination and distribution parameters for the pharmacokinetic models depicted in Fig. 3A and 3B are given in Table 3 where the transfer constant k_{el} represents the sum of all elimination processes including metabolism and urinary excretion [35]. For VM-26 the mass transfer constants k_{12} and k_{21} are in all cases about equal, but k_{13} tends to be considerably larger than k_{31} which indicates turnover in compartment 3 or the "deep" compartment, to be very slow. On the other hand, k_{12} and k_{21} for VP-16 seem quite erratic with no clear trend in their relative magnitude. k_{el}, which is the sum of all linear elimination processes is nearly the same for both drugs. However, the body clearance of VP-16 is clearly 3 times greater than VM-26. This difference in plasma clearance is a reflection of the larger central compartment volume for VP-16 than for VM-26. The difference in plasma clearance between VM-26 and VP-16 is related to an even larger difference in renal clearance for the two agents where the mean fractional renal clearance of VP-16 is nearly 6 times greater than for VM-26.

This contrast in renal clearance in turn represents the 3-4 times-larger excretion rate constant for VP-16 over VM-26 which is only partially compensated for by the somewhat larger fraction of VM-26 metabolized. The estimation of the fraction of drug metabolized both for VM-26 and VP-16 agrees exceedingly well with the estimation of metabolism based upon recovery of unchanged drug in the urine but does not agree with the figure for actual metabolite recovered in the urine. The relative tissue binding ($V_T u$) was evaluated from knowledge of albumin binding and steady-state distribution volumes [47, 48]. It can be seen in Table 5 that both VM-26 and VP-16 show a high tendency towards tissue storage. If this also occurs with the metabolites it could account for their low recovery in urine.

The integral coefficients in Table 5 can be used to rank chemotherapeutic agents on a pharmacokinetic basis as has been done for a series of semisynthetic penicillins [49]. It can be seen that for the central compartment the integral coefficients for VP-16 are larger than those for VM-26. Whereas the VP-16 integral coefficient for the peripheral compartment is nearly the same as that for the "shallow" peri-

Table 3. First-order mass transfer rate constants to fit plasma VM-26 and VP-16 levels

Subject	Body weight (kg)	k_{12} (hr^{-1})	k_{21} (hr^{-1})	k_{13}* (hr^{-1})	k_{31}* (hr^{-1})	k_{el} (hr^{-1})	Cl† (ml/min)
VM-26							
D.S.	56	0·496	0·494	0·217	0·035	0·264	16·30
J.CS	45	0·474	0·510	0·086	0·076	0·243	13·77
E.M.	83	0·600	0·739	0·101	0·054	0·230	18·31
J.C.	51	1·633	2·041	0·308	0·092	0·308	15·53
C.D.	76	0·485	0·354	0·109	0·043	0·264	12·68
F.J.	47·5	0·739	0·696	0·141	0·058	0·340	19·11
Mean		0·571	0·806	0·160	0·060	0·272	15·95
(± S.D.)		(0·105)	(0·621)	(0·086)	(0·021)	(0·043)	(2·50)
VP-16							
C.D.	74	1·311	0·806			0·487	40·75
N.G.	62	0·667	0·372			0·340	32·31
K.R.	56	0·545	1·033			0·397	63·90
A.A.	89	2·249	1·051			0·462	29·01
T.S.	48	0·727	0·498			0·292	43·36
J.B.	75	0·066	0·074			0·269	47·13
R.J.	62	0·259	0·144			0·339	106·35
P.R.	81	0·113	0·104			0·220	45·89
H.R.	48	0·075	0·113			0·285	32·72
K.R.	56	0·072	0·067			0·203	35·21
L.B.J.	57	0·083	0·151			0·220	35·21
Mean		0·560	0·401			0·319	47·10
(± S.D.)		(0·685)	(0·389)			(0·096)	(21·85)

*Not appolicable to VP-16 model.

†Plasma clearance $Vc \times k_{el}$; body clearance (Cl_B) = dose/area under plasma concentration curve, 0–∞ hr; $\overline{Cl}_B$ = 15·9 ± 2·5 ml/min for VM26 and 47·2 ± 21·9 ml/min for VP 16.

Table 4. Excretion and distribution parameters for VM-26 and VP-16 with reference to plasma levels and urine excretion

Subject	Renal* clearance, Cl_r (ml/min) (±S.D.)	k_e (hr^{-1})	k_m† (hr^{-1})	Fraction metabolized, Fm‡ (found)§¶
VM-26				
D.S.	3·16 (2·70)	0·051	0·213	0·81 (0·93, 0·42)
J.CS	1·39 (0·14)	0·025	0·218	0·90 (0·91, 0·31)
E.M.	3·14 (0·43)	0·040	0·190	0·83 (0·82, 0·34)
J.C.	1·29 (0·27)	0·026	0·282	0·92 (0·92, 0·24)
C.D.	2·08 (0·042)	0·041	0·205	0·83 (0·88, 0·37)
F.J.	2·26 (0·27)	0·040	0·300	0·88 (0·90, 0·22)
Mean (±S.D.)	2·22 (0·81)	0·037 (0·010)	0·234 (0·045)	0·86 (0·89, 0·32) (0·04) (0·04, 0·08)
VP-16				
C.D.	20·16 (3·82)	0·241	0·246	0·50 (0·62, 0·15)
N.G.	7·16 (0·41)	0·075	0·265	0·78 (0·82, 0·16)
K.R.	21·19 (4·21)	0·214	0·183	0·46 (0·57, 0·15)
A.A.	8·05 (0·52)	0·095	0·367	0·79 (0·81, 0·32)
T.S.	13·35 (0·44)	0·134	0·158	0·54 (0·58, 0·37)
J.B.	17·44 (3·34)	0·092	0·177	0·66 (0·68, 0·10)
R.J.	12·44 (1·57)	0·089	0·250	0·74 (0·80, 0·16)
P.R.	9·81 (1·65)	0·046	0·174	0·79 (0·80, 0·21)
H.R.	14·38 (2·05)	0·089	0·196	0·69 (0·73, 0·16)
K.R.	11·70 (0·53)	0·073	0·130	0·64 (0·71, 0·11)
L.B.J.	13·50 (0·75)	0·084	0·136	0·62 (0·64, 0·13)
Mean (±S.D.)	13·56 (4·56)	0·112 (0·061)	0·207 (0·069)	0·66 (0·71, 0·18) (0·12) (0·09, 0·09)

*Based on mean fractional renal clearances calculated from amount of unchanged drug excreted divided by integral of plasma concentration curve to the midpoint of urine collection interval, $Cl_r = \Sigma AU/\int_0^t CpdT$.
†$k_e = Cl_r/Vc$; $k_m = k_{el} - k_e$
‡$Fm = k_m/k_{el}$.
§First column based on difference between dose and cumulative unchanged drug recovered in urine after 72 hr.
¶Second column actual cumulative metabolite recovered in urine after 72 hr.

pheral compartment of VM-26 it is markedly less than the integral coefficient for the other, "deep", peripheral compartment of VM-26.

It was found that the area under the plasma concentration curve of VP-16 for those patients reported here and those patients for which only plasma levels were obtained shows a linear relation to dose (Fig. 4) but does not go through zero presumably because protein binding tends to influence the plasma levels much more significantly somewhere below 100 mg. An insufficiently wide range of doses of VM-26 precluded an analysis of the plasma concentration integral versus dose relationship for this drug.

In vitro analysis of protein binding to human serum albumin has demonstrated that the binding of VP-16 has a $Ka = 3{\cdot}54 \times 10^4\ M^{-1}$ with an $N = 0{\cdot}686$ and that VM-26 has a $Ka = 3{\cdot}72 \times 10^5\ M^{-1}$ with an $N = 0{\cdot}760$. At typical plasma concentrations of VP-16 and VM-26, these affinity constants equate to an average per cent binding of 94 and 99·4% respectively [50]. We found no evidence for *in vitro* binding of VM-26 to calf thymus DNA.

DISCUSSION

Although the biological activity and mechanisms of action of VM-26 and VP-16 are similar, we have noted the following principal differences between the pharmacokinetics of these drugs: volumes of distribution, body and plasma clearance, protein binding, renal clearance, fraction of drug metabolized, and integral coefficients.

VP-16 behaves according to a two-compartment open model and VM-26 to a three-compartment open model with both a "shallow"

Table 5. Distribution in tissue compartments with reference to body water and integral coefficients for central and peripheral compartments for VM-26 and VP-16

Subject	Body water* (l.)	$(V_Tu)_\beta$† (l.)	$(V_Tu)_\gamma$† (l.)	Integral coefficients‡ D1 (hr)	D2 (hr)	D3§ (hr)
VM-26						
D.S.	33·6	205·0	8405·0	1·44	3·80	23·48
J.CS.	27·0	251·7	2295·0	1·88	3·82	4·66
E.M.	49·8	215·0	4573·3	1·26	3·53	8·13
J.C.	30·6	----¶	3371·7	1·01	2·60	10·87
C.D.	45·6	458·3	4163·3	0·00	5·57	10·30
E.J.	28·5	338·3	4463·3	1·25	3·12	7·15
Mean	35·9	293·7	4545·3	1·14	3·74	10·77
(± S.D.)	(9·5)	(106·0)	(2075·3)	(0·63)	(1·01)	(6·62)
VP-16						
C.D.	44·4	169·3		0·89	3·34	
N.G.	37·2	231·3		1·71	5·27	
K.R.	33·6	67·5		1·60	1·33	
A.A.	53·4	206·8		0·70	4·63	
T.S.	28·8	183·3		2·36	5·00	
J.B.	45·0	704·8		2·77	3·31	
R.J.	37·2	506·5		2·11	5·31	
P.R.	48·6	556·5		3·43	4·94	
H.R.	28·8	391·8		2·84	2·33	
K.R.	33·6	550·0		3·82	5·29	
L.B.J.	34·2	122·7		2·66	2·50	
Mean	38·6	335·5		2·26	3·93	
(± S.D.)	(8·1)	(214·2)		(0·98)	(1·42)	

*60% of body weight.
†See [47, 48]: When referenced to body water, it is an index of tissue binding.
‡See [49]: D1, D2, D3 are for compartments 1, 2, and 3, respectively; has been corrected for serum albumin binding, 99·4 & 94% respectively.
§Not applicable to VP-16 pharmacokinetic model.
¶$Vd_\beta < Vc$.

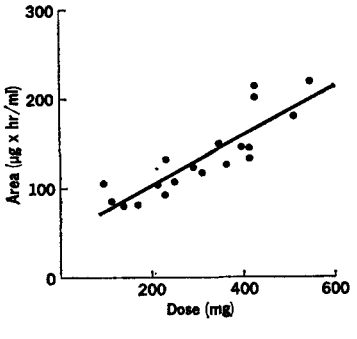


Fig. 4. Area under Plasma Concentration Curve from 0–72 hr for doses of VP-16 between 100–550 mg. Solid line is weighted least-squares fit free to pass through any intercept. Area determined from integral of plasma concentration.

and "deep" peripheral compartment. It appears that the central compartment for VP-16 which has a volume of 7·71 l. corresponds to both the central compartment (3·55 l.) and shallow peripheral compartment (3·48 l.) of VM-26. This difference which may be related to differences in magnitude of serum albumin binding seems to govern the large difference in plasma clearance of the two drugs by decreasing the amount of free drug. Furthermore, the difference in plasma clearance is in the same direction as the maximally tolerated doses for these two agents, namely, the plasma clearance of VP-16 is nearly 3 times that of VM-26 and the difference in the maximally-tolerated dose is correspondingly 3 times larger for VP-16 [12, 14].

The turnover in compartment 2 of VP-16 is nearly twice that of VM-26 in the same compartment but the turnover in compartment 3 of VM-26 is close to 10 times slower than compartment 2 of VP-16 which probably accounts for the much longer elimination half-life time of VM-26. An additional kinetic factor governs VM-26 elimination, namely renal clearance. The excretion rate constant for VP-16 is 3 times larger and the renal clearance 6 times greater than for VM-26.

Although the theoretical fraction of drug metabolized based on kinetic analysis is larger for VM-26 than VP-16, the much larger tolerated dose of VP-16 can compensate for this difference. As stated above, the fraction of VM-26 or VP-16 metabolized based on cumulative unchanged drug recovered in the urine agrees exceedingly well with the theoretical fraction of drug metabolized. However, the fraction of drug recovered as metabolite in the urine is only a small fraction of these estimates. This discrepancy can be due to several factors: a significant proportion of drug is eliminated by way of the feces (calculated to be: 57% of VM-26 and 53% of VP-16) or the metabolite and/or unchanged drug is sequestered in various tissues and subsequently excreted only very slowly if at all. The fecal excretion in rodents has been reported to be about 50% of the dose [10, 51] however, we found the fecal levels of VP-16 and VM-26 in humans to be in most cases much less than 10% [24, 25]. Furthermore, because the body clearance (dose divided by area under plasma concentration curve) is equal to the plasma clearance, neither VP-16 nor VM-26 is eliminated simultaneously from the peripheral compartment (i.e. tissue clearance = 0). Therefore, unless one invokes a special mechanism for specific sequestering of unchanged drug only within the central compartment, then the pharmacokinetics themselves mitigate against depot of unchanged drug.

The volumes of the tissue compartments (V_Tu) referenced to body water during the β-phase of the plasma decay kinetics for VM-26 and VP-16 are several times larger than body water and approximate the magnitude of pentobarbital [48]. In addition, the volume of the tissue compartments during the γ-phase for VM-26 is several orders of magnitude larger than body water and 10 times larger than its β-phase volume. Therefore, both drugs demonstrate a great propensity for tissue storage, one even greater than that for some of the long-acting barbiturates. This phenomena may be significant in terms of the unique mechanism of action of VM-26 and VP-16 at the G_2-phase of the cell cycle. It has been stated that it may be essential for VM-26 and presumably VP-16 to be in contact with sensitive cells for a protracted period in order to interrupt the cell cycle at G_2 [15]. Furthermore, the tendency for extensive tissue storage and a much slower turnover at the "deep" peripheral compartment with VM-26 may lead to a more pronounced effect on the chromosomes than was observed with VP-16 [20].

The binding of VP-16 and especially VM-26 to serum albumin *in vitro* is of sufficient pharmacological magnitude ($> 10^4\ M^{-1}$) to influence distribution and elimination of these drugs *in vivo* [52]. Consequently, the integral coefficients for the central compartments of VM-26 and VP-16 have been corrected to reflect this binding where the binding affinity of VM-26 is about 10 times greater than VP-16. The slightly larger integral coefficient for the central compartment of VP-16 than that for the central compartment of VM-26 suggests that the bioavailability of VP-16 is slightly superior to VM-26 based solely on differences in the pharmacokinetics of these two drugs.

The integral coefficients for the peripheral compartment of VP-16 and the "shallow" compartment of VM-26 are about the same and in turn suggest about equal bioavailability. In contrast, the "deep" compartment of VM-26 has a very large, although somewhat variable, integral coefficient suggesting a high bioavailability index. The relative bioavailabilities of course assume that peripheral tissue binding does not remove drug from the biophase in those compartments. Furthermore, because the binding to serum albumin is reversible it may serve to prolong drug action, especially for VM-26.

In conclusion, the pharmacokinetic analysis of VM-26 and VP-16 has suggested a rationale for the difference in the equitoxic doses of these two agents by the nearly 3-times-larger excretion rate constant for VP-16 than VM-26 and the related 6-times-larger renal clearance. Furthermore, the analysis has tentatively identified the protein binding component of VM-26 distribution as responsible for the basic difference in the plasma decay kinetics and consequently pharmacokinetic models for VP-16 and VM-26 in man. The analysis of tissue distribution with reference to body water has shown that both VM-26 and VP-16 are extensively stored in tissue and VM-26 to have a slower turnover than VP-16. The pharmacokinetic integral coefficients have facilitated theoretically ranking VM-26 and VP-16 according to their bioavailabilities in different body compartments.

Acknowledgements—The expert technical assistance of Mrs. D. Alford in the collection and assaying of the biological specimens for these analyses is gratefully acknowledged as well as is the assistance of Mr. Frank Pelsor and Mrs. Carol Marcks in the protein and DNA binding experiments and Mrs. Barbara Nespoli for preparation of this manuscript. Special appreciation is extended to Dr. Carl Metzler for use of his computer program *NONLIN*.

REFERENCES

1. R. Bentley, New American remedies 1: Podophyllum peltatum. *Pharm. J. Trans.* **3,** 456 (1861).
2. I. W. Kaplan, Condylomata acuminata. *New Orleans med. surg. J.* **94,** 388 (1942).
3. L. King and M. Sullivan, Colchicine-like effect of podophyllotoxin. *Science* **10,** 244 (1946).
4. H. Savel, The metaphase arresting plant alkaloids and cancer chemotherapy. *Progr. exp. Tumor Res (Basel)* **8,** 189 (1966).
5. M. G. Kelly and J. L. Hartwell, The biological effects and the chemical composition of podophyllin. A review. *J. nat. Cancer Inst.* **14,** 967 (1954).
6. G. Falkson, A. G. Sandison and J. van Zyl, Podophyllin derivatives SPG-827 and SPI-77 in the treatment of advanced cancer. *S. Afr. J. Radiol.* **2,** 1 (1964).
7. V. K. Vaitkevicins and M. L. Reed, Clinical studies with podophyllum compounds SPI-77 (NSC-72274) and SPG-827 (NSC-42076). *Cancer Chemother. Rep.* **50,** 565 (1966).
8. C. Keller-Juslen, M. Kuhn, A. von Wartburg and H. Stähelin, Synthesis and antimitotic activity of glycosidic lignan derivatives related to podophyllotoxin. *J. med. Chem.* **14,** 963 (1971).
9. P. Dombernowsky, N. I. Nissen, V. Larsen, Clinical investigation of a new podophyllum derivative, epipodophyllotoxin, 4'-demethyl-9-(4,6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819) in patients with malignant lymphomas and solid tumors. *Cancer Chemother. Rep.* **56,** 71 (1972).
10. I. Nissen, V. Larsen, H. Pedersen and K. Thomsen, Phase I clinical trial of a new antitumor agent 4,-demethylepipodophyllotoxin-9-(4,6-0-ethylidene-β-D-glucopyranoside (NSC-141540; VP-16-213). *Cancer Chemother. Rep.* **56,** 769 (1972).
11. R. W. Sonntag, H. J. Senn, G. Nage, K. Giger and P. Alberto, Experience with 4'-demethylepipodophyllotoxin 9-(4,6-0-2-theynlidene-β-D-glucopyranoside); VM-26; NSC-122819 in the treatment of malignant lymphosis. *Europ. J. Cancer* **10,** 93 (1974).
12. F. M. Muggia, O. S. Selawry and H. H. Hansen, Clinical studies with a new podophyllotoxin derivative, epipodophyllotoxin, 4'-demethyl-9-(4,6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819). *Cancer Chemother. Rep.* **55,** 575 (1971).
13. B. D. Sklansky, R. S. Mann-Kaplin, A. F. Reynolds, M. L. Rosenblum and M. D. Walker, 4'-demethyl-epipodophyllotoxin-β-D-thenylidene-glucoside (PTG) in the treatment of malignant intracranial neoplasms. *Cancer (Philad.)* **33,** 460 (1974).
14. P. J. Creaven, S. J. Newman, O. S. Selawry, M. H. Cohen and A. Primack, Phase I clinical trials of weekly administration of 4'-demethylepipodophyllotoxin-9-(4,6-0-ethylidene-β-D-glucopyranoside) (NSC-14-540; VP-16-213). *Cancer Chemother. Rep.* **58,** 901 (1974).
15. H. Stähelin, 4'-demethyl-epipodophyllotoxin thenylidene glucoside (VM-26), a podophyllum compound with a new mechanism of action. *Europ. J. Cancer* **6,** 303 (1970).
16. H. Stähelin, Activity of a new glycoside lignan derivative (VP-16-213) related to podophyllotoxin in experimental tumors. *Europ. J. Cancer* **9,** 215 (1973).
17. T. L. Avery, D. Roberts and R. A. Price, Delayed toxicity of 4'-demethyl-epipodophyllotoxin 9-(4,6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819) in mice. *Cancer Chemother. Rep.* **57,** 165 (1973).
18. A. Grieder, R. Maurer and H. Stähelin, Dissimilar effects on thymidine incorporation and DNA synthesis of the podophyllotoxin derivative VP-16-213. *Experientia* **29,** 772 (1973).
19. A. Grieder, R. Maurer and H. Stähelin, Effect of an epipodophyllotoxin derivative (VP-16-213) on macromolecular synthesis and mitosis in mastocytoma cells *in vitro*. *Cancer Res.* **34,** 1788 (1974).
20. C. C. Huang, Yu Hou and J. J. Wang, Effects of a new antitumor agent, epipodophyllotoxin on growth and chromosomes in human hematopoietic cell lines. *Cancer Res.* **33,** 3123 (1973).

21. C. Naresh Misra and De Wayne Roberts, Inhibition by 4′-demethyl-epipodophyllotoxin 9-(4,6-0-2-thenylidene-β-D-glucopyranoside) of human lymphoblast cultures in G_2-phase of the cell cycle. *Cancer Res.* **35,** 99 (1975).
22. M. Gosalvez, J. Perez-Garcia and M. Lopez, Inhibition of NADH-linked respiration with the anticancer agent 4′-demethyl-epipodophyllotoxin thenylidene glucoside (VM-26). *Europ. J. Cancer* **8,** 471 (1972).
23. J. H. Burchenal and S. K. Carter, New cancer chemotherapeutic agents. *Cancer (Philad.)* **30,** 1639 (1972).
24. P. J. Creaven and L. M. Allen, Clinical pharmacology of 4′-demethyl-epipodophyllotoxin ethylidene glucoside. *Proc. XI Intl. Cancer Congress, Florence, Italy*, October, 1974. In Press.
25. P. J. Creaven and L. M. Allen, Clinical pharmacology of the new anti-cancer drug VM-26. *Clin. Pharmacol. Ther.* **17,** 232 (1975).
26. L. M. Allen and P. J. Creaven, Pharmacokinetics of an epipodophyllotoxin derivative (VP-16-213) in man. *Proc. Amer. Assoc. Cancer Res.* **16,** 808 (1975).
27. L. M. Allen and P. J. Creaven, VM-26 and VP-16, Contrasts in the human pharmacokinetic parameters of two antineoplastic epipodophyllotoxin derivatives. *Sixth Intl. Congress of Pharmacology*, Helsinki, Finland, July (1975).
28. P. J. Creaven, L. M. Allen, F. M. Muggia, Plasma camptothecin (NSC 100880) levels during a 5-day course of treatment: Relation to dose and toxicity. *Cancer Chemother. Rep.* **56,** 573 (1972).
29. N. Bert LaDu, H. George Mandel and E. Leong Way, *Fundamentals of Drug Metabolism and Drug Disposition*, p. 63, Williams & Wilkins, Baltimore (1972).
30. L. M. Allen and P. J. Creaven, The effect of microsomal activation on the interaction between isophosphamide and DNA. *J. pharm. Sci.* **61,** 2009 (1972).
31. L. M. Allen and P. J. Creaven, Inhibition of macromolecular biosynthesis in cultured L 1210 mouse leukemia cells by thalicarpine (NSC 68075). *Cancer Res.* **33,** 3112 (1973).
32. L. M. Allen and P. J. Creaven, Binding of an antitumor agent, thalicarpine, to DNA, *J. pharm. Sci.* **63,** 475 (1974).
33. A. Rescigno and G. Segre, *Drug and Tracer Kinetics*, Blasidell, Waltham, Massachusetts (1966).
34. M. Rowland, L. Z. Benet and S. Riegelman, Two compartment model for a drug and its metabolites: Application to acetylsalicylic acid pharmacokinetics. *J. pharm. Sci.* **59,** 364 (1970).
35. J. G. Wagner, Biopharmaceutics and Relevant Pharmacokinetics. In *Drug Intelligence Publications*, Hamilton, Illinois (1971).
36. H. O. Hartley, The modified Gauss–Newton method for fitting of non-linear regression functions by least squares. *Technometrics* **3,** 269 (1961).
37. C. M. Metzler, *NONLIN*, Tech. Rep. No. 7292/69-7293/005, Upjohn, Michigan (1969).
38. L. G. Atkins, Weighting functions and data truncation in the fitting multi-exponential functions, *Biochem. J.* **138,** 125 (1974).
39. F. J. Anscombe and J. N. Tukey, The examination and analysis of residuals. *Technometrics* **5,** 14 (1963).
40. N. R. Draper and H. Smith, *An introduction to non-linear estimation in applied regression analysis.* p. 263. Academic Press, New York (1966).
41. J. W. Westlake, Problems associated with analysis of pharmacokinetic models. *J. pharm. Sci.* **60,** 882 (1971).
42. H. G. Boxenbaus, S. Riegelman, R. M. Elashoff, Statistical estimations in pharmacokinetics. *J. Pharmacokin. Biopharmac.* **2,** 123 (1974).
43. G. D. Knott and D. K. Reece, MLAB: A civilized curve-fitting system. *Proc. On-Line.* **1,** 497 (1972).
44. G. D. Knott and R. I. Shrager, On-Line modelling by curve fitting. *Proc. Siggraph.* **6,** 138 (1972).
45. J. C. K. Loo and S. Riegelman, Assessment of pharmacokinetic constants from postinfusion blood curves obtained after i.v. infusion. *J. pharm. Sci.* **59,** 53 (1970).
46. W. J. Jusko and M. Gilbaldi, Effects of change in elimination on various parameters of the two-compartment open model. *J. pharm. Sci.* **61,** 1270 (1972).

47. E. R. GARRETT and H. J. LAMBERT, Pharmacokinetics of trichlorethanol and metabolites and interconversions among variously referenced pharmacokinetic parameters. *J. pharm. Sci.* **62,** 550 (1973).
48. M. EHRNEBO, Pharmacokinetics and distribution properties of pentobarbital in humans following oral and intravenous administration. *J. pharm. Sci.* **63,** 1114 (1974).
49. W. J. JUSKO, G. P. LEWIS, L. W. DITTERT, Integral coefficients of multicompartment pharmacokinetic models, application to chemotherapy. *Chemotherapy* **17,** 109 (1972).
50. H. M. SOLOMAN and G B. THOMAS, A rapid method for the estimation of drug-albumin affinity constants in human plasma. *Clin. Pharmac. exp. Therap.* **12,** 445 (1970).
51. *Pre-Clinical Brochure VP*-16-213, Research Department, Sandoz-Pharmaceuticals, Sandoz-Wandor, Inc., East Hanover, New Jersey, June (1971).
52. J. R. GILLETTE, Overview of drug-protein binding in *Ann. N.Y. Acad. Sci.* **236,** 6 (1973).

Europ. J. Cancer Vol. 11, pp. 709–715. Pergamon Press 1975. Printed in Great Britain

Delayed Pyridine Nucleotide Reoxidation Induced by the Anticancer Agent VM-26 as measured *In Vivo* and *In Situ* by NADH Microfluorimetry*

MARIO GOSALVEZ,† R. GARCÍA-CAÑERO† AND HUBERT REINHOLD‡
†*Dept. of Experimental Biochemistry, Clínica Puerta de Hierro, Facultad de Medicina, Universidad Autónoma, Madrid, Spain*
and
‡*The Radiobiological Institute of the Organization for Health Research, TNO, Rijswik, The Netherlands*

Abstract—*Anoxia cycles have been performed in rats bearing rhabdomyosarcoma while focusing an alpha-diphosphopyridine nucleotide (NADH) microfluorimeter in the surface of the tumor tissue. A delayed re-oxidation of the pyridine nucleotides upon re-oxygenation of the animal was observed after the animal was injected with chemotherapeutic doses of the anticancer agent 4'-demethyl-epipodophyllotoxin thenylidene glucoside (NSC-122819) (VM-26). These results confirm* in vivo *and* in situ *that VM-26 is a respiratory inhibitor.*

INTRODUCTION

IN PREVIOUS papers, we reported that the anticancer agent VM-26 [1, 2] inhibits NADH-linked respiration at low concentrations [3] and that this respiratory inhibition seems to be somewhat more specific for certain tumour cells than for certain normal cells [4]. Conversely, we have also reported that rotenone, a classical inhibitor of NADH-linked respiration, is able to inhibit tumour growth *in vivo* and therefore we have postulated that an anticancer chemotherapy based on specific inhibitors of NADH-linked respiration may be worth investigating [5].

We have shown that i.p. chemotherapeutic treatment with VM-26 results in respiratory inhibition of the cells [4], a fact favoring our proposition that the mechanism of action and/or toxicity of VM-26 may lie in its effect on respiration. However, as the abdominal cavity of an ascites tumour might behave as an *in vitro* chamber if the drug is not rapidly absorbed, it will be convenient to demonstrate *in vivo* and *in situ* that VM-26 inhibits the respiration of parentally-treated solid tumours, in order to ascertain the biological significance of the effects of the drug in cellular respiration.

In this report we illustrate *in vivo* and *in situ* that VM-26, injected i.p. at chemotherapeutic doses, is able to delay the reoxidation of pyridine nucleotides of solid rat rhabdomyosarcomas growing in the skin of rats. This effect is interpreted as an inhibition of tissue respiration by VM-26 and provides additional evidence supporting the proposed mechanism of action and/or toxicity of VM-26 as based on its effect on respiration. To perform *in vivo* and *in situ* the measurement of redox cycles of pyridine nucleotides, we have taken advantage of the NADH microfluorimetric technique for tissue surfaces [6–9] and the so-called "sandwich

Accepted 30 May 1975.

*This work was supported by Grant 12-913-74 of the Spanish Instituto Nacional de Prevision and by Contract N01-CM-53792 from the Division of Cancer Treatment, National Cancer Institute, H.E.W. (U.S.A.)

tumor" technique [10]. The NADH microfluorimetry in the sandwich tumor is defined as a very suitable technique for the precise evaluation of the possibilities of an anticancer chemotherapy based on respiratory inhibitors.

MATERIAL AND METHODS

Tumor system

The tumor under investigation was the rhabdomyosarcoma BA112 [11–14] growing in a "sandwich" chamber implanted in the subcutis of the back of a rat. Female WAG/Rij rats, isologues to the tumor, were used when 5 weeks old. The average weight was 120 g. The "sandwich" tumor system was a modification of the mouse-sandwich system [10]. In essence, a thin sheet of subcutis is fixed between a mica base plate and a glass cover slip. The tumor is implanted in this thin sheet of subcutis, next to a skin nerve, and grows as a circular sheet of transparent tumor tissue. The diameter of such a tumor is about 4 mm and its thickness ranges from 200 to 300 (μm) [15]. Through the glass cover slip the tumor can be observed *in vivo* and investigated by focusing the microfluorimeter on it.

NADH microfluorimetry

The microfluorimeter was a modified version of the microfluorimeter for NADH surface fluorescence described by Chance [6–7], and the technique was essentially as described by us in the mouse-sandwich tumor [8, 9]. The microfluorimeter consisted of a standard type Leitz Orthoplan microscope with MPV I. This microscope has an advantage in that the microscope stage is large enough to carry the rat in a simple holder. The lamp was an HBO 100 W, the filter system consisted of a BG 12 pre-filter, a "Ploem" Dichroic system (BG 38/4 + UGI/4 Selection filters, H-400 dichroic mirror and barrier filter K-400), a K430 u.v. filter and a 500 mm wide bad interference filter.

The photomultiplier operated on 0·8 kV, cooling to −28°C reduced the dark current to about 0·02 nA. The signal was recorded on a chart recorder that was connected to the photomultiplier via a digital voltmeter and a low-frequency band filter (1/2 Hz). The latter served as a noise-suppressing filter.

The objective used was a Leitz 11 × Ultropal without condensor. The measuring aperture equaled 100 μm at the tissue level.

To study the sandwich tumors under the microfluorimeter, the animals were anaesthetized with a composite mixture consisting of the following anaesthetics: α chloralose 1 g%, urethane 1·25 g%, pentobarbital 0·12 g%, tribromo ethanol 0·5 g%, valium 0·1 g%, to which, besides the appropriate solvents (poli thylen glycol and tert. amyl alcohol), 8.4 g % $NaHCO_3$ was added to counteract the respiratory acidosis that may result from hypoxia cycles. This mixture was given i.v., slowly; 4 ml/kg. The animal was then intubated via a larynx canula and received artificial respiration via a Starling-type respiratory pump. The respiratory rate was 60/min, the respiratory volume was adjusted for every individual animal depending on the weight of the animal, according to Guyton's formula [16]. The gas mixtures given consisted of a mixture of pure nitrogen and oxygen which was fed with an overflow into the respiratory pump. Calibrated flow meters and electro-magnetic gas valves facilitated the adjustment and changes in oxygen content when required. Rotenone and VM-26 were administered to the rat at the indicated doses, by i.p. injection, using poli ethylen glycol plus amyl alcohol, and a mixture of ricinus-oil plus ethyleneoxide [2] as solvents, respectively.

For the microfluorimetric determination of rat brain, normal animals were subjected to a trepanation of 3 mm of diameter. All other aspects of the technique were otherwise as in tumors except that the microfluorimeter was focused on the brain surface.

To ascertain that the effects of rotenone and VM-26 on the redox level of tissue pyridine nucleotides, were not due to circulation changes induced in the tumors by spurious effects of the drugs in the rat blood pressure, blood pressure was measured separately in rats treated with drugs at different doses. Blood pressure measurements were taken from the carotic artery and recorded via a pressure transducer with a Schwarzer strip-chart recorder. Rotenone, at 1·5 mg/Kg, i.p., either did not affect or decreased the blood pressure by 10%, depend-on the rat. VM-26 did not affect blood pressure at any of the tested doses in any rat.

Oxygen uptake and redox measurements in mitochondria

Rat-liver, rat-rhabdomyosarcoma and rat-brain mitochondria were prepared essentially as described by Schneider [17], Devlin [18] and Basford [19], respectively. Mitochondrial oxygen uptake was measured with a Clark-type oxygen electrode at 22° C in a 3 ml chamber. The assay medium for mitochondrial respiration was composed of 225 mM sucrose, 20 mM KCl, 7 mM $MgCl_2$, 10 mM Tris HCl and 5 mM Tris-Pi, at pH 7·2. The redox states of

mitochondrial pyridine nucleotides were measured with a dual wavelength spectrophotometer (Perkin–Elmer Hitachi 356) as described by Chance and Williams [20, 21]. Substrates, salts and co-factors were purchased from Sigma Company. Mitochondrial protein was determined by the Biuret reaction [22].

RESULTS

Figure 1 shows typical results of microfluorimetric experiments in sandwich rhabdomyosarcoma and rat brain of rats injected with VM-26 or rotenone.

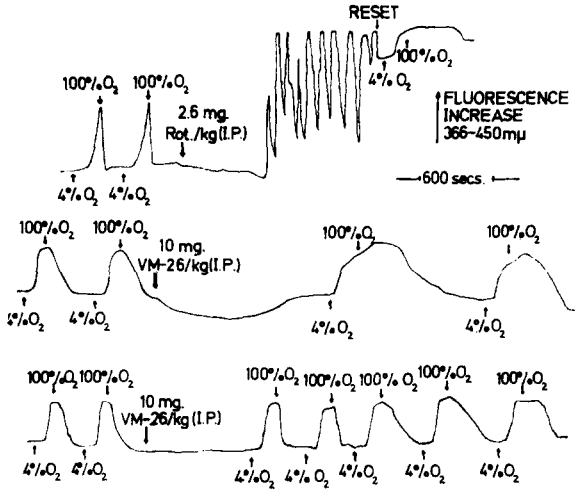


Fig. 1. Pyridine nucleotide redox changes monitored by NADH microfluorimetry in vivo *and* in situ, *performed in rats subjected to anoxia cycles during the i.p. rotenone and VM-26 treatments. The upper recording is an experiment done by focusing the microfluorimeter on an arterial region of the sandwich rhabdomyosarcoma. The middle recording is an experiment with another rhabdomyosarcoma, focusing the microfluorimeter on a venous region. The lower recording shows an experiment recorded from the rat brain surface.* 100% O_2: *the animal was switched to breathing pure oxygen.* 4% O_2: *the animal was switched to breathing a mixture of* 4% *oxygen and* 96% *nitrogen.* 2·6 *mg* of Rot./*Kg I.P.*) *i.p. injection of this dosage to the tumor-bearing rat.* 10 *mg. VM-26/Kg (I.P.) i.p. injection of this dosage to the tumor-bearing rat (middle recording) or to a normal rat (lower recording). The traces in the figure represent a smooth line drawn through the actual recordings; the level of electronic "noise" ranged from* 3 *to* 8% *of the changes.*

The upper recording of Fig. 1 shows an experiment performed in a sandwich rhabdomyosarcoma. After anesthesia and intubation, the sandwich-tumor-bearing animal was placed on the stage of the microfluorimeter which was then focused on a tumor surface area free of blood vessels but near an arterial capillary. After a few minutes, allowed for the equilibration of the animal, the NADH tissue fluorescence was recorded by switching the prisma from the visual-observation position to the photomultiplier position. The recording of fluorescence (far left) starts with the animal breathing 100% oxygen. Immediately after the starting of the recording, the animal is subjected to anoxia by forcing it to breathe 4% oxygen in nitrogen. As shown in the recording, after a brief delay, this procedure induces an increase in NADH tissue fluorescence due to the reduction of mitochondrial pyridine nucleotides by intracellular substrates in the absence of intracellular oxygen [8, 9]. Two hundred seconds after the induction of anoxia, the anoxic period is terminated by switching the animal back to breathing 100% oxygen, and an immediate decrease of fluorescence is observed due to the recuperation of the aerobic-level redox state of intracellular pyridine nucleotides. The fluorescence recovery from anoxia is very rapid as the recorded tissue point lies very near to an arterial capillary. Afterwards, the recording shows a second anoxia cycle followed by the i.p. injection of rotenone, to the rat (2·6 mg/kg of weight). Upon the injection of rotenone, there is a slight decrease in the tumor NADH fluorescence followed, after a few minutes, by a great increase in fluorescence and fluorescence oscillations, until the fluorescence is stabilized at a new, highly reduced, aerobic level. The cause of the fluorescence oscillations after the rotenone treatment is not yet clear but could be due either to reduction of cytoplasmic pyridine nucleotides, which has been reported to take place in an oscillating fashion under certain circumstances [23–25], or to small variations induced by rotenone, in the rat arterial pressure. After the fluorescence is equilibrated, a new 200-second anoxic cycle is performed and, in this case, the recovery from anoxia is considerably delayed. In this case, after inducing the respiration with 100% oxygen, the fluorescence remains at the anaerobic level for a few minutes and later on rapidly decreases to almost the aerobic level of fluorescence attained upon the rotenone injection. This delay in the recovery from anoxia is interpreted as a rotenone-induced inhibition of the tissue respiratory rate. Intracellularly, rotenone would partially block the mitochondrial respiratory chain and then the reoxidation of mitochondrial pyridine nucleotides, in the presence of oxygen, would take place slowly. The fluorescence decrease would then occur the moment pyridine nucleotides are completely re-oxidized. It is important to note that the extent of the fluorescence change of the anoxic cycle performed after rotenone, is smaller than the fluorescence

change of the previous control anoxic cycles. Therefore, rotenone would have induced a mitochondrial reduction of pyridine nucleotides not re-oxidable by oxygen, which is in agreement with previous reports [26]. On the other hand, the high level of fluorescence attained in aerobiosis after rotenone administration, is higher than would be expected if only the total reduction of mitochondrial pyridine nucleotides were to be involved. Thus a reduction of cytoplasmic pyridine nucleotides must collaborate in the attaining of such a highly reduced state of intracellular nucleotides, induced indirectly by rotenone. This would be supported by the increase in the glycolytic rate of tumor cells treated with rotenone as reported by us [27].

The middle recording of Fig. 1 shows an experiment performed on a sandwich rhabdomyosarcoma, but in this case the microfluorimeter was focused on a free area near a venous capillary. The recording shows first, two control anoxic cycles. Here the recovery from the anaerobic level of fluorescence takes place more slowly than in the previous experiment, due to the slower difusion of oxygen in the tissue, in venous regions. After the control cycles, the i.p. injection of VM-26 to the rat (10 mg/kg of weight) induces a slight oxidation of pyridine nucleotides (fluorescence decreases) that levels off in a few minutes. Two subsequent similar 200-second anoxic cycles performed, show a long delay in the recovery of anoxia with the administration of 100% oxygen. This delay is interpreted as a VM-26 induced inhibition of the tissue respiratory rate, by the same reasons stated in the previous experiment. The second anoxic cycle, after the VM-26 injection, is not as wide as the first one; however, cycles that followed the second one, remained at similar widths (not shown). If one could assume that the width of the cycle is proportional to the extent of the tissue respiratory inhibition, VM-26 would induce first, a strong respiratory inhibition which later stabilizes itself. *The lower recording of Fig.* 1, shows a microfluorimetric experiment performed on the brain surface of a rat. Here again, two control anoxic cycles recorded from a free area near a venous capillary, are shown, followed by the i.p. injection of VM-26 to the rat (10 mg/kg of weight). The injection of VM-26 does not induce any change in the aerobic level of fluorescence but when performing, after a few minutes, successive anoxic cycles, a progressive widening of the cycles is observed due to the delay in the recovery from anoxia after the administration of 100% oxygen. Again, the delay in the recovery is interpreted as an inhibition of the respiratory rate of the brain tissue by VM-26; and as the widening of the rat brain cycles is less apparent than the widening of the tumor cycles, it would seem that VM-26 has less effect in brain respiration than in rhabdomyosarcoma respiration.

To provide a basis for the interpretations given to the microfluorimetric experiments, anoxia cycles were performed in isolated mitochondria, measuring the redox changes of pyridine nucleotides. Figure 2 shows two cycles in isolated mitochondria, the lower cycle being recorded in the absence of inhibitor. In both recordings the far left indicates the redox level of the mitochondrial pyridine nucleotides in the absence of substrate. In the lower cycle, with the addition of succinate and after a brief delay, the oxygen in the cell is exhausted and a great anaerobic reduction appears. This

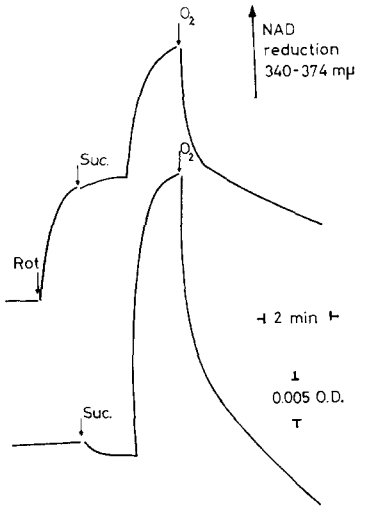


Fig. 2. *Changes in the redox state of pyridine nucleotides of isolated mitochondria* (2 *mg/ml*) *in the presence and absence of rotenone* (0·1 *μM*). *Suc.*: 5 *mM succinate; O_2: bubbling oxygen.*

reduced level is reoxidized by bubbling oxygen. The reoxidation is very rapid at first but slows down before attaining complete reoxidation. In the upper cycle, rotenone is added to the mitochondria prior to the addition of succinate. In this case, the second and last phase of reoxidation are much slower due to the inhibition of respiration by rotenone. This experiment in mitochondria supports the concept that a delay of reoxidation of pyridine nucleotides can be caused by inhibiting respiration with an inhibitor of NADH-linked substrates.

As the microfluorimetric experiments in Fig. 1 seem to indicate that rat brain tissue is less sensitive to VM-26 inhibition than is rat rhabdomyosarcoma, we compared, in Fig. 3,

the effect of VM-26 on the respiration of mitochondria isolated from rat rhabdomyosarcoma with both rat brain and rat liver. The concentrations of each type of mitochondria were adjusted to yield equivalent respiration [4, 28] to facilitate the comparison. Using this criteria, it appears that rat brain mitochondrial respiration is less sensitive to VM-26 than is rat rhabdomyosarcoma mitochondria.

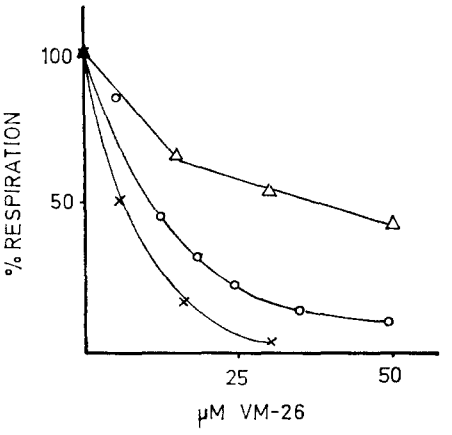


Fig. 3. Effect of increasing concentrations of VM-26 on the respiration of mitochondria isolated from rat rhabdomyosarcoma, rat liver and rat brain. The concentration of the different mitochondria was adjusted to yield equivalent respirations in order to facilitate the comparison (crosses: *rat rhabdomyosarcoma mitochondria, 2 mg/ml;* circles: *rat liver mitochondria, 0·9 mg/ml;* triangles: *rat brain mitochondria, 0·9 mg/ml*).

DISCUSSION

Erecinska *et al.* [29, 30] have demonstrated that delayed reoxidation in mitochondria can be produced by addition of respiratory inhibitors which block partially the respiratory chain. This blocking acts as a bottle-neck for the reoxidation of the pool of carrier molecules behind the blocked point [29, 30]. Although the experiments of Erecinska *et al.* were done with terminal inhibitors, they have provided the basis for the interpretation of our experiments. Thus, the delay of the reoxidation of pyridine nucleotides found in the treatments with VM-26 and rotenone is secondary to a blocking of the mitochondrial respiratory chain caused by the respiratory inhibitors. NADH microfluorimetry, therefore, appears to be a suitable technique for the *in vivo* and *in situ* qualitative estimation of the effect of drugs on tissue respiratory rates. However, for the precise quantitative use of this technique it would be convenient to correlate the microfluorimetric experiments with data collected with micro-oxygen electrodes suitable for *in vivo* and *in situ* tissue determinations. However, presently NADH microfluorimetry offers the possibility of relative qualitative estimation of tissue respiratory rates *in vivo* and *in situ*.

From our results, it can be concluded that 10 mg/kg of VM-26 administered i.p.—a dose well within the range of the chemotherapeutic dose for rodent tumors [1]—affects considerably the respiration of the rabdomyosarcoma. Rotenone, a well known potent inhibitor of NADH-linked respiration [26], in a dose of 2·6 mg, affects the respiration of the rhabdomyosarcoma to the same extent as 10 mg of VM-26. Our results tend to confirm the proposed mechanism of action and toxicity of VM-26 as based on its effects and relative specificity for tumor respiration [3, 4]. On the other hand, we do not exclude the possibility that VM-26 might have effects in other biochemical parameters. VM-26 has been reported to inhibit *in vitro* the entering of the cell into mitosis and, in short incubation periods, is also able to arrest the cells in metaphase [1].

Within this context, it is interesting to point out that a respiratory inhibitiion would prevent the formation of the energy necessary in the premitotic period [31] and in the metaphase and anaphase transitions [32].

The application of NADH microfluorimetry to the *in vivo* and *in situ* evaluation of the mechanism of action of VM-26, provides a model study for the use of this technique in the precise determination of the possibilities of an anticancer chemotherapy based on respiratory inhibitors [5, 28].

Acknowledgements—Dr. M. Gosalvez acknowledges the personal contributions of Mr. J. Barrett (Torrance, California) and Mr. G. Blanco (Madrid), and the discussions and comments of Prof. B. Chance. Dr. H. Reinhold acknowledges the assistence of Mrs. M. Sahadat (rat preparations) and Mrs. J. Verwey, Mrs. G. H. Buisman and Miss M. Poessé (blood pressure measurements).

REFERENCES

1. H. Stahelin, 4'-Demethyl-epipodophyllotoxin thenylidene glucoside (VM-26), a podophyllum compound with a new mechanism of action. *Europ. J. Cancer* **6**, 303 (1970).
2. Per Dombernowsky, N. I. Nissen and V. Larsen, Clinical investigation of a new podophyllum derivative, epipodophyllotoxin, 4'-demethyl-9-(4, 6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819), in patients with malignant lymphomas and solid tumors. *Cancer Chemother. Rep.* **56**, 71 (1972).

3. M. Gosalvez, J. Pérez-García and M. López, Inhibition of NADH-linked respiration with the anti-cancer agent 4'-demethyl-epipodophyllotoxin thenylidene glucoside (VM-26). *Europ. J. Cancer* **8,** 47 (1972).
4. M. Gosalvez, M. Blanco, J. Hunter, M. Miko and B. Chance, Effects of anticancer agents on the respiration of isolated mitochondria and tumor cells. *Europ. J. Cancer* **10,** 567 (1974).
5. M. J. Figueras and M. Gosalvez, Inhibition of the growth of Ehrlich ascites tumors by treatment with the respiratory inhibitor rotenone. *Europ. J. Cancer* **9,** 529 (1973)
6. B. Chance, F. Cohen, L. Jobsis and B. Schoener, Localized fluorometry of oxidation-reduction states of intracellular pyridine nucleotide in brain and kidney cortex of the anesthetized rat. *Science* **136,** 325 (1962).
7. B. Chance, J. R. Williamson, D. Jamieson and B. Schoener, Properties and kinetics of reduced pyridine nucleotide fluorescence of the isolated and *in vivo* rat heart. *Biochem. Journal* **341,** 357 (1965).
8. M. Gosalvez, R. G. Thurman, B. Chance and H. Reinhold, Regional variation in the oxygenation of mouse mammary tumours *in vivo* demonstrated by fluorescence of pyridine nucleotide. *Brit. J. Radiol.* **45,** 510 (1972).
9. M. Gosalvez, R. G. Thurman, B. Chance and H. Reinhold, Indication of hypoxic areas in tumours from *in vivo* NADH fluorescence. *Europ. J. Cancer* **8,** 267 (1972).
10. H. Reinhold, Improved microcirculation in irradiated tumors. *Europ. J. Cancer* **7,** 273 (1971).
11. H. Reinhold, A cell dispersion technique for use in quantitative transplantation studies with solid tumours. *Europ. J. Cancer* **1,** 67 (1965).
12. H. Reinhold and C. de Bree, Tumour cure rate and cell survival of a transplantable rat rhabdomyosarcoma following X-irradiation. *Europ. J. Cancer* **4,** 367 (1968).
13. H. Reinhold, Quantitative evaluation to the radiosensitivity of cells of a transplantable rhabdomyosarcoma in the rat. *Europ. J. Cancer* **2,** 33 (1966).
14. J. J. Fischer and H. Reinhold, The cure of Rhabdomyosarcoma BA 112 after fractionated radiotherapy. *Radiology* **105,** 429 (1972).
15. H. Reinhold. In preparation.
16. A. C. Guyton, Measurement of the respiratory volume of laboratory animals. *Amer. J. Physiol.* **150,** 194 (1947).
17. W. C. Schneider, Intracellular distributions of enzymes, III. The oxidation of octanoic acid by rat liver fractions. *J. biol. Chem.* **176,** 259 (1948).
18. T. M. Devlin, Neoplastic tissue mitochondria. In *Methods in Enzymology.* (Edited by R. W. Estabrook and M. E. Pullman) Vol. X, p. 110. Academic Press, New York (1967).
19. R. E. Basford, Preparation and properties of brain mitochondria. In *Methods in Enzymology.* (Edited by R. W. Estabrook and M. E. Pullman) Vol. X, p. 96. Academic Press, New York (1967).
20. B. Chance and B. Hess, Metabolic control mechanisms. I. Electron transfers in the mammalian cell. *J. biol. Chem.* **234,** 2404 (1959).
21. B. Chance and G. R. Williams, Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. biol. Chem.* **217,** 409 (1955).
22. A. G. Gornall, C. J. Bardawill and M. N. David, Determinations of serum proteins by means of the Biuret Reaction. *J. biol. Chem.* **177,** 751 (1949).
23. A. Ghosh and B. Chance, Oscillations of glycolytic intermediates in yeast cells. *Biochem. biophys. Res. Comm.* **16,** 174 (1964).
24. A. Betz and B. Chance, Phase relationship of glycolytic intermediates in yeast cells with oscillatory metabolic control. *Arch. Biochem.* **109,** 585 (1965).
25. B. Chance, B. Hess and A. Betz, DPNH oscillations in a cell-free extract of *S. carlsbergensis. Biochem. biophys. Res. Comm.* **16,** 182 (1964).
27. M. Gosalvez, L. López-Alarcón, S. García-Suarez, A. Montalvo and S. Weinhouse, Stimulation of respiration of Ehrlich ascites tumor cells by inhibitors of pyruvate kinase. *Europ. J. Biochem.* **55,** 315 (1975).
26. B. Chance and G. Hollunger, Inhibition of electron and energy transfer in mitochondria. I. Effects of amytal, thiopental, rotenone, progesterone and methylene glycol. *J. biol. Chem.* **278,** 418 (1963).
28. M. Gosalvez, R. García-Cañero, M. Blanco and C. Gurucharri-Lloyd, Effects and specificity of anticancer agents on the respiration and energy metabolism of tumor cells. *Cancer Chemother. Reports.* In press (1975).

29. M. WAGNER and M. ERECINSKA, Experimental and theoretical studies of the electron transport chain. I. *Arch. Biochem.* **147,** 666 (1971).
30. M. WAGNER, M. ERECINSKA and M. PRING, Theoretical and experimental studies of the electron transfer reactions in the cytochrome chain. II. *Arch. Biochem.* **147,** 675 (1971).
31. D. MAZIA, Mitosis and the physiology of cell division. In *The Cell.* (Edited by J. BRACHET and A. MIRSKY), Academic Press, New York (1961).
32. R. A. TOBEY, D. F. PETERSEN and E. C ANDERSON, Energy requirements for mitosis in Chinese hamster cells. In *Biochemistry of Cell Division* (Edited by R. BASERGA) 39 Ch. Thomas, Springfield, Illinois (1969).

Europ. J. Cancer Vol. 11, pp. 717–719. Pergamon Press 1975. Printed in Great Britain

Putrescine as Inhibitor of 3,4-Benzopyrene Cancerogenesis

GEORGE KALLISTRATOS, with the technical assistance of URSULA KALLISTRATOS
Research Institute for Experimental Biology and Medicine, D-2061 Borstel. F.R. Germany

Abstract—*The cancerogenic action of 3,4-benzopyrene (3,4-BP) can be reduced by substances physiologically occurring in organism of several animal species and human beings, as well as by a number of synthetically-prepared compounds. Experimental investigations on female mice (IMRI-strains, 4–5 weeks old, 20–25 g of body weight) have revealed that when 10 mg putrescine was added to the 3,4-BP solutions (2·52 mg 3,4-BP in 0·5 ml tricaprylin) the local development of tumors, after a single s.c. injection, was considerably decreased. In comparison to the groups treated with only 3,4-BP solutions, the cancerogenesis was almost 100%.*

INTRODUCTION

THE CANCEROGENIC action of 3,4-benzopyrene (3,4-BP) has been described in numerous detailed publications since 1933, after the isolation and identification of the compound by Cook, Hewett and Hieger [1]. A few years later the first papers appeared, which reported possibilities for inactivating cancerogenic properties of 3,4-BP by means of several chemical compounds. Meanwhile, a number of natural and synthetic anticancerogens have been found, which are able to reduce the 3,4-BP-cancerogenesis up to complete inhibition. The chemical composition of these anticancerogens varies considerably and they belong to different chemical groups such as a,β-unsaturated aliphatic di- and tricarboxylic acids and their derivatives, i.e., maleic and citraconic anhydrides [2], dimethyl acetylene dicarboxylate [3], cis-aconitic acid [4], etc; also, some flavons [5], quinones [6], peroxides [7], mono- and dithiols [8], (i.e., mercaptosuccinic acid, L-cysteine ethyl ester, bismuthiol I., dithiothreitol) as well as some biogenic amines and their decarboxylation products, (putrescine) [9], etc. In addition, the degradation of the 3,4-BP molecule by soil microflora, results in the loss of its cancerogenic activity [10]. Theoretically, the inhibition of the 3,4-BP-cancerogenesis by bacteria and their metabolic products could also take place in the digestive tract of several animal species which are "not susceptible" to cancerogens [11].

Among the compounds produced by the bacteria and having an important role on the metabolism of cancer cells is, in our opinion, the diamine putrescine [12]. Putrescine (1,4-diaminobutane) was selected for our studies on the "mechanism of the biological inactivation of cancerogens in mammals", because it reacts with several active groups of cell components, such as DNA, RNA, etc. and can thus inhibit them to react with the cancerogen. Therefore, putrescine could block indirectly the reaction between 3,4-BP and several cell components, thus avoiding the cytological alterations caused by 3,4-BP which are responsible for cancerogenesis.*

MATERIAL AND METHODS

To prove the effect of putrescine on the 3,4-BP-cancerogenesis, female IMRI mice

Accepted 28 May 1975.

*The mechanism of the inhibition of the 3,4-BP cancerogenesis by putrescine is still unknown. We suppose that putrescine probably acts as "isolator" between 3,4-BP and cell components, *hindering their binding* with the cancerogen.

strains, 4–5 weeks old and 20–25 g of body weight, were treated with 2·52 mg 3,4-benzopyrene + 10 mg putrescine dissolved in 0·5 ml tricaprylin and injected s.c. (Group B + P). In a preliminary study using a group of 10 mice we noticed the strong inhibitory action of putrescine on the 3,4-BP-cancerogenesis. To confirm our observations, we checked the results again with 30 laboratory animals, for a statical evaluation. For comparative studies, 30 more mice were treated under the same experimental conditions, but with only 2·52 mg 3,4-BP (Group B). In group C, 30 animals served as controls without any treatment.

3,4-Benzopyrene, $C_{20}H_{12}$, mol.wt. 252·32, melting point 176·5°C, > 99% purity, was purchased from the Fa.Schuchardt, München, Germany. Putrescine (1,4-diaminobutane) $C_4H_{12}N_2$, mol.wt. 88·15 (grade of purity: purissimum) F 25–27, Kp_{11} 60–61°, d_4^{25} 0·881, n_D^{25} 1.457 was purchased from Fa.Fluka, Buchs-Swiss, as well as, tricaprylin (glycerin-tricaprylate, grade of purity: technical). The mixture of 2·52 mg 3,4-BP + 10 mg putrescine in 0·5 ml tricaprylin was injected immediately after preparing the solution to avoid the formation of a "gell" which is difficult to inject.

RESULTS AND DISCUSSION

The experimental results are graphically summarized in Fig. 1. All animals of group B, where only 3,4-BP was injected, developed a tumor after 80–100 days and died because of the malignant process between 100–200 days after the 3,4-BP injection. The tumors were mainly fibrosarcomas, but also rhabdomyosarcomas and carcinomas (Fasske). On the contrary, in group B + P the tumor development was almost completely inhibited (photographs). From this group, 2 mice died few days after the injection probably due to the toxicity of putrescine (injected putrescine amount 400–600 mg/kg of body weight). Four more animals died during the first year of observation and ten mice between 365–523 days. None of these sixteen mice dead developed a tumor locally. Only in one mouse dead 361 days after the 3,4-BP + P-injection, did we find a tumor located in the stomach. This is an interesting finding, because usually experimental tumors are developed locally in the place where the cancerogen was injected, and metastasis is relatively rare. Another possibility is that probably the tumor was developed spontaneously, independent from the treatment with 3,4-BP + putrescine.

Thirteen mice from the group B + P are still alive (age of animals 540 days) and only 2 developed a tumor.

From the control untreated group C, 5 mice died of accidents or diseases not related to tumors.

The high mortality of the group B, as well as the high rate of tumor development in comparison to the group B + P, indicates that the presence of putrescine in non-toxic doses can inhibit the 3,4-benzopyrene cancerogenesis and prolong the life of mice treated with 3,4-BP.

Acknowledgement—I thank Dr. E. Fasske for the histological examinations of the tumors.

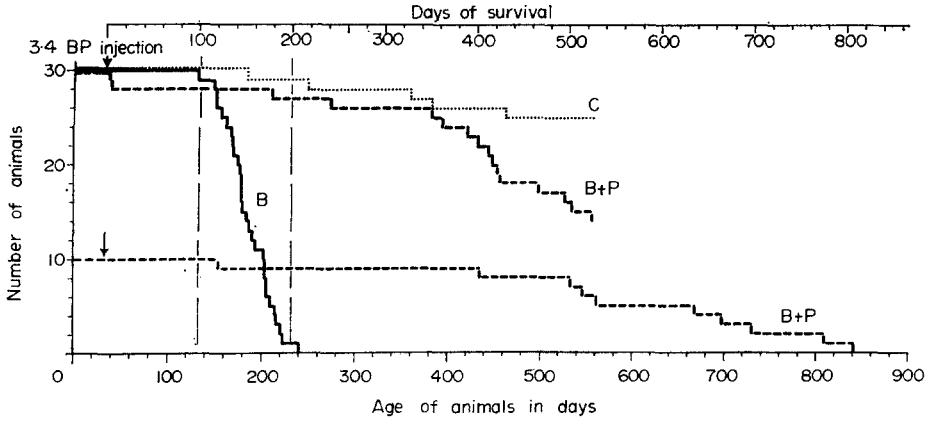


Fig. 1. The effect of putrescine on the 3,4-benzopyrene cancerogenesis. Group B: The mortality rate of 30 mice treated with 2·52 mg 3,4-BP. Animals died within 7 months after a single s.c. injection of 3,4-BP due to the tumor development which was 100%. Group B + P: Prolongation of life of the mice treated similar to B + 10 mg putrescine added to 3,4-BP solution. Inhibition of tumor development in presence of putrescine. Group C: Control untreated mice.

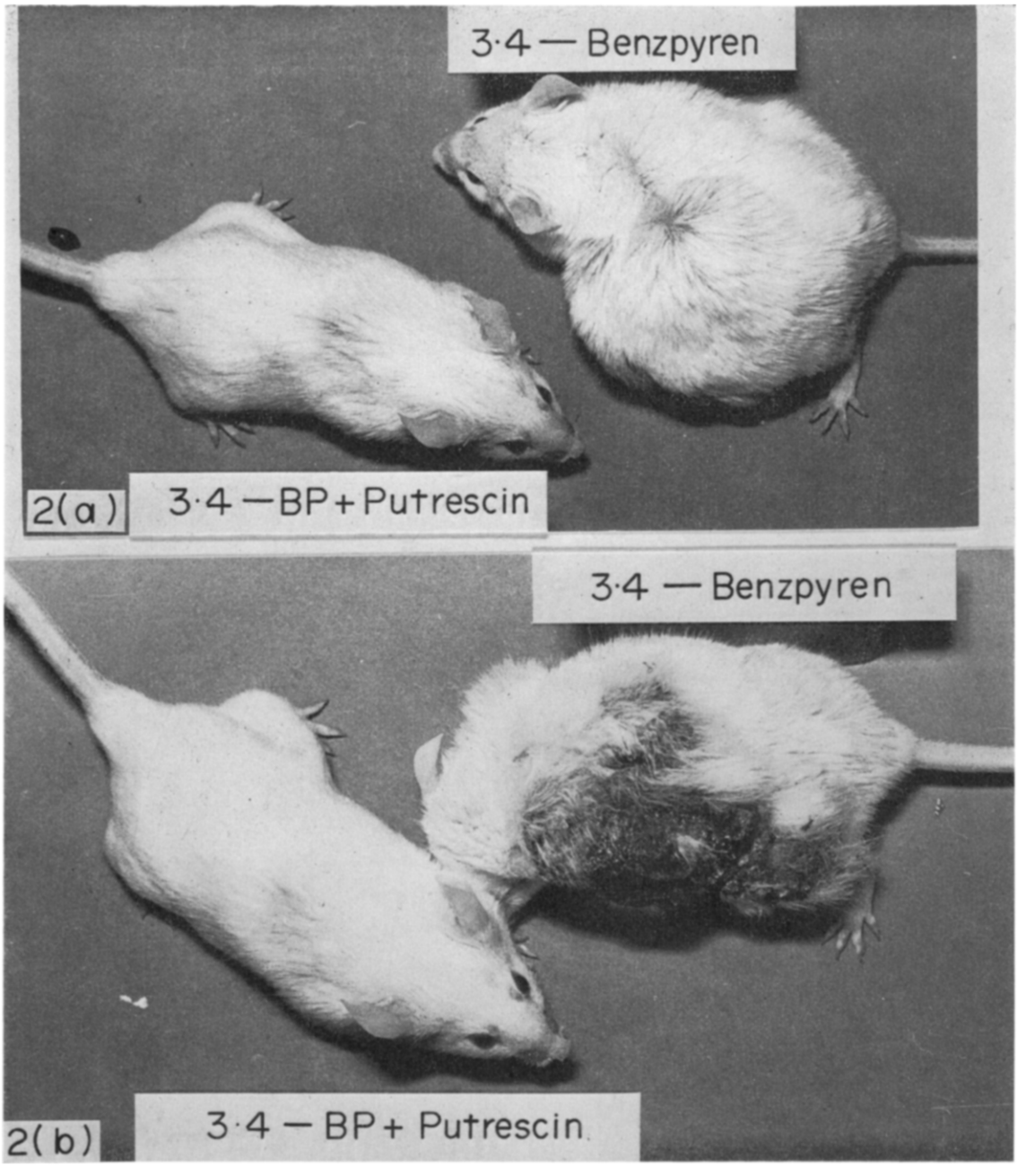


Fig. 2. Two characteristic photographs demonstrating the inhibition of 3,4-*BP-cancerogenesis in presence of putrescine. Right: Tumor development* 6 *months after a single s.c. injection of* 2·52 *mg of* 3,4-*BP in* 0·5 *ml tricaprylin. Left: Complete inhibition of tumor development in the presence of* 10 *mg of putrescine to the injected* 3,4-*BP solution. (Female IMRI mice).*

(to face p. 718)

REFERENCES

1. J. W. Cook, C. L. Hewett and I. Hieger, The isolation of a cancer producing hydrocarbon from coal tar. *J. chem. Soc.* **135,** 395 (1933).
2. H. G. Crabtree, Influence of unsaturated dibasic acids on the induction of skin tumors by chemical carcinogens. *Cancer Res.* **5,** 346 (1945).
3. Y. Iwanami and S. Odashima, Inactivation of Benzo(a)pyrene. *Naturwiss.* **61,** 509 (1974).
4. G. Kallistratos and U. Kallistratos, Die Bedeutung der cis-Aconitsäure für die biologische Inaktivierung der 3,4-Benzopyren Kanzerogenese. *Experientia* **31,** 490 (1975).
5. L. W. Wattenberg and L. Leong, Inhibition of the cancerogenic action of Benzo(a)pyrene by Flavons. *Cancer Res.* **30,** 1922 (1970).
6. V. von Brand, Chinon und cancerogene Wirkung. *Naturwiss.* **42,** 300 (1955).
7. C. Nagata, Y. Tagashira and M. Kodama, Effect of hydrogen peroxide, Fenton's reagent and irons ions on the carcinogenicity of 3,4-Benzopyrene. *Gann* **64,** 277 (1973).
8. G. Kallistratos and U. Kallistratos, Verhinderung der 3,4-Benzopyren-Kanzerogenese durch natürliche und synthetische Verbinydungen. *Münch. med. Wschr.* **117,** 391 (1975).
9. G. Kallistratos and U. Kallistratos, Hemmung der 3,4-Benzopyren Kanzerogenese bei Mäusen duch Putrescin. *Naturwiss.* **61,** 459 (1974).
10. A. Khesina, N. P. Shcherbak, L. M. Shabad and I. S. Vostrov, Destruction of Benzo(a)pyrene by soil microflora. *Byull. éksp. biol. Med. USSR* **68,** 70 (1969).
11. G. Kallistratos and A. Pfau, Zur Wirkung von 3,4-Benzpyren bei großen Säugetieren. *Naturwiss.* **58,** 222 (1971).
12. G. Kallistratos, A. Pfau and A. Timmermann, Suggestions and experimental studies on cancer biochemistry. *Chim. Chron.* (*Athens*) **31A,** 4 (1966).

Europ. J. Cancer Vol. 11, pp. 721–724. Pergamon Press 1975. Printed in Great Britain

Prophylaxis of Chemically Induced Epithelial Tumors with an Aromatic Retinoic Acid Analog (Ro 10-9359)

WERNER BOLLAG

Department of Experimental Medicine, F. Hoffmann-La Roche & Co., Ltd., CH-4002 Basel, Switzerland.

Abstract—*An aromatic analog of retinoic acid, ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (= Ro 10-9359), has been found to possess a prophylactic effect on the development of skin papillomas and carcinomas of mice induced by 7,12-dimethylbenzanthracene and croton oil. The daily oral application of 30 mg/kg Ro 10-9359—given during the promotion phase of carcinogenesis—delayed the appearance and retarded the growth of papillomas and reduced the incidence of carcinomas. Considering the low toxicity of Ro 10-9359, its preventive action on epithelial tumors is very remarkable.*

INTRODUCTION

RETINOIC acid exerts a prophylactic as well as a therapeutic effect on carcinogen induced benign and malignant skin tumors of mice [1, 2]. In experimental and clinical investigations the toxic effects of the so-called hypervitaminosis A syndrome limit the application of high doses for prophylactic and therapeutic purposes. Therefore analogs of retinoic acid were sought, which would possess more favourable ratios between anti-tumor effect and toxicity. Recently aromatic analogs of retinoic acid have been found to have a far better therapeutic quotient than retinoic acid [3, 4]. In this paper the prophylactic effect of such a retinoic acid analog on the development of chemically induced benign and malignant epithelial tumors of the skin is described.

MATERIAL AND METHODS

Female Swiss albino mice of the random-bred Füllinsdorf strain, weighing 20–22 g, were fed the mouse diet Nafag 199, containing 2500 international units of vitamin A per kg food.

Accepted 6 June 1975

Papillomas and carcinomas were induced by painting 7,12-dimethylbenz(α)anthracene (DMBA) as initiating agent and croton oil as promoting agent on the skin of mice. One hundred and fifty mcg DMBA dissolved in 0·2 ml acetone were applied on days 1 and 8 on a 5 cm^2 area of shaved skin on the back of the mice. One-half mg croton oil dissolved in 0·2 ml acetone was painted on the skin twice a week from day 42 on. The aromatic retinoic acid analog Ro 10-9359 = Ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (Fig. 1) was

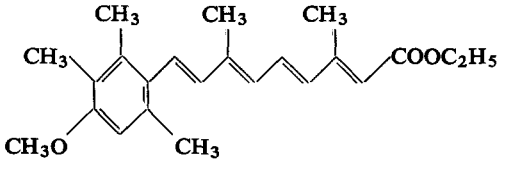


Fig. 1. Chemical structure of the aromatic retinoic acid analog Ro 10-9359.

suspended in arachis oil and given during the promotion phase of carcinogenesis from day 42 until the end of the experiment (day 293). Ro 10-9359 was administered by stomach tube to 66 mice, in a dose of 30 mg/kg daily on 5 days of the week. The control group of

66 mice received only the vehicle. The appearance and development of papillomas and carcinomas were recorded. The volumes of papillomas were calculated with the formula $\frac{4}{3}r^3\pi$, r being the mean radius. The mean number of papillomas per animal and the mean volume of papillomas per animal as well as the cumulative number of carcinomas were determined.

RESULTS

In the control group 47 of 66 mice were still surviving at day 293 of the experiment. In the treated group 56 out of 66 animals were still living. In the control group, at day 293, 9 mice had died from carcinoma and 10 from other causes. In the treated group 2 animals had died from carcinoma and 8 from other conditions. Thus Ro 10-9359 in the doses used exerted no serious or even lethal toxic effects. The only symptom of hypervitaminosis A was a slight desquamation of the skin which appeared in 18 of the animals treated with Ro 10-9359.

Papillomas

The aromatic retinoic acid analog Ro 10-9359 has a very marked inhibiting effect on the development of papillomas (Table 1, Fig. 2, Fig. 3). On day 153 after the first application of DMBA the mean number of papillomas per animal was 3·59 in the control group, whereas in the mice treated with Ro 10-9359 the average number was only 1·49 per animal. In the

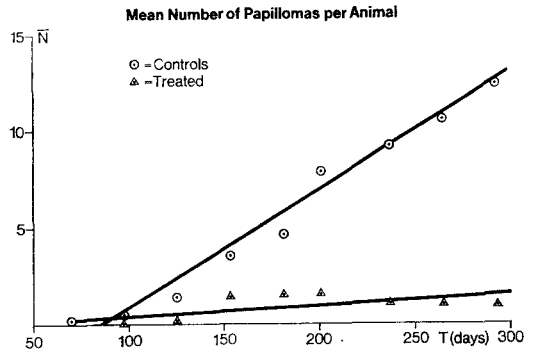


Fig. 2. Mean number of papillomas per animal in controls [⊙] and Ro 10-9359 treated mice [△]. $\bar{N}$ = Mean number of papillomas per animal; T = Days after first application of carcinogen.

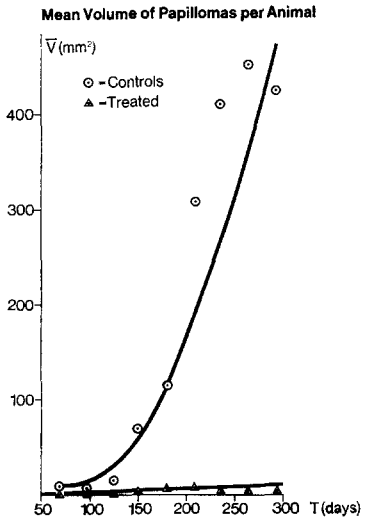


Fig. 3. Mean volume of papillomas per animal in controls [⊙] and Ro 10-9359 treated mice [△]. $\bar{V}$ = Mean volume of papillomas per animal (mm^3); T = Days after first application of carcinogen.

Table 1. Prophylaxis of epithelial tumors with the aromatic retinoic acid analog Ro 10-9359

	Mean number of papillomas per animal									
Days after first application of carcinogen	0	69	97	125	153	181	201	237	265	293
Controls	0	0·2	0·55	1·48	3·59	4·67	7·93	9·23	10·6	12·43
Mice treated with Ro 10-9359 3 0 mg/kg daily, orally	0	0·09	0·14	0·34	1·49	1·58	1·63	1·10	1·07	1·05
	Mean volume of papillomas per animal (mm^3)									
Days after first application of carcinogen	0	69	97	125	153	181	209	237	265	293
Controls	0	8·3	5·42	16·18	70·7	116·27	310·47	412·09	453·17	426·28
Mice treated with Ro 10-9359 30 mg/kg daily, orally	0	1·82	0·76	1·28	3·07	7·36	8·7	5·72	5·9	6·82
	Incidence of carcinomas (cumulative number)									
Days after first application of carcinogen	0	69	97	125	153	181	209	237	265	293
Controls	0	0	1	1	1	3	5	13	26	46
Mice treated with Ro 10-9359 30 mg/kg daily, orally	0	0	0	1	1	1	1	3	3	4

further course of the experiment the controls showed a steady rise of their papilloma count to a mean number of 12·43 at day 293. On the other hand, in the treated group the mean number of papillomas per animal even declined slightly to an average of 1·05 papillomas per animal at the end of the experiment. As can be seen from Fig. 2 the mean number of papillomas per animal in the control group grows as a function of time. The regression coefficient $b = 0{\cdot}06$ is highly significantly greater than 0 with $\alpha < 0{\cdot}001$. For the treated group a regression coefficient $b = 0{\cdot}0024$ has been obtained. This value is not significantly different from 0 ($\alpha > 0{\cdot}05$). That means, in the treated group there is no statistically significant increase of the mean number of papillomas per animal within the considered period between day 69 and day 293. The comparison of the mean volumes of papillomas gave a still more marked difference between the values of the controls and the treated animals. On day 153 the controls had a mean volume of papillomas per animal of 70·7 mm^3 whereas the corresponding value for the treated mice was only 3·07 mm^3. During the next phase of the experiment the difference was still growing. On day 293, the mean papilloma volume per animal had risen to 426·28 mm^3 whereas this value for the treated mice reached only 6·82 mm^3. This signifies a ratio of more than 60 to 1 in favour of the animals treated with Ro 10-9359. From Fig. 3 can be seen that the mean volume of papillomas per animal in the control group grows much stronger than in the treated group. The difference of the regression coefficient of the 2 lines ($b_1 = 2{\cdot}136$ for the controls and $b_2 = 0{\cdot}028$ for the treated group) is statistically significant, as tested by the t-test with $t = 9{\cdot}82$ and $\alpha < 0{\cdot}001$.

Carcinomas

The effect of Ro 10-9359 on the induction of carcinomas can be seen from Table 1 and Fig. 4. In the control animals a cumulative number of 46 carcinomas was reached during the experiment of 293 days. The treatment with Ro 10-9359 had a clear-cut preventive effect, as only 4 carcinomas appeared. This is an approximately ten fold smaller value for the treated group than that for the unprotected controls.

Animals free of papillomas and carcinomas

At the end of the experiment only 5 of 47 surviving animals of the control group—10·6%—were free of papillomas and carcinomas. In the group treated with Ro 10-9359, however, 32 of the 56 surviving mice—57·1%—had neither papillomas nor carcinomas.

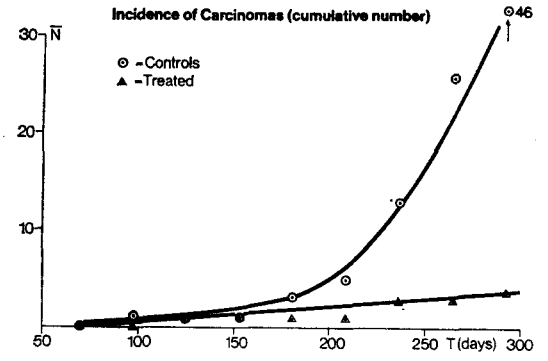


Fig. 4. Incidence of carcinomas (cumulative number) in controls [⊙] *and Ro* 10-9359 *treated mice* [△]. $\bar{N}$ = *Number of carcinomas; T = Days after first application of carcinogen.*

DISCUSSION

Ro 10-9359, an aromatic analog of retinoic acid, systemically applied during the promotion phase of chemical carcinogenesis, has a very marked prophylactic effect on the development of papillomas and carcinomas of mouse skin. It delays the appearance and retards the growth of papillomas and it reduces also the incidence of carcinomas. It was known from previous experiments that the therapeutic quotient of this compound—correlating anti-tumor effect and toxicity—was very favourable [3]. Chemically induced skin papillomas had been made to regress with doses of Ro 10-9359 which did not provoke a hypervitaminosis A. The doses applied in the present prophylactic experiment induced only a slight desquamation of the skin, but not the other toxic symptoms of the hypervitaminosis A syndrome consisting of alopecia, alterations of mucous membranes and bone fractures; nevertheless a very marked preventive effect on papilloma and carcinoma induction was achieved. Therefore it may be justified to consider the application of this compound for the prevention of malignant epithelial tumors in groups of human beings who run a high risk of cancer incidence. The underlying principle of the action of this compound probably does not differ from that of retinol, its esters and retinoic acid which have been shown to possess a prophylactic effect on chemically induced epithelial tumors [2, 5–10]. The advantage of Ro 10-9359 lies in its dissociation of anti-tumor effect and toxicity which had been detected in a screening procedure, described elsewhere [3].

In organ cultures Ro 10-9359 and the corresponding free acid like other retinoic acid analogs have been found to prevent or counteract hyperplastic and metaplastic changes of tracheal and prostate epithelium induced by vitamin A deficiency or methylcholanthrene [11–14]. *In vivo* the metaplasia of the vaginal epithelium induced by hormones has been prevented [15]. Ro 10-9359 may therefore exert its tumor prophylactic effect by the prevention of such preneoplastic changes. However, the fact that Ro 10-9359 is also therapeutically active against well established papillomas and carcinomas [3] is an argument in favour of a mechanism of action identical for preneoplastic as well as neoplastic lesions. As we still know very little of the biochemistry of vitamin A and its analogs in relation to their regulatory and differentiating effect on epithelial tissues, their biochemical action on neoplastic epithelial tissues also still remains to be elucidated.

REFERENCES

1. W. Bollag, Effect of vitamin A acid (NSC-122758) on transplantable and chemically induced tumors. *Cancer Chemother. Rep.* **55,** 53 (1971).
2. W. Bollag, Prophylaxis of chemically induced benign and malignant epithelial tumors by vitamin A acid (retinoic acid). *Europ. J. Cancer* **8,** 689 (1972).
3. W. Bollag, Therapeutic effects of an aromatic retinoic acid analog on chemically induced skin papillomas and carcinomas of mice. *Europ. J. Cancer* **10,** 731 (1974).
4. W. Bollag, Therapy of epithelial tumors with an aromatic retinoic acid analog. *Chemotherapy* **21,** 236 (1975).
5. E. W. Chu and R. A. Malmgren, An inhibitory effect of vitamin A on the induction of tumors of forestomach and cervix in the Syrian hamster by carcinogenic polycyclic hydrocarbons. *Cancer Res.* **25,** 884 (1965).
6. M. V. Cone and P. Nettesheim, Effect of vitamin A on 3-methylcholanthrene-induced squamous metaplasia and early tumors in the respiratory tract of rats. *J. nat. Cancer Inst.* **50,** 1599 (1973).
7. R. E. Davies, Effect of vitamin A on 7,12-dimethylbenz(α)anthracene-induced papillomas in rhino mouse skin. *Cancer Res.* **27,** 237 (1967).
8. N. A. Rowe and R. J. Gorlin, The effect of vitamin A defficiency upon experimental oral carcinogenesis. *J. dent. Res.* **38,** 72 (1959).
9. U. Saffiotti, R. Montesano, A. R. Sellakumar and S. A. Borg, Experimental cancer of the lung. Inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous cell tumors. *Cancer (Philad.)* **20,** 857 (1967).
10. R. J. Shamberger, Inhibitory effect of vitamin A on carcinogenesis. *J. nat. Cancer Inst.* **47,** 667 (1971).
11. I. Lasnitzki, Personal communication.
12. I. Lasnitzki and D. S. Goodman, Inhibition of the effects of methylcholanthrene on mouse prostate in organ culture by vitamin A and its analogs. *Cancer Res.* **34,** 1564 (1974).
13. M. B. Sporn, Personal communication.
14. M. B. Sporn, G. H. Clamon, N. M. Dunlop, D. L. Newton, J. M. Smith and V. Saffiotti, Activity of vitamin A analogues in cell cultures of mouse epidermis and organ cultures of hamster trachea. *Nature (London)* **253,** 47 (1975).
15. H. Weiser, Personal communication.

Europ. J. Cancer Vol. 11, pp. 725–732. Pergamon Press 1975. Printed in Great Britain

Effects of Mixtures and Complexes of Chlorambucil and Antibody on a Human Melanoma Cell Line

C. VENNEGOOR, D. VAN SMEERDIJK and Ph. RÜMKE
Department of Immunology, Antoni van Leeuwenhoekhuis, The Netherlands Cancer Institute, Sarphatistraat 108, Amsterdam 1004, The Netherlands

Abstract—*Cultured human melanoma cells were incubated with chlorambucil, antibody, a mixture of the two, or a complex of chlorambucil and antibody. Antibody increased the cytotoxic action of chlorambucil when the incubation was simultaneous. The effect was dependent on the concentration of both drug and antibody. When incubation with antibody was followed by incubation with chlorambucil, or the sequence of incubations was reversed, no synergistic effect on the tumour cells was noticed. The cytotoxic effect of the complex of chlorambucil and antibody was not higher than that of the mixture of chlorambucil and antibody.*

INTRODUCTION

CYTOTOXIC drugs are frequently used in the treatment of cancer. However, there are often limitations in their application as a result of an accompanying systemic toxicity. For this reason attempts have been made to bind cytotoxic agents to plasma proteins [1–4] which seem to be taken up rather selectively by cancer cells without prior extracellular hydrolysis [5–7]. A particularly attractive concept is the binding of a cytotoxic drug to specific, tumour-directed antibodies. Several reports have now been made of favourable effects of chlorambucil bound to antibodies in animal tumour systems *in vivo* [8–10], and it has also been possible to study the effect *in vitro* [11]. In addition, in a patient with disseminated melanoma, regression of all the metastatic nodules has been described after treatment with chlorambucil-labelled antibody directed to the tumour [9].

For linkage of drug to antibody the authors applied a method described by Linford *et al.* [12] for physical binding of chlorambucil to serum proteins without loss of its alkylating activity. However, recent studies indicated that it is unlikely that chlorambucil is sufficiently firmly attached to immunoglobulins under these conditions [13, 14]. Moreover, it has been shown that a mixture of drug and antibody or a separate administration of these agents can already be effective *in vivo* [11, 15–17] as well *in vitro* [14].

In this paper, we report our results of the binding of chlorambucil to gamma-globulin, and also the effect of chlorambucil and antibody on human melanoma cells *in vitro*.

Accepted 6 June 1975.

MATERIAL AND METHODS

Phosphate buffered saline

Phosphate buffered saline (PBS), pH 7·2, consisted of NaCl, 8·0 g/l; KCl, 0·2 g/l; KH_2PO_4, 0·2 g/l and $Na_2HPO_4 \cdot 2H_2O$, 1·15 g/l,

Melanoma cells

Human melanoma cells, NKI-2 and NKI-4, have both been developed to cell lines in our laboratory [18]. The NKI-2 cells were used for immunisation. The cytotoxicity tests were performed with NKI-4 cells which were subcultured

in a CO_2-incubator at 37°C. The medium consisted of MEM-Eagle containing 10% (v/v) foetal bovine serum (Gibco Biocult, Glasgow, Great Britain), and a mixture of antibiotics, namely Na–penicillin G (100 U/ml), streptomycin (0·1 mg/ml), pimaricin (2·5 mg/ml) and kanamycin (0·1 mg/ml). All antibiotics were obtained from Mycofarm (Delft, The Netherlands).

Antiserum preparation

The cultured melanoma cells (NKI-2) to be used for immunisation were detached from the tissue culture flasks by incubation with 0·54 mM EDTA in PBS for 3 min at 37°C. Immediately after the incubation an equal volume of PBS containing 0·65 mM $CaCl_2$ and 0·54 mM $MgSO_4$ was added. The cells were washed five times in PBS. A suspension of 10^8 living cells in 1 ml PBS (without adjuvant) was injected three times, with weekly interfalls, into the ear vein of a rabbit. One week after the last injection blood was collected by cardiac puncture.

Sera and gamma-globulin fractions

Sera. Sera were inactivated by incubation at 56°C for 30 min, and stored in small aliquots at −20°C.

The antiserum was used unabsorbed, since absorption with normal tissue reduced the titer to such an extent that antibodies against melanoma cells could hardly be detected. Moreover, for the present *in vitro* studies it was unimportant whether the antibodies were directed to normal or to tumour associated antigens. Detection of antibodies was performed with the membrane immunofluorescence test. With NKI-4 cells the titer of the antiserum, expressed as the serum dilution that stained 50% of the cells, was 800. The control serum diluted 1:10 was negative.

Preparation of gamma-globulin. All steps were carried out at 0−4°C. The sera were diluted two-fold with PBS. Fractions containing largely IgG were prepared by adding $(NH_4)_2SO_4$ to a final concentration of 1·6 M $(NH_4)_2SO_4$ (pH 7·2). The precipitate was washed two times with a 1·6 M $(NH_4)_2SO_4$ solution, dissolved, and dialysed against PBS.

In some cases the gamma-globulin fraction was further purified by passage through a column of DEAE-Sephadex A-50 equilibrated in 0·41% NaCl, 0·08% $Na_2HPO_4 \cdot 2H_2O$ and 0·02% $NaH_2PO_4 \cdot H_2O$, pH 7·4 [19]. The protein containing fractions, which appeared to be pure IgG after examination with immunoelectrophoresis, were concentrated by ultrafiltration under high pressure (membrane PM 10, Amicon Co., Lexington, Mass., U.S.A.) to concentrations of 10 mg protein per ml. The gamma-globulin fractions were stored in small aliquots at −70°C.

Physical binding of chlorambucil to gamma-globulin

Chlorambucil was linked to the gamma-globulin fraction of antiserum by a procedure described by Blakeslee and Kennedy [20]. Chlorambucil (Sigma, St. Louis, Mo., U.S.A.) was dissolved in 0·06 M NaOH and immediately added to an equal volume of either the gamma-globulin or the more purified IgG fraction of antiserum in PBS, previously diluted 1:2 in distilled water. The concentration of protein in the reaction mixture was 5 mg/ml and the protein to chlorambucil ratio (w/w) was 1:1·52. In case of pure IgG, this ratio corresponds to a molar ratio of protein to chlorambucil of 1:750. The pH of the mixture was adjusted to 9·0 or 10·5 with 0·1 N NaOH. After incubation for 10 min at 37°C the sample was subjected to gel-filtration on a column of Sephadex G-25 (coarse), equilibrated in 0·15 M NaCl and 0·01 M Tris–HCl (pH 8·5) at 4°C. The protein containing fractions were dialysed against PBS at 4°C for 24 hr and stored in small quantities at −70°C.

Determination of the active and hydrolysed form of chlorambucil

A method described by Linford was applied [21]. Samples containing free or physically bound chlorambucil were extracted with ethanol. After centrifugation the supernantant fractions were dried by evaporation under reduced pressure. The residues were dissolved in a 0·1 M potassium phosphate buffer (pH 3·6) and extracted with either benzene, for isolation of the active form of the drug, or with ethyl acetate, in which both the active and hydrolysed (inactive) forms of chlorambucil are isolated. The extracts were evaporated till dry under reduced pressure and the residues were dissolved in absolute ethanol. The estimation of the dissolved residues was measured in the spectrophotometer in the region 230–280 nm with 2 nm interfalls.

The amount of chlorambucil was determined from the absorbance at 258 nm after correction for protein [21], scattering [22] and recovery of chlorambucil after extraction [21]. As a reference a solution of 30 μg chlorambucil/ml ethanol was used with an absorbance of 1·55 corrected for scattering.

In a number of experiments the active form of chlorambucil was measured by its alkylating activities in a modified Epstein technique [23]. Both methods for determination of the active form of chlorambucil gave the same results.

Cytotoxicity tests

These tests were carried out under sterile conditions. Melanoma cells (NKI-4) were detached from the tissue culture flasks with the aid of a phosphate buffered saline solution containing 0·54 mM EDTA as described under the immunisation procedure. The cells were centrifuged, resuspended in tissue culture medium and seeded in microtest plates No. 3034 (Falcon, Oxnard, Ca., U.S.A.) so that each well would have 100–150 cells attached to the bottom. After incubation for 18 hr at 37°C the culture medium was removed and freshly prepared solutions of chlorambucil and/or rabbit globulin fractions in culture medium which had been sterilised by passage through Millipore filters (pore size 0·22 μm, Millipore, Bruxelles, Belgium) were added. The volumes of the incubation mixtures were 20 μl. The number of wells in a test or a control was usually 8, and occasionally 4 or 12. After incubation for 24 or 48 hr at 37°C the plates were carefully washed with PBS containing 0·65 mM $CaCl_2$ and 0·54 mM $MgSO_4$. The cells which had neither died nor become detached were fixed in methanol, stained with crystal violet and counted under a microscope.

When chlorambucil was tested, the drug was dissolved to a concentration of 10 mg/ml in 0·5 M $NaHCO_3$. To this solution 4 vol of 0·07 M NaCl were added and 5 vol of medium for tissue culture containing 20% foetal bovine serum. Control experiments with a 0·5 M solution of $NaHCO_3$, diluted like those containing chlorambucil, showed no significant reduction in cell number.

Membrane immunofluorescence

Membrane immunofluorescence was carried out essentially as described by Lewis and Phillips [24], except that incubations were at 37°C for 30 min. As conjugate FITC-labelled horse anti rabbit immunoglobulin was used (PK-17, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The percentage of stained cells was scored and as an end-point of titration the concentration of the test serum which gave 50% positive cells was used. As the percentage of positive cells was proportional to the logarithm of the serum concentration in 80–20% of positive cells (see Fig. 1), the titer could be easily

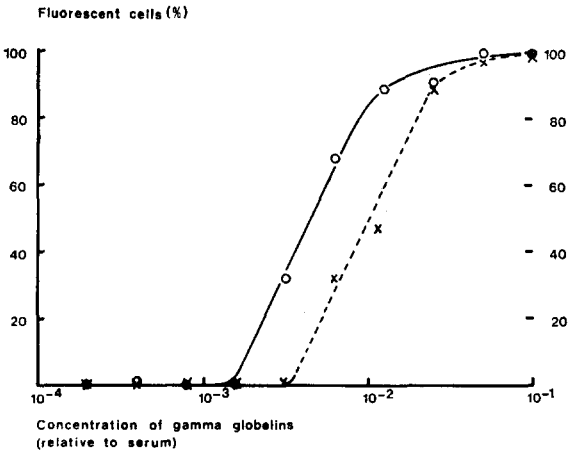


Fig. 1. Membrane immunofluorescence of a partially purified gamma-globulin fraction directed against melanoma cells on the cell line NKI-4.
○ —— ○ *untreated gamma-globulin*
× – – – × *chlorambucil linked to gamma-globulin*

evaluated. The percentage of loss of antibody activity due to the chlorambucil binding procedure was calculated using the formula $(1-B/A)\times 100$, in which A represents the concentration of the globulin fraction, incubated with chlorambucil, which gives 50% fluorescent cells and B the concentration of the untreated globulin fraction giving 50% staining.

Statistical evaluation

To test the significance of the difference in cell number between the test and the control in each microplate, we applied Student's t-test. A value of $P < 0{\cdot}05$ was considered significant.

RESULTS

Binding active chlorambucil to gamma-globulin and to serum proteins

Attempts to link chlorambucil to the gamma-globulin fraction of serum, without loss of the alkylating activity of the drug, were made according to three methods described in the literature. These procedures have in common that linkage is acquired by incubation of protein with drug, whereafter the uncombined drug is removed by dialysis or gel filtration. The incubations were performed at a protein–chlorambucil ratio of 10:1 in the presence of 0·1 M $NaHCO_3$ without adjusting the pH (pH 9·7) [11, 12, 25], the same after adjusting the pH to 3·5 [11], or at a protein–chlorambucil ratio of 1:1·52 at pH 9·0–10·5 [20]. The results are summarised in Table 1. Physically bound chlorambucil was

Table 1. *Binding of chlorambucil to gamma-globulin and serum*

Chlorambucil incubated with	Ratio protein to chlorambucil (μg/ml)	Incubation			Chlorambucil		
		Time	Temperature (°C)	pH	Physically bound Active (%)	Hydrolysed (%)	Binding by alkylation (%)
Partially purified gamma globulin fraction	10:1	1 hr	4	9·7	0	N.D.*	N.D.
		2 hr	20	9·7	0·08	N.D.	N.D.
		2 hr	37	9·7	0	0	49
		30 min	20	3·5	5·2†	0	27
	1:1·52	10 min	37	10·5	8·9	0	2·2
IgG	1:1·52	10 min	37	10·5	13·5	0·3	N.D.
		10 min	37	9·0	9·2	0	N.D.
Serum	10:1	1 hr	4	9·7	23	N.D.	N.D.
		2 hr	4	9·7	14	0	35
		10 min	37	9·7	17	0	35
		2 hr	37	9·7	14	0	61

*Not determined.
†This figure is too high since chlorambucil precipitated at low pH.

recovered after extraction with benzene, indicating that it was present in its active form. This was confirmed by determination of chlorambucil with the Epstein technique. In the buffer used for dialysis only the total content of chlorambucil was measured, without discrimination between the active or hydrolysed (inactive) form. The amount of the added chlorambucil that could not be removed by dialysis, nor be extracted with ethyl acetate from the dialysed incubation mixtures, was considered covalently bound to protein by alkylation. Occasionally, samples were investigated before and after they had been stored at −20°C for 12 days. Virtually the same contents of active chlorambucil were found. This indicates that chlorambucil when physically bound to protein is preserved from both hydrolysis and alkylation at temperatures below zero.

Comparison of the results of binding the active form of chlorambucil to gamma-globulin with that to whole serum, using a protein–chlorambucil ratio of 10:1 at pH 9·7 (Table 1), indicated that chlorambucil could not be bound to the gamma-globulin fraction of serum by the procedure used for whole serum [12]. We also attempted to bind chlorambucil to gamma-globulin at low pH [11]. The major difficulty encountered consisted in the poor solubility of chlorambucil at low pH.

When the gamma-globulin fraction of serum was incubated at a considerably lower protein–chlorambucil ratio 1:1·52 at high pH, binding of active chlorambucil to the protein occurred. The data presented were obtained after gel filtration of the incubation mixture on Sephadex G-25. After subsequent dialysis against PBS some 80% of the chlorambucil initially bound to the gamma-globulin was still present in the active form. However, a considerable proportion of the protein precipitated in the majority of the samples, apparently in the form of a chlorambucil–protein complex since on centrifugation both the chlorambucil and protein contents of the samples were lowered, while the chlorambucil to protein ratio of the supernatant remained constant.

Determination of antibody activity

The titer of antibody in the unabsorbed gamma-globulin fraction of the antiserum before and after incubation with chlorambucil was measured in the indirect membrane immunofluorescence test. In case chlorambucil was bound to gamma-globulin loss of antibody activity was about 50% (Fig. 1). The loss of antibody activity appeared not to be due to the presence of chlorambucil in the protein but to the exposure to high pH.

Effects of chlorambucil and antibody on melanoma cells in vitro

Effect of chlorambucil alone. We first examined the cytotoxic effect of chlorambucil on human melanoma cells. Figure 2 shows the dose–response. After incubation for 1 hr with chlorambucil no reduction in cell number was noticed. After prolongation of incubation to 24 hr or more, significant effects of chlorambucil were detectable. When the results were plotted on log-probability paper [27], the concentration of

chlorambucil resulting in 20% reduction in cell number was found to be 100 μg ml in case the incubation time after addition of the drug was 24 hr, and 50 μg/ml when the incubation time with the drug was 48 hr.

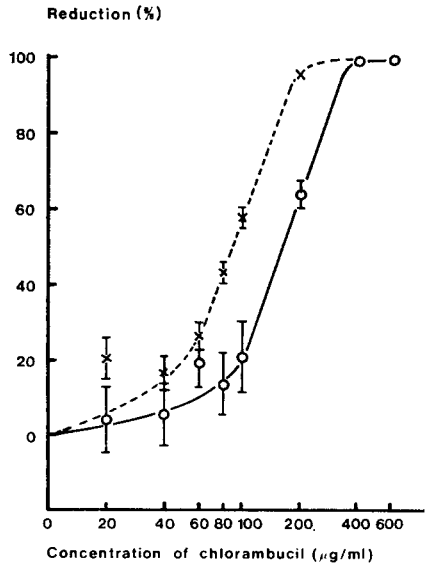


Fig. 2. Dose response on human melanoma cells (NKI-4) of chlorambucil. The vertical lines indicate twice the s.e. of the mean in both directions [26]
○——○ *effect after 24 hr of incubation in the presence of chlorambucil*
×---× *effect after 48 hr of incubation in the presence of chlorambucil.*

Effect of chlorambucil bound to antibody. Chlorambucil linked to the unabsorbed antimelanoma gamma-globulin fraction was tested on melanoma cells in a 48 hr incubation period. As controls chlorambucil, gamma-gobulin, and the combination of free chlorambucil and gamma-globulin were used at the same concentrations as in the complex of chlorambucil and gamma-globulin (Table 2). Often no cytotoxicity of the complex was found and the effect was always equal to, or less than, that of the mixture of chlorambucil and antibody. In some experiments the reduction in cell number obtained with the mixture of drug and gamma-globulin was significantly higher than that of each of the controls of chlorambucil and gamma-globulin. For this reason we further studied the effect of different combinations of chlorambucil and gamma-globulin on melanoma cells.

Effect of co-incubation with chlorambucil and antibody. Table 3 summarises the results when the tumour cells were incubated in the presence of different concentrations of chlorambucil and/or gamma-globulin. Though normal gamma-globulin fraction never showed any immunofluorescence staining or complement dependent cytotoxicity, a cytotoxic effect was recorded in the combination with chlorambucil. The effect of the combination of chlorambucil and gamma-globulin from antiserum instead of normal serum was only slightly higher. To compare reductions in cell number in the different combinations, we calculated the difference between the reduction in cell number after incubation with antibody and the reduction with control globulin. To obtain information as to whether the effects of chlorambucil and antibody were synergistic or only additive, we compared the effects of interaction between chlorambucil and gamma-globulins. The effect of interaction was calculated by substracting the sum of the data of the controls (i.e., reduction in cell number when the tumour cells were respectively incubated with only chlorambucil and with only gamma-globulin) from the data obtained after incubation with the mixture of drug and gamma-globulin. Table 4 shows that, at a dilution of the gamma-globulin fraction of antiserum comparable with a serum dilution of 1:80, only a concentration of chlorambucil of 12·5 μg/ml showed a significant effect on the tumour cells as compared with the controls, i.e. gave a significant value both for

Table 2. Effect of the complex of chlorambucil and antibody on human melanoma cells (NKI-4); time of incubation 48 hrs

Agent	Chlorambucil concentration (μg/ml)	Antibody concentration (μg protein/ml)	Cell number ± s.d.	*P*-value*
Medium control			66·1 ± 17·6	
Chlorambucil	21		54·1 ± 10·2	> 0·10
Antibody		75	85·7 ± 20·2	> 0·10
Chlorambucil + antibody†	21	75	24·6 ± 3·7	< 0·001
Chlorambucil/antibody‡	21	75	65·0 ± 9·3	> 0·50

*Compared with the medium control.
†Mixture of chlorambucil and antibody.
‡Chlorambucil linked to antibody.

Table 3. Summary of the observed reduction in cell number of human melanoma cells after incubation with chlorambucil, gamma-globulin or a mixture of chlorambucil and gamma-globulin; time of incubation 48 hr

Incubation with	Variation in reduction in cell number compared with the untreated control % ± s.d.	
Chlorambucil (12·5 μg/ml to 50 μg/ml)	16·6 ± 3·6*	20·3 ± 5·1*
Normal gamma-globulin (diluted 1:80 to 1:20)	−10·7 ± 6·0	3·7 ± 6·8
Gamma-globulin from antiserum (diluted 1:80 to 1:20)	− 8·1 ± 6·2	19·9 ± 4·7*
Mixture of chlorambucil and normal gamma-globulin	21·3 ± 4·6*	39·6 ± 2·6*
Mixture of chlorambucil and gamma-globulin from antiserum	31·1 ± 3·3*	53·3 ± 2·5*

*Significant; $P < 0·05$

Table 4. Effect of interaction of chlorambucil with antibody during incubation with human melanoma cells; time of incubation 48 hr

Chlorambucil concentration (μg/ml)	Dilution of the gamma-globulin fraction of antiserum 1:80 Interaction (% ± s.d.)	1:80 Difference in reduction* (% ± s.d.)	1:40 Interaction (% ± s.d.)	1:40 Difference in reduction (% ± s.d.)	1:20 Interaction (% ± s.d.)	1:20 Difference in reduction (% ± s.d.)
0		2·6 ± 7·7		3·2 ± 7·2		26·8 ± 6·7†
12·5	25·9 ± 7·5†	10·5 ± 5·0†	24·6 ± 7·2†	9·7 ± 4·6†	2·1 ± 6·5	13·4 ± 4·8†
25	21·0 ± 7·6†	9·8 ± 5·2	17·1 ± 7·3†	10·2 ± 5·0†	3·4 ± 6·4	7·8 ± 4·4
50	27·4 ± 7·4†	2·5 ± 4·4	31·2 ± 6·9†	15·9 ± 4·2†	13·1 ± 6·1†	20·8 ± 4·2†

*Difference in reduction in cell number compared with the control containing normal gamma-globulin.
†Significant; $P < 0·05$.
Interested readers may get the statistical treatment by writing the author C. Vennegoor directly.

difference in reduction and for interaction. With gamma-globulin from antiserum diluted to 1:40 significant effects were obtained at all three concentrations of chlorambucil tested. When the gamma-globulin fraction was diluted to 1:20 only significant results were obtained with a concentration of chlorambucil of 50 μg/ml. The effect of chlorambucil and antibody appears therefore to be dependent on the concentration of both chlorambucil and antibody.

Effect of separate incubation with chlorumbucil and antibody. To obtain more information as to the necessity of simultaneous presence of drug and antibody, we examined the effects of incubation with chlorambucil, followed by incubation with gamma-globulin, and also the reversed sequence. In one type of experiment the melanoma cells were incubated for 24 hr with chlorambucil. Hereafter the medium was removed and replaced by the gamma-globulin fraction from either normal serum or antiserum. After a second incubation for 24 hr the cells were washed, fixed and counted. In other types of experiments the incubations were first with the gamma-globulin fraction from either normal or antiserum for 24 hr and then with chlorambucil for 24 or 48 hr respectively. In all these experiments the concentrations of chlorambucil and gamma-globulin were as used in the experiment represented in Table 4. The reductions in cell number were in both types of experiments comparable with those obtained after co-incubation with drug and antibody (Table 3). However, there was no indication of any interaction between chlorambucil and gamma-globulin from antiserum when the incubations were separately. This indicates that the reduction in cell number. in the latter experiments could only be the result of additive effects of the drug and antibody.

DISCUSSION

Serum proteins readily bind or envelope chlorambucil and other alkylating agents without loss of their alkylating ability [4, 12]. The results of Linford and coworkers [12] suggested that the forces which bind chlorambucil to the serum proteins have their origin in the mutual attraction of benzene rings in the drug and the protein. The nature of a distinct protein will therefore determine the quantity of chlorambucil which can non-covalently be bound in an active form. In our experiments active chlorambucil could only be linked to gamma-globulin or IgG when a considerably higher drug to protein ratio was used than is necessary for binding total serum proteins. The molar ratio of active chlorambucil linked to IgG varied from 55 to 100, which is approximately five to ten times higher than reported [20]. In the *in vitro* test system, however, the complex of chlorambucil and antibody never showed an increase in cytotoxicity compared with the control containing a mixture of drug and antibody. Several causes could underlie the failure of the complex to augment cytotoxicity in our test. It is most likely that during incubation with tumour cells the drug has become inactive as a result of alkylation with the carrier protein. Our results (Table 1) indicated that alkylation of chlorambucil to gamma-globulins occurred easily and we were not able to prevent this by masking the sulphydryl groups in the gamma-globulin fraction with etyleneimine [28]. Further possible events which might have occurred are dissociation of drug and antibody or hydrolysis of the active groups of chlorambucil during incubation with the tumour cells.

When melanoma cells were incubated with a mixture of chlorambucil and antibody, a reduction in cell number could be observed, which appeared to be significant compared with the controls. This indicates that the cytotoxicity of chlorambucil was augmented specifically by antibody. The results are comparable with the *in vitro* studies of Rubens and Dulbecco [14], who used polyoma virus-transformed baby hamster kidney cells, and of Davies and O'Neill [11] with theta antigens of the mouse. It is an interesting feature that a combination of free chlorambucil and free antibody can also increase the life span of mice which are injected with lethal doses of tumour cells [11, 17].

In conclusion we can say that mixtures of chlorambucil and antibody were more cytotoxic for melanoma cells than complexes of chlorambucil and antibody. When, however, inactivation of the drug by alkylation with the carrier could be prevented, better results than reported in this paper might be obtained.

Acknowledgements—The authors are obliged to Ir. G. Hart for statistical evaluations, to Mrs. S. Swen for providing facilities for tissue culture, to Dr. C. Benckhuijsen for determinations of chlorambucil with the Epstein technique and for fruitful discussions and to J. de Vries for gift of the sera and the melanoma cell line NKI-4.

REFERENCES

1. H. Isliker, J. C. Cerottini, J. C. Jaton and G. Magnenat, Specific and non-specific fixation of plasma proteins in tumors. In *Chemotherapy of Cancer* 1964, Elsevier, Amsterdam.
2. R. Wade, M. E. Whisson and M. Szekerke, Some serum protein nitrogen mustard complexes with high chemotherapeutic selectivity. *Nature (London)* **215,** 1303 (1967).
3. M. Szekerke, R. Wade and M. E. Whisson, The use of macromolecules as carriers of cytotoxic groups (Part I). Conjugates of nitrogen mustards with proteins, polypeptidyl proteins and polypeptides. *Neoplasma (Bratisl.)* **19,** 199 (1972).
4. M. Szekerke, R. Wade and M. E. Whisson, The use of macromolecules as carriers of cytotoxic groups (Part II). Nitrogen mustard–protein complexes. *Neoplasma (Bratisl.)* **19,** 211 (1972).
5. H. Busch, E. Fujiwara and D. C. Firszt, Studies on the metabolism of radioactive albumin in tumor-bearing rats. *Cancer Res.* **21,** 371 (1961).
6. I. L. Spar, W. F. Bale, R. L. Goodland and M. J. Izzo, Preparation of purified antibody which reacts with human fibrin. Preliminary tracer studies on tumor patients. *Cancer Res.* **24,** 286 (1964).
7. T. Ghose, R. C. Nairn and J. E. Fothergill, Uptake of proteins by malignant cells. *Nature (London)* **196,** 1108 (1962).
8. T. Ghose, M. R. C. Path and S. P. Nigam, Antibody as carrier of chlorambucil. *Cancer (Philad.)* **29,** 1398 (1972).

9. T. Ghose, S. T. Norvell, A. Guclu, D. Cameron, A. Bodurtha and A. S. MacDonald, Immunochemotherapy of cancer with chlorambucil-carrying antibody. *Brit. med. J.* **3,** 495 (1972).
10. I. Flechner, The cure and concomitant immunization of mice bearing Ehrlich ascites tumors by treatment with an antibody–alkylating agent complex. *Europ. J. Cancer* **9,** 741 (1973).
11. D. A. L. Davies and G. J. O'Neill, *In vivo* and *in vitro* effects of tumour specific antibodies with chlorambucil. *Brit. J. Cancer* **28,** *Suppl. I,* 285 (1973).
12. J. H. Linford, J. Legal and E. Zacharias, The construction of an ultrafiltration unit to measure the binding of certain nitrogen mustards to serum protein. *Canad. J. Biochem.* **40,** 137 (1962).
13. W. C. J. Ross, The interaction of chlorambucil with human γ-globulin. *Chem.-Biol. Interactions* **8,** 261 (1974).
14. R. D. Rubens and R. Dulbecco, Augmentation of cytotoxic drug action by antibodies directed at cell surface. *Nature (Lond.)* **248,** 81 (1974).
15. D. A. L. Davies, The combined effect of drugs and tumor-specific antibodies in protection against a mouse lymphoma. *Cancer Res.* **34,** 3040 (1974).
16. D. A. L. Davies, A. J. Manstone and S. Buckham, Protection of mice against syngeneic lymphomata: I. Use of antibodies. *Brit. J. Cancer* **30,** 297 (1974).
17. D. A. L. Davies, S. Buckham and A. J. Manstone, Protection of mice against syngeneic lymphomata: II. Collaboration between drugs and antibodies. *Brit. J. Cancer* **30,** 305 (1974).
18. J. E. de Vries, Ph. Rümke and J. Bernheim, Cytotoxic lymphocytes in melanoma patients. *Int. J. Cancer* **9,** 567 (1972).
19. H. Brummelhuis, Het isoleren van specifieke antistoffen met behulp van immuunadsorbentia. Thesis, 1972, Amstelstad, Amsterdam, p. 25–26.
20. D. Blakeslee and J. C. Kennedy, Factors affecting the noncovalent binding of chlorambucil to rabbit immunoglobulin G. *Cancer Res.* **34,** 882 (1974).
21. J. H. Linford, The recovery of free chlorambucil from solution in blood serum. *Biochem. Pharmacol.* **11,** 693 (1962).
22. W. M. Allen, A simple method for analyzing complicated absorption curves, of use in the colorimetric determination of urinary steroids. *J. clin. Endocr.* **10,** 71 (1950).
23. C. Benckhuijsen, Acid-catalysed conversion of triethyleneimine thiophosphoramide (thio-TEPA) to an SH compound. *Biochem. Pharmacol.* **17,** 55 (1968).
24. M. G. Lewis and T. M. Phillips, The specificity of surface membrane immunofluorescence in human malignant melanoma. *Int. J. Cancer* **10,** 105 (1972).
25. L. G. Israels and J. H. Linford, Some observations on the reactions of chlorambucil, azo-mustard (CB 1414), and cyclophosphamide. *Proc. Vth. Canad. Cancer Res. Conf.* **5,** 399 (1963).
26. M. G. Kendall and A. Stuart, *The Advanced Theory of Statistics I,* 1969, Griffin, London.
27. H. E. Skipper and L. H. Schmidt, A manual on quantitative drug evaluation in experimental tumor systems, Part I. *Cancer Chemotherapy Rep.* **17,** 1 (1962).
28. M. A. Raftery and R. D. Cole, On the aminoethylation of proteins. *J. biol. Chem.* **241,** 3475 (1966).

Europ. J. Cancer Vol. 11, pp. 733–737. Pergamon Press 1975. Printed in Great Britain

Serum Insulin and Somatomedin Levels in the Tumour-bearing Rat*

G. A. J. GOODLAD, ANGELA J. H. MITCHELL, L. McPHAIL and CATHERINE M. CLARK

Department of Biochemistry, Irvine Building, University of St. Andrews, St. Andrews, U.K.

Abstract—*In the serum of rats bearing the Walker 256 carcinoma post-absorptive levels of insulin and somatomedin were decreased. This was accompanied by a lowered serum glucose level and an elevation in serum unesterified fatty acids. The administration of insulin to these rats produced falls in serum glucose and unesterified fatty acids similar to those observed in insulin-treated normal rats.*

INTRODUCTION

THE COMPLEX interrelationship which exists between the tissue requirements for various essential metabolites and circulating hormone levels is profoundly disturbed in the tumour-bearing host not only by the demands of the growing tumour for essential metabolites but possibly by the direct action of tumour secretions on the endocrine organs of the host [1]. In rats bearing the Walker 256 carcinoma we have observed a decreased level of muscle protein synthesis [2–4] accompanied by a significant reduction in the post-absorptive level of serum glucose. A persistent lowering of blood glucose, induced by the energy requirements of a growing tumour, could result in diminished levels of circulating insulin and also of somatotrophin [5, 6]. Both these hormones are involved in the maintenance of protein anabolism in skeletal muscle; insulin by direct action on muscle [7] and somatotrophin by promoting the production and release of somatomedin by liver and kidney [8]. Somatomedin in turn causes an increased incorporation of amino acids into muscle protein [9]. Hypoglycaemia has also been observed in a number of human cancer cases where the tumour is of non-pancreatic origin [10–12]. In some cases this has been attributed to the secretion by the tumour of "insulin-like" substances. In other instances, however, no such substances have been detected [10, 13]. In the present paper, we have estimated the post-absorptive levels of insulin and somatomedin in rats bearing the Walker 256 carcinoma and found that in this case diminished serum glucose levels are accompanied by a marked decrease in serum insulin and somatomedin. The significance of these findings in relation to the more specific changes observed in muscle protein synthesis due to tumour growth is discussed.

MATERIAL AND METHODS

Treatment of animals

Three-month old 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 at 16:00 hr 14 g ground rat cake (North Eastern Cooperative Ltd., Aberdeen) made into a paste with water, all of which was consumed by both groups of rats throughout the experimental period. All animals were kept on a 12 hr light-dark cycle (lights on 07:00 hr). After 10–12 days, when the tumour had grown to 20–30 g, peripheral

Accepted 6 June 1975.

*This work was supported by grants from the Scottish Hospital Endowments Research Trust and the Medical Research Council.

blood was collected at 10:00–11:00 hr from the neck vessels for the estimation of serum glucose, fatty acids and insulin and cardiac blood was collected for the estimation of somatomedin. In some experiments control and tumour-bearing rats were injected with 1 unit insulin 30 min before sacrifice.

Strips of costal cartilage were prepared from young rats aged between 22 and 27 days as described by Alford, Bellair, Burger and Lovett [14].

Estimation of serum glucose and unesterified fatty acids

Serum glucose was estimated enzymatically with glucose oxidase as described by Raabo and Terkildsen [15] and serum unesterified fatty acids by the method of Duncombe [16].

Estimation of serum insulin

This was carried out by a radio-immunoassay technique [17] using purified rat insulin as standard.

Estimation of somatomedin activity of serum

The relative somatomedin content of serum of tumour-bearing and control rats was compared by assaying the enhancing effect of 0·1 ml serum on the incorporation of ^{35}S-SO_4 into two 2 mm lengths of rat costal cartilage in an *in vitro* system described by Alford, Bellair, Burger and Lovett [14] containing 20 μCi ^{35}S-SO_4. Blank incubations were also carried out where the addition of serum was omitted. After incubation the pieces of cartilage were removed, dried with paper tissue, weighed and digested at 50° for 16 hr in 0·3 ml 100 mM phosphate buffer, pH 6·5, containing 0·5 mg papain per ml and 2 mM with respect to cysteine and ethylenediamine tetraacetic acid. 0·1 ml aliquots of the digest were applied to Whatman No. 1 filter paper strips (2 × 8 cm). The strips were washed in 0·1 N HCl in 95% ethanol three times then air-dried and counted in NE233 liquid scintillator.

All assays were carried out in triplicate. The incorporation observed in the absence of serum was subtracted and the results are expressed as counts/min per mg cartilage per 0·1 ml serum. The adequacy of the method was tested by adding increasing amounts of serum from a normal rat to the assay system. Figure 1 shows that the amount of ^{35}S incorporated was proportional to the amount of serum added over the range 0·05–0·20 ml.

Radioactive measurements

All radioactive measurements were carried out on an Intertechnique SL30 counter using NE233 scintillator.

Statistical analysis

Differences between means of tumour-bearing and control groups of rats were tested for signicance by Student's *t*-test.

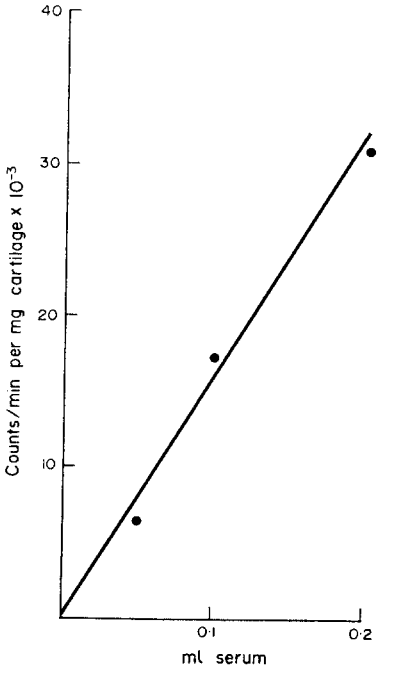


Fig. 1. Incorporation of ^{35}S-sulphate into rat costal cartilage strips in the presence of increasing amounts of rat serum.

MATERIAL

Reagents for the assay of serum glucose were obtained from Sigma Chemical Co. Ltd., and those for the assay of unesterified fatty acids from the Boehringer Corporation Ltd (London). A sample of purified rat insulin was kindly gifted by Dr. Schlichtkrull of the Novo Research Institute, Copenhagen.

Zinc insulin was obtained from Boots Pure Drug Co. Ltd., Nottingham. A radio-immuno-(assay kit for insulin and sodium ^{35}S-sulphate 39Ci/mole) were obtained from the Radiochemical Centre Ltd., Amersham, Bucks. NE233 liquid scintillator was obtained from Nuclear Enterprises Ltd., Edinburgh (U.K.)

RESULTS AND DISCUSSION

As seen from Tables 2 and 3 there was no significant loss of carcase weight in the tumour-

Table 1. *The effect of the Walker* 256 *carcinoma on serum glucose, and unesterified fatty acids in the post-absorptive rat*

	Insulin	Serum glucose mg/100 ml	Serum unesterified fatty acids mEq/l
*Control	−	108·1 ± 2·50	0·568 ± 0·054
	+	46·6 ± 5·07	0·396 ± 0·077
†Tumour-bearing	−	83·1 ± 7·90	0·698 ± 0·047
	+	44·8 ± 6·17	0·390 ± 0·012

Results are the mean ± S.E. of 5 rats.
1·0 unit of insulin was injected intraperitoneally 30 min before animals were killed.
*Mean initial body weight of rats, 175 ± 5·6 g.
†Mean weight of tumour, 24 ± 3·0 g.

bearing rats at the end of the period studied. At this stage, animals still consumed the fixed amount of food offered and their mobility was unaffected. Nevertheless previous studies have shown that at this stage of tumour growth an impairment of muscle protein metabolism had occurred [2–4].

Table 1 shows the levels of glucose and unesterified fatty acids in the serum of tumour-bearing rats. Serum glucose was decreased significantly by tumour growth ($P < 0{\cdot}02$). Unesterified fatty acid levels tended to be elevated although the extent of the increase did not attain statistical significance ($0{\cdot}1 > P > 0{\cdot}05$). Elevated levels of unesterified fatty acids have also been observed in cancerous human subjects, the magnitude of the increase being related to the severity of the neoplastic condition [18].

The lowered serum glucose in the tumour-bearing rat is not accompanied by an increase in circulating insulin (Table 2). Indeed there is a marked reduction in the level of this hormone ($P < 0{\cdot}001$). The possibility that the tissues of the tumour-bearing rat might not be responsive to the action of insulin was also investigated. Administration of insulin caused significant falls ($P < 0{\cdot}01$) in both serum glucose and unesterified fatty acids in tumour-bearing rats of similar magnitude to those observed in insulin-treated control rats (Table 1). Somatomedin levels were also significantly reduced ($P < 0{\cdot}001$) in sera of tumour-bearing rats (Table 3).

These results indicate that the fall in serum glucose observed in tumour-bearing rats was not due to an overproduction of insulin by either the tumour or the tissues of the host, but is more likely a reflection of the general disturbance in energy metabolism associated with tumour growth [19–23]. The depression in somatomedin levels may be associated with the lowered glucose levels but might also be a result of the tendency for unesterified fatty acids to be elevated since somatotrophin and hence, presumably somatomedin release is inhibited in subjects with raised serum unesterified fatty acid levels [24].

To a certain extent, lowered levels of insulin

Table 2. *The effect of the Walker* 256 *carcinoma on serum insulin in the post-absorptive rat*

	Body weight (g)		Tumour weight (g)	Insulin ng/ml serum
	Initial	Final		
Control	181 ± 3·5	187 ± 2·4	—	2·2 ± 0·33
Tumour-bearing	184 ± 3·7	179 ± 4·0*	30 ± 2	0·6 ± 0·09

Results are the mean ± S.E. of 11 rats in each group.
*Tumour weight excluded.

Table 3. *The effect of the Walker* 256 *carcinoma on serum somatomedin in the post-absorptive rat*

	Body weight (g)		Tumour weight (g)	cpm × 10^{-3} per mg costal cartilage
	Initial	Final		
Control	169 ± 6·2	176 ± 4·2	—	24·3 ± 3·02
Tumour-bearing	175 ± 7·3	182 ± 3·1*	36 ± 3·2	14·5 ± 2·55

Results are the mean ± S.E. of 7 rats in each group.
*Tumour weight excluded.

and somatomedin would be consistent with the diminished protein synthesis observed in the muscles of tumour-bearing rats [2–4]. However, it must be noted that the effect of the tumour on phasic muscles is much greater than on tonic muscles [2] and insulin is known to have a similar stimulatory effect on protein synthesis in red and white fibres [25]. Furthermore, in the diabetic rat, the decreased ability of muscle ribosomes to synthesise protein has been associated with a defect in the larger ribosomal subunit [7] whereas in the tumour-bearing rat, the smaller ribosomal subunit would appear to be the more likely location of the defect [4]. At the present time little is known either about the types of muscle affected by somatomedin or the detailed mechanism of action of this factor. Clearly, in the tumour-bearing rat, there is a complex disturbance of endocrine function mediated possibly by the ability of the tumour to effect alterations in the utilisation of metabolites and perhaps secrete factors which act directly on the tissues of the host [26, 27] or cause the production of some signal factor which has a regulatory effect on protein synthesis [28].

REFERENCES

1. G. A. J. Goodlad, *Protein Metabolism and Tumour Growth, in Mammalian Protein Metabolism*, Vol. 2 (Edited by H. N. Munro and J. B. Allison). pp. 415–444, Academic Press, New York (1964).
2. C. M. Clark and G. A. J. Goodlad, Depletion of protein of phasic and tonic muscles in tumour-bearing rats, *Europ. J. Cancer* **7,** 3 (1971).
3. G. A. J. Goodlad and C. M. Clark, Activity of gastrochemius and soleus polyribosomes in rats bearing the Walker 256 carcinoma, *Europ. J. Cancer* **8,** 647 (1972).
4. C. M. Clark and G. A. J. Goodlad, Muscle protein biosynthesis in the tumour-bearing rat: a defect in a post-initiation stage of translation, *Biochim. biophys. Acta* **378,** 230 (1975).
5. S. Reichlin, The physiology of growth hormone regulation: pre- and post-immunoassay eras, *Metabolism* **22,** 987 (1973).
6. D. N. Kipnis, F. Hertelendy and L. D. Machlin, Studies of growth hormone secretion, *International Congress Series* No. 184, P. 601, Excerpta Medica, Amsterdam, 1968.
7. I. R. Wool, Insulin and the regulation of protein synthesis in muscle, *Proc. Nutr. Soc.* **31,** 185 (1972).
8. J. M. Tanner, Human growth hormone, *Nature (Lond.)* **237,** 433 (1972).
9. W. D. Salmon, Jr. and M. R. DuVall, *In vitro* stimulation of leucine incorporation into muscle and cartilage protein by a serum factor with sulphation activity; differentiation of effects from those of growth hormone and insulin, *Endocrinology* **87,** 1168 (1970).
10. C. B. Hobbs and A. C. Miller, Review of endocrine syndromes associated with tumours of non-endocrine origin, *J. clin. Pathol.* **19,** 119 (1966).
11. R. H. Unger, Riddle of tumour hypoglycaemia, *Amer. J. Med.* **40,** 325 (1966).
12. B. F. Bower and G. S. Gordan, Hormonal effects of non-endocrine tumours, *Ann. Rev. Med.* **16,** 83 (1966).
13. F. Chowdhury and S. J. Bleicher, Studies of tumour hypoglycaemia, *Metabolism* **22,** 663 (1973).
14. F. P. Alford, J. T. Bellair, H. G. Burger and N. Lovett, A simplified assay for somatomedin, *J. Endocrinol.* **54,** 365 (1972).
15. E. Raabo and T. C. Terkildsen, On the enzymatic determination of blood glucose, *Scand. J. clin. Lab. Invest.* **12,** 402 (1960).
16. W. G. Duncombe, The colorimetric micro-determination of non-esterified fatty acids in plasma, *Clin. chim. Acta* **9,** 122 (1964).
17. C. N. Hales and P. J. Randle, Immunoassay of insulin with insulin-antibody precipitate, *Biochem J.* **88,** 137 (1963).
18. P. S. Mueller and D. M. Watkin, Plasma unesterified fatty acid concentrations in neoplastic disease, *J. Lab. clin. Med.* **57,** 95 (1961).
19. G. B. Mider, Some aspects of nitrogen and energy metabolism in cancerous subjects: a review, *Cancer Res.* **11,** 821 (1951).
20. R. W. Begg, Tumour-host relations, *Adv. Cancer Res.* **5,** 1 (1968).
21. D. J. Ingle, Urinary glucose and tumour growth in partially depancreatised force-fed rats, *Endocrinology* **62,** 78 (1958).

22. D. J. INGLE, Suppression of steroid diabetes by Walker carcinoma in the rat, *Endocrinology* **66,** 289 (1960).
23. D. J. INGLE, Effect of Jensen tumour on pancreatic diabetes in non-adrenalectomised and adrenalectomised rats, *Proc. Soc. Exp. Biol.* (*N.Y.*) **120,** 225 (1965).
24. G. A. BRAY and L. A. CAMPFIELD, Metabolic factors in the control of energy stores, *Metabolism* **24,** 99 (1975).
25. F. A. SHORT, Protein synthesis by red and white muscle *in vitro*: effect of insulin and animal age, *Amer. J. Physiol.* **217,** 307 (1969).
26. W. NAKAHARA, A chemical basis for tumour–host relations, *J. nat. Cancer Inst.* **24,** 77 (1960).
27. G. A. J. GOODLAD and M. J. RAYMOND, The action of the Walker 256 carcinoma and toxohormone on amino-acid incorporation into diaphragm protein, *Europ. J. Cancer* **9,** 139 (1973).
28. M. TOPOREK, Effect of whole blood or albumin fraction from tumour-bearing rats on liver protein synthesis, *Cancer Res.* **33,** 2579 (1973).

Europ. J. Cancer Vol. 11, pp. 739–742. Pergamon Press 1975. Printed in Great Britain

Beta-glucuronidase in Human Cutaneous Tumours*

C. VAQUERO,† C. MASSON,‡ M. GUIGON§ and J. HEWITT‡
†*Attachée de Recherche à l'INSERM*
‡*Laboratoire de Recherches Dermatologiques, Hôpital Tarnier, 89 rue d'Assas, 75006 PARIS*
§*Chargée de Recherche à l'INSERM.*

Abstract—*β-glucuronidase activity in human malignant melanomas (34 cases) is compared with other benign and malignant pigmentary or non-pigmentary cutaneous tumours. The results show good agreement between histochemical and biochemical study and indicate a statistically significant rise of β-glucuronidase activity in human malignant melanomas. The significance of this observation is not yet explained.*

INTRODUCTION

In a previous work, β-glucuronidase activity, studied by histochemical and biochemical methods, proved to be much greater in malignant melanomas than in other malignant cutaneous tumours [1]. Similar observations were made on cultures of human malignant melanomas [2] prepared from metastases (2 cases) and from a primary tumour. However, Braun-Falco and Burg's histochemical study on 25 cases of human melanomas [3] (9 superficial, 8 nodular, and 8 metastases) showed only a low β-glucuronidase activity.

Therefore, we felt it necessary:

(1) to confirm our previous results in a larger number of cases: human malignant melanomas at various stages of their courses, benign pigmentary tumours, benign and malignant non pigmentary cutaneous tumours and normal skin;

(2) to give a statistical comparison of β-glucuronidase biochemical mean activity in these tumours and in normal skin.

Accepted 10 April 1975.

This work was done with the help of the "Institut National de la Recherche et de la Santé Médicale" and of the "Fondation pour la Recherche Médicale Française".

MATERIAL AND METHODS

The tumoral and normal skin samples are listed in Table 1.

The samples, removed by "biopsy excision" or during surgery, were frozen immediately in liquid nitrogen.

Each specimen was studied both histochemically and biochemically:

β-glucuronidase—*histochemical study* by two methods, both applied to cryostat sections: the method of Seligman and co-workers [4], and that of Fishman and Goldman [5], adapted to skin specimens and described in a previous publication [1]; according to the type of tumour, a supplementary study of pigmentation was made with demonstration of the melanic pigment by Masson's silver staining procedure, and of DOPA-oxidase activity [6].

β-glucuronidase—*biochemical assay* was made by the Talalay and Fishman method [7]; the units of biochemical activity are related to protein level [8]. To facilitate comparison with biochemical assay results, the histochemical results were assessed in number of crosses, from 0 to + + +.

Statistical study—the results of biochemical assays were used to establish statistics on the basis of variance analysis as a method of comparison of mean values.

Table 1. β-glucuronidase activity, as studied by histochemical and biochemical methods in human cutaneous and in normal skin

		Number (of cases)	Histochemistry	Biochemistry*
Malignant melanomas	—Primary tumours	10	+ to + + +	79·1 ± 19·7
	—Cutaneous metastases	14	± to + + +	68 ± 23·5
	—Lymph node metastases	10	± to + + +	67·7 ± 23·6
Benign pigmented-cell nevi		20	± to + ±	33·8 ± 3·4
Malignant non-pigmentary tumours	—Basal-cell carcinomas	12	± to + +	47·2 ± 9·3
	—Squamous-cell carcinomas	6	± to +	32·3 ± 9·5
Benign non-pigmentary tumours		14	±	23·7 ± 6·9
Normal skin		10	±	25·5 ± 3·6

*Mean ± s.e. ($P < 0·05$)
(Results expressed in units/mg of protein).

RESULTS

The results of the histochemical and biochemical comparative study of β-glucuronidase activity are given in Table 1.

Analysis of the results of the comparative study shows:

a good agreement between histochemical assessment and corresponding biochemical value;

a very low β-glucuronidase activity in normal skin;

a moderately increased activity in benign pigmented-cell nevi, while malignant melanomas show a considerably increased activity.

Among the non-melanotic tumours, benign ones have a very low activity and malignant ones a relatively higher activity; moreover, though basal-cell epitheliomas have a higher activity than epidermoid carcinomas, the level is clearly lower than in malignant melanomas.

The comparison of mean levels led to the establishment of four groups with significantly different activity levels. These groups are listed in order of decreasing activity in Table 2, which shows that:

in malignant melanomas there is no significant difference between primary tumours, cutaneous and lymph node metastases; they form one single group;

basal-cell epitheliomas, with an activity which is significantly different from that of epidermoid carcinomas, form an isolated group among malignant non-melanotic tumours;

epidermoid carcinomas and benign pig-

Table 2. Statistical study of biochemical β-glucuronidase activity by groups of activity

	Number	Biochemical activity*	Comparison of mean values
Malignant melanomas	34	71·2 ± 13·3	$\alpha = 5\%$ significant difference
Basal-cell carcinomas	12	47·2 ± 9·3	
Benign pigmented-cell nevi Squamous-cell carcinomas	26	33·1 ± 3·5	$\alpha \to 1‰$ very significant difference
Benign non-pigmentary tumours Normal skin	24	24·6 ± 4·4	$\alpha \to 1‰‰$ highly significant difference

*Mean ± s.e. ($P < 0·05$).

mented-cell nevi form one group, as there is no significant difference in their levels of activity;

finally, normal skin and benign non-melanotic tumours form one group, with the same activity level.

In Fig. 1, all the biochemical assay values reported in the histogram show two important points:

a great variability in malignant melanomas so that they overlap with other distributions;

a relative increase in β-glucuronidase activity in basal-cell epitheliomas, which does not reach, however, the highest values of malignant melanomas.

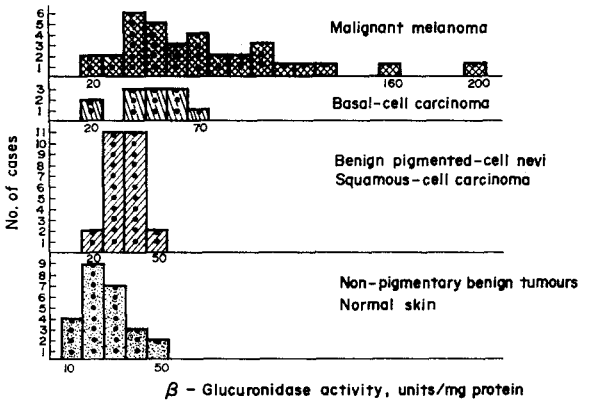


Fig. 1. Histogram of β-glucuronidase activity in human cutaneous tumours and in normal skin. The abscissa values are the biochemical assay values expressed in units per mg of protein. The mean activities of four groups are significantly different (see Table 2).

DISCUSSION

This work, centering on the study of β-glucuronidase by histochemical and biochemical methods, shows that this enzymatic activity increases significantly in malignant melanomas, compared with other human cutaneous tumours.

There is a statistical relationship between malignant degeneration of pigmentary nevi and β-glucuronidase activity. Normal melanocytes are highly inactive; benign pigmentary nevi show a moderately increased activity in nevi cells; in a case of Dubreuilh's melanosis (Hutchinson's melanotic freckle) the intra-epidermal zone likewise shows only a moderate β-glucuronidase activity increase, while in the nodular zone of degeneration the invasive malignant melanoma derived from it has a considerable activity. A few melanomas however, had a rather low activity, approaching that of certain very active benign nevi: β-glucuronidase activity cannot be used, therefore, as a criterion of malignancy.

Statistical calculation confirms the very significant enzymatic activity increase of malignant melanomas, as we had noted in our previous study [1]. On the other hand, there is no significant difference in activity between the different histological types of primary tumours [9], nor between primary tumours and cutaneous and lymph node metastases; as primary tumours are usually removed before any therapy is applied, while metastases are usually removed after various treatments, this lack of difference may be due to the possible incidence of such treatments on β-glucuronidase activity. No difference in activity could be observed according to the malignancy in the metastatic tumours.

One could question whether the relatively low activity of certain malignant melanomas was not related to a lower pigmentary activity. Indeed, achromic forms of lymph node metastases seem to be rather less active than pigmented forms. Besides, it is usually in the more pigmented thecae of benign nevi and in the more pigmented part of Hutchinson's melanotic freckle that enzyme activity is highest. However, the correlation between β-glucuronidase activity [Fig. 2(*a*)] and DOPA-oxidase activity [Fig. (2*b*)] is not constant, and discrepancy was total in the case of one malignant melanoma, which was achromic, DOPA-negative and highly β-glucuronidase-active.

Among the malignant non-melanotic tumours, only the basal-cell epitheliomas show a relative β-glucuronidase activity increase. As is known, these tumours are formed by proliferation of indifferentiated cells from hair buds. This particular origin may account for the β-glucuronidase activity increase. On the other hand, enzyme activity is stronger in subnecrotic areas. One may wonder if necrosis does not distort results through lysosomial enzyme release, and in particular β-glucuronidase. However, enzyme activity in basal-cell epitheliomas never reaches the higher levels of malignant melanomas.

The few difficulties of interpretation of β-glucuronidase activity variations in human malignant melanoma induced us to look for an explanation in animal experimentation and to use an experimental model [8]. The work, which dealt with melanoma B 16 and s.c. non-pigmentary malignant Ehrlich's tumour in the mouse, showed that β-glucuronidase biochemical activity in melanoma B 16 was not significantly different from that in Ehrlich's tumour.

Moreover, in histochemical studies, melanoma B 16 seems to have a different activity from that of the human melanoma, showing only β-glucuronidase activity with the substrate used in Fishman and Goldman method [5]. This difference in the enzyme activity may be related to the variation in the melanosome ultrastructure as reported by Rodriguez and McGavran [6]. Another explanation of these differences with human tumours could be the existence of β-glucuronidase isoenzymes, as has been observed for other enzymes in human malignant melanomas [10]. This hypothesis might also explain why low results were obtained with both methods: specific tumoral isoenzymes might not respond adequately to the substrates used. On the contrary, the rare discrepancies observed in human malignant melanomas between the results of histochemical and biochemical methods might well be connected with the different locations from where the specimens were taken due to the heterogeneous cutaneous material.

It might be of interest to probe into the significance of these observations. Further study of this enzymatic activity might help elucidate the mechanism whereby β-glucuronidase activity is increased in malignant melanomas and demonstrate the connection between this phenomenon and the malignant nature of pigmentation. Perhaps an explanation will be given when β-glucuronidase can be located in the cell by electron microscopy and when its relationship with the melanocyte's own structure is better defined.

Acknowledgements—We would like to thank Pr. Cl. Dufourmentel and Pr. Banzet for their kind supply of surgical samples and Dr. C. Aubert for his help in the experimental part of this work.

REFERENCES

1. J. Hewitt, M. Guigon, J. Bolubasz and C. Masson, Activité de la β-glycuronidase dans les tumeurs pigmentaires de la peau humaine. Etude histochimique et biochimique. *Rev. eur. Clin. Biol.* **15,** 416 (1970).
2. P. Janiaud, J. Le Calvez and Ch. Aubert, Caractérisation cytoenzymologique des cellules de mélanomes cutanés humains en culture. *Ann. Derm. Syph.* (Paris) **100,** 536 (1973).
3. O. Braun-Falco and G. Burg, Zur Enzym-Histochemie des malignen Melanoms. Untersuchungen an Primärtumoren und Metastasen. *Arch. Dermatol. Forsch.* **246,** 303 (1973).
4. A. M. Seligman, M. M. Nachlas, L. H. Manheimer, O. M. Friedman and G. Wolf, Development of new methods for the histochemical demonstration of hydrolytic intracellular enzymes in a program of cancer research. *Ann. Surg.* **130,** 333 (1949).
5. W. H. Fishman and S. S. Goldman, A post-coupling technique for β-glucuronidase employing the substrate naphtol AS-BI-β-D-glucosiduronic acid. *J. Histochem. Cytochem.* **13,** 441 (1965).
6. H. A. Rodriguez and M. H. McGavran, A modified Dopa reaction for the diagnosis and investigation of pigment cells. *Amer. J. clin. Path.* **52,** 219 (1969).
7. B. Talalay, W. H. Fishman and C. Huggins, Chromogenic substrates. II: Phenolphtalein glucuronic acid as substrate for the assay of glucuronidase activity. *J. biol. Chem.* **166,** 757 (1946).
8. C. Vaquero, M. Guigon, C. Masson, J. Lecordier and J. Hewitt. Activité de la β-glycuronidase dans le mélanome B 16 de la souris. *C.R. Soc. Biol.* (Paris) **165,** 1217 (1971).
9. V. J. McGovern, M. C. Mihm, Jr., C. Bailly, J. C. Booth, W. H. Clark, Jr., A. J. Cochran, E. G. Hardy, J. D. Hicks, A. Levene, M. G. Lewis, J. H. Little and G. W. Milton, The classification of malignant melanoma and its histologic reporting. *Cancer (Philad.)* **32,** 1446 (1973).
10. R. Prasad, M. M. Romsdahl, C. R. Shaw, D. M. Mumford and J. L. Smith, Jr., Isozyme variations in human malignant melanoma. *Cancer Res.* **34,** 1435 (1974).

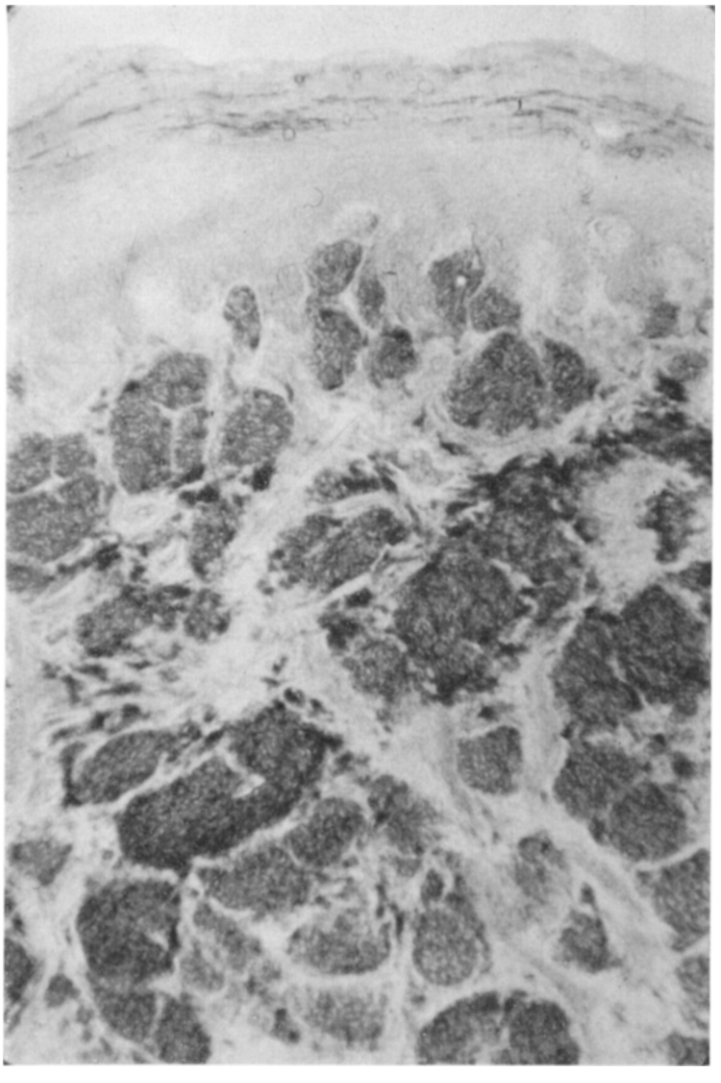


Fig. 2(*a*)

Fig. 2. *Malignant melanoma.* (*a*) *Activity of β-glucuronidase studied with Seligman's method* (× 20) (*b*) *Activity of DOPA oxidase* (× 20).

(to face p. 742)

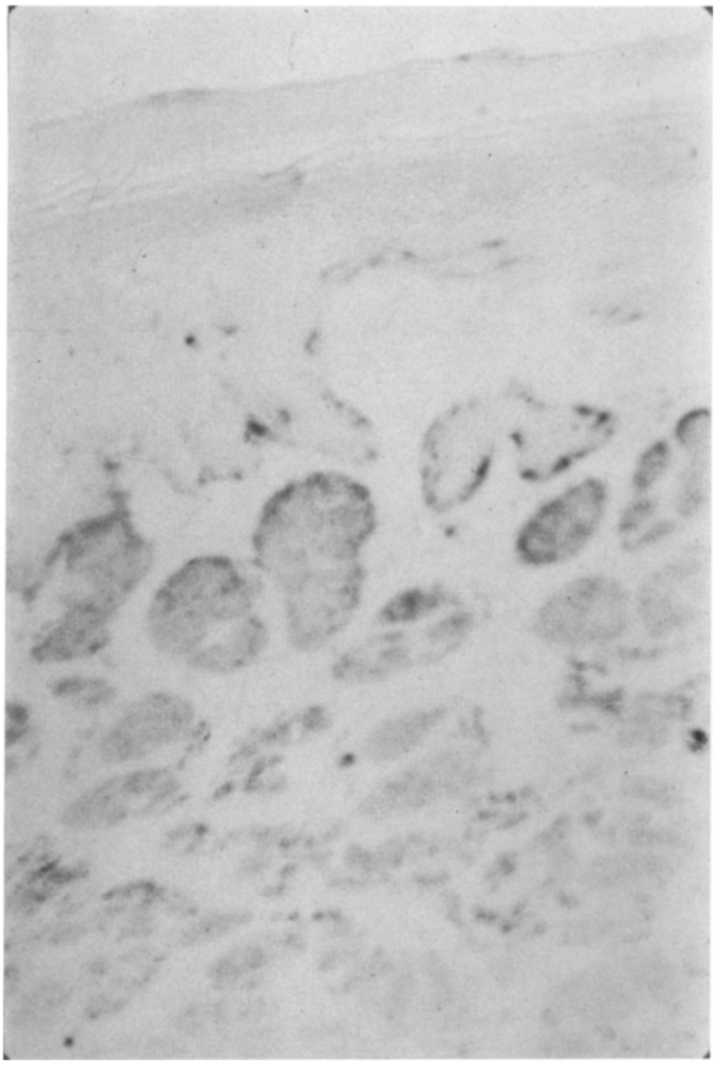


Fig. 2(*b*)

Europ. J. Cancer Vol. 11, pp. 743–749. Pergamon Press 1975. Printed in Great Britain

Ineffectiveness of Anticoagulation in Experimental Radiation Therapy*

ERWIN M. RÖTTINGER,† ROBERT SEDLACEK and HERMAN D. SUIT
Department of Radiation Medicine, Massachusetts General Hospital, Boston, MA 02114, *U.S.A.*

Abstract—*A spontaneous mammary carcinoma, a methylcholanthrene induced fibrosarcoma, and a methylcholanthrene induced squamous carcinoma have been examined in syngeneic C3H$_f$/Sed mice to assess the modification of tumor response to local radiation therapy by the administration of warfarin. For the fibrosarcoma (demonstrably antigenic in this animal system) recipient mice were normal or previously sensitized to the tumor. The transplant take rate and the growth rate of the mammary carcinoma and the fibrosarcoma were determined following subcutaneous injections of single cell suspensions. The radiation dose required for local control of* 50% *of the tumors (TCD_{50}) was determined for single and ten equal radiation doses. The dosage of warfarin in the drinking water resulted in a 2–3-fold prolongation of the prothrombin time.*

Warfarin administration before and following inoculation of tumor cells did not alter the transplantability. Warfarin administration either during the course of fractionated irradiation, or at and following single doses, did not affect tumor response to irradiation.

INTRODUCTION

WITH the growing experience in the use of anticoagulants, increasing attention has been focused on their effect on growth and spread of malignant tumors. Wood [1, 2] described the formation of an enveloping microthorombus around tumor cells as a decisive part in the formation of metastases. Anticoagulants were found to reduce the frequency of metastases in allogeneic and syngeneic systems. The hypotheses explaining these findings were recently discussed [3].

Apparently encouraging clinical results in the treatment of patient with advanced tumors have been reported by Elias [4], Thornes [5], and Larson [6]. Ries *et al.* [7], in a non-randomized clinical study, treated patients with carcinoma of the cervix with and without anticoagulants during intracavitary and external orthovoltage irradiation. In patients receiving anticoagulants the local recurrence rate between 5 and 18 months after treatment was in stage II disease 10·7% (82/763) as compared with 18·9% (128/676) in patients treated without anticoagulants. The comparable figures for stage III are 14·2% (129/911) and 26·6% (191/717) respectively. The corresponding 5 yr survival rates in patients treated with and without anticoagulants were in stage II 76% (185/243) versus 71% (298/420), and in stage III 55% (136/247) vs 41% (181/439) respectively.

The experience of Ries *et al.* provided a clinical indication that improvement in results of radiation therapy might be achieved by concomitant anticoagulant medication. If this were true, there would be important implications for radiation therapy in general. This report is a description of experiments which we have performed to study this question in several experimental animal tumor systems.

MATERIAL AND METHODS

Experimental animals

C3H^f/Sed mice were used in these experiments. These mice were produced and main-

Accepted 12 June 1975.
*This work was supported in part by the U.S. Public Health Service Grant CA 13311.
†Present address: Strahlenklinik, Universitätsklinikum Essen, 43 Essen 1, Germany.

tained in a defined flora, pathogen-free mouse colony (flora consisting of Bacillus sp., Clostridium sp., Fusobacterium sp, *Staphylococcus epidermidis*). Mice used in these experiments were initially 8–12 weeks old. Recipient mice were isohistocompatible with the mice in which the tumors had originated. This was demonstrated by the survival of second set skin grafts in perfect condition for more than 200 days. Male mice were employed in the assays with the fibrosarcoma and mammary carcinoma and female mice were used for the work with the squamous cell carcinoma. For one assay with the mammary carcinoma, female mice were used.

Tumor system

The mouse mammary carcinoma [8] and the fibrosarcoma [9, 10] have been described previously. The well differentiated keratinizing squamous carcinoma has been induced recently in a $C3H_f$/Sed mouse by repeated painting with a solution of methylcholanthrene in benzene. The tumors used were a third to sixth generation isotransplants. Transplantation of mammary carcinoma and fibrosarcoma was by means of cell suspensions and a total of 10^5–10^6 viable cells (trypan blue exclusion test). In cell transplantation assays tumor cell suspensions were diluted two-fold to five-fold and an inoculum containing the appropriate cell number was injected in a 0·1 ml volume into the left flank. For radiation dose respose assays, tumors were transplanted into the leg muscle on the right. The squamous carcinoma was transplanted by the trocar technique using tumor fragments of approximately 1 mm dia.

Anticoagulation

Sodium warfarin (Coumadin; Endo Laboratories, Inc., Garden City, N.Y.) was added to the drinking water with an initiation dosage of 1·0 mg/l for a period of four days (Fig. 1). For continuous administration a dose of 0·65–0·75 mg/l was sufficient to maintain the prothrombin time within the desired limits from 2 to 3 times the average normal value in male mice. The double amount of 1·3 mg/l was required for female mice in order to produce the same effect. The effect of sodium warfarin depended on the sterilizing cycle for the feeding pellets. Wayne Sterilizable Lab. Blox (Allied Mills, Chicago, Ill.) were sterilized 4 min at 136°C in a high vacuum, high pressure steam

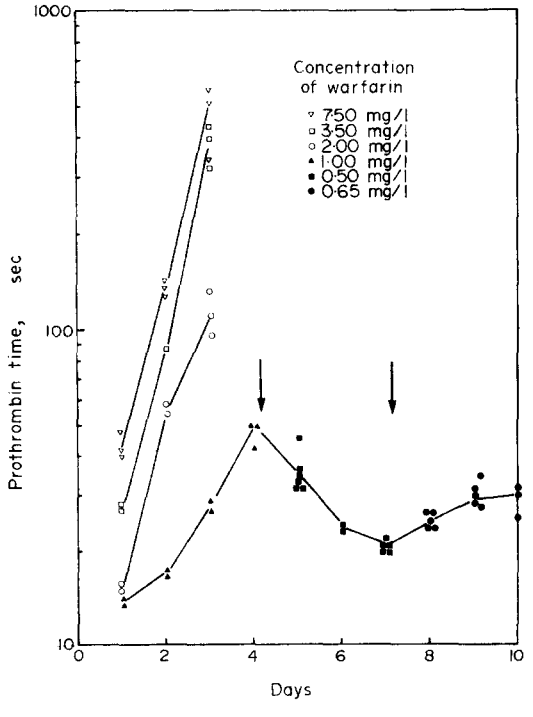


Fig. 1. Effect of concentration of warfarin in drinking water on prothrombin time in male $C3H^f$/Sed. mice. The normal prothrombin time ranged between 10 and 15 sec. Each day one mouse of a cage with warfarin in the drinking water of the indicated concentration was sacrificed following two aspirations of 0·5 cm^3 blood with sodium oxalate by cardiac puncture. Arrows indicate a change of the warfarin concentration.

sterilizer. For continuous anticoagulation by sodium warfarin, the drinking water was changed as in controls once weekly. Housing of five mice per cage did not interfere with the experiment providing the anticoagulation did not cause excessive bleeding.

The degree of warfarin induced anticoagulation was monitored by the one stage prothrombin time. Anticoagulated and control mice with and without tumors were removed randomly from the experiment at least twice weekly. Animals were anesthetized, and blood collected by ventricular puncture with sodium oxalate. After centrifugation, 0·05 ml plasma was mixed with 0·1 ml thromboplastin (Simplastin; Warner-Chilcott Laboratories, Morris Plains, N.J.), and the prothrombin time was determined by means of a wire loop, under standard conditions. The normal prothrombin time ranged between 10 and 15 seconds.

Immunization procedure

Subcutaneous implantation of 1 mm^3 pieces of living fibrosarcoma tissue was used to initiate tumor growth for immunization. An incision was made in the skin of the flank, and a tumor piece was placed under the skin by means of a trocar. The incision was closed with a wound clip.

Tumors 8–12 mm dia were removed, 12 days later, under Nembutal anesthesia. A circular incision was made around the edge of the tumor, and the tumor was removed by blunt dissection. The incision was closed with wound clips.

Irradiation procedure

Local irradiation of the tumor-bearing site was affected by use of a pair of 3-cm dia 137 Cs photon beams which were parallel opposed [11]. Mice were placed on a brass tray with the tumor bearing site over a 3-cm cutout. The tray permitted immobilization of the unanesthetized mouse by taping the foot of the tumor bearing leg on a brass plate. The tray was then inserted in an irradiation chamber and placed into the irradiator. Design of this unit made certain that the position of the tumor and cutout coincided precisely with that of the photon beam. Radiation dose rate to the tumor bearing site was approximately 1000 rad/min. The dose was uniform throughout the tumor bearing site; errors from all causes are estimated to be ± 4%.

Experimental design

This study is based on a series of TD_{50} and TCD_{50} assays. TD_{50} represents the number of tumor cells which would, on the average, be expected to transplant the tumor into half the recipients; TCD_{50} is the radiation dose which, on the average, achieves local control of half of the irradiated tumors. Five mice were housed per cage. Cages were randomly assigned to drinking water with or without warfarin. Subsequently mice were randomly assigned to different dose levels of radiation in the TCD_{50} assays or to different numbers of viable cells in the TD_{50} assays. In the TD_{50} assays, warfarin was added to the drinking water four days prior to the inoculation of tumor cells and continued for 140 days in the experiment with the mammary carcinoma and for 80 days in the experiment with the fibrosarcoma. Tumors were irradiated when they reached the specified diameter. Warfarin treatment was initiated when the first tumors in one assay reached this specified diameter. Radiation dose was given either as a single dose or as 10 equal doses with 24 hr intervals between fractions. The latter represents a closer approximation to the actual clinical practice and should increase our chance of detecting a better response in the test animals. The warfarin treatment was terminated in the experiments with 10 fractions at the completion of the irradiations. In the experiments with single doses warfarin administration was terminated at a later time. The duration of warfarin therapy, the number of mice per assay and the range of days within which the tumors reached the specified diameter are presented in Table 1.

Scoring and analysis of results

In cell transplantation assays mice were examined weekly after transplantation for appearance of tumor at the site of inoculation. Subsequently the tumors were measured three times per week until death of the majority of the animals, in order to compare the growth of the tumors with and without warfarin. Volumes of the usually ellipsoid tumors were computed by the equation:

$$V = \frac{4}{3}\pi r_1 r_2 r_3$$

Following irradiation mice were examined weekly for regrowth of tumor. Tumor recurrence was followed until the tumor reached about 15 mm dia. Logit regression lines were fitted through the transplant take and the

Table 1. *Radiation dose-tumor control response assays: effect of warfarin treatment on* TCD_{50}

Tumor system	Diameter of tumor (mm)	Number of dose fractions	Mice per assay	Start of irradiation*	Time of warfarin treatment*
Mammary carcinoma	8	1	34	18–30	—
			38	18–26	18–90
	8	10	38	19–34	—
			44	19–33	17–40
Fibrosarcoma	8	1	35	7–11	—
			45	7–11	7–107
Fibrosarcoma sensitized†	8–12	1	46	9–17	—
			33	9–17	10–120
Fibrosarcoma	8	10	47	10–16	—
			25	10–23	10–50
Squamous carcinoma	8	1	18	11–16	—
			26	11–15	11–81

*Days after transplantation of tumor.
†Hosts immunized before tumor transplantation by live transplants.

tumor control results in each assay. From this line the TD_{50} or TCD_{50} value with 95% confidence limits was calculated [8].

RESULTS

TD_{50} values for the mammary carcinoma and fibrosarcoma are listed in Table 2. There was no effect of warfarin in the dose schedule employed to modify tumor transplant take rate. The data do document that the immunization procedure resulted in a significant increase in resistance to transplantation of the fibrosarcoma and that warfarin did not suppress that immunity significantly.

TCD_{50} values for the mammary carcinoma as a function of time after irradiation are plotted in Figs 2 and 3 for single doses and for fractionated irradiation respectively. Warfarin did not affect local results. In these assays the dose levels were slightly low and hence TCD_{50} values could not be computed at 120 days. However, the curves relating TCD_{50} with time

Table 2. TD_{50} *assays for mammary carcinoma and fibrosarcoma transplanted into control or warfarin-treated recipients*

Animal tumor system	Host	Duration of experiment	TD_{50} ($\times 10^5$) (95% confidence limits) Control	Warfarin
Mammary carcinoma†	Normal	120 days	3·9 (0·94–16)	3·9 (0·94–16)
Fibrosarcoma‡	Normal	100 days	1·3[a] (0·36–5·0)	0·74[b] (0·26–2·1)
	Immunized	100 days	24[c] (3·1–180)	12[d] (3·4–89)

Warfarin administration continued from:
4 days prior to transplantation to 120 days (†), respectively 80 days (‡) afterward
Significant differences: a $\ll$ c and b $\ll$ d; $p \leqq 0.05$.

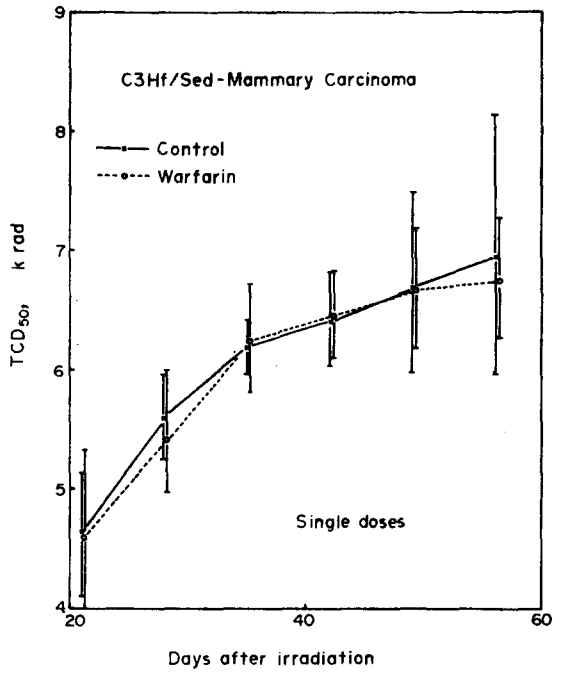


Fig. 2. TCD_{50} vs time after single dose irradiation of mammary carcinoma tumors in normal and warfarin treated mice. Warfarin administration was initiated when the first tumors had reached the specified size of 8 mm dia. Vertical bars indicate the 95% confidence limits.

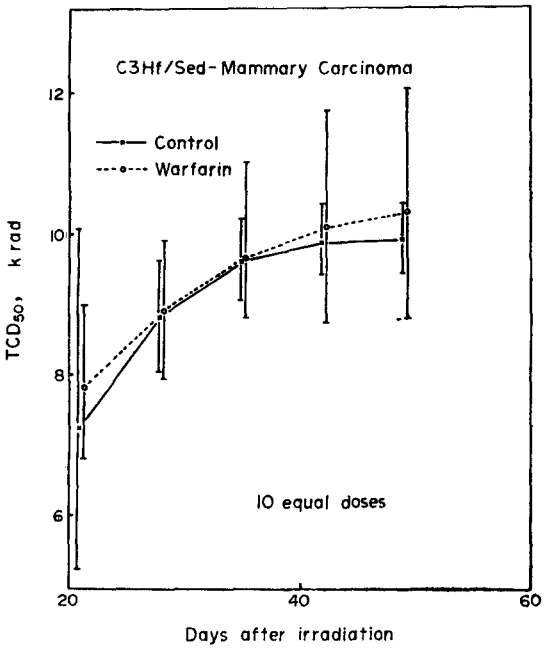


Fig. 3. TCD_{50} vs time after irradiation (10 equal doses) of mammary carcinoma tumors in normal and warfarin treated mice. Warfarin administration was initiated 2 days before the first tumors reached the specified diameter of 8 mm for irradiation. Vertical bars indicate the 95% confidence limits.

Table 3. TCD_{50} assays for fibrosarcoma growing in control or warfarin-treated recipients

Diameter of tumor at irradiation (mm)	Status of host	Number of equal dose Fractions	TCD_{50} (k rad) after 100 days (95% Confidence limits) Control	Warfarin
8	Normal	1	3·31[a] (2·7–4·1)	3·68[b] (2·9–4·7)
	Normal	10	7·31 (6·6–8·1)	7·32 (6·9–7·7)
10	Immunized	1	4·01 (3·4–4·7)	3·80 (3·2–4·5)
12	Immunized	1	3·97 (3·6–4·3)	4·32 (3·8–4·9)
8–12	Immunized	1	3·91[c] (3·6–4·2)	3·93[d] (3·6–4·3)

Significant differences: a ≪ c and b ≪ d; $p \leqq 0{\cdot}05$.

for the first 50 days after irradiation are reliable indicators of local response.

TCD_{50} values for the fibrosarcoma are listed in Table 3. Of interest was the higher rather than lower TCD_{50} in immunized mice. This has been discussed in a separate report [9]. Again, no effect of warfarin was noted.

TCD_{50} values for the squamous carcinoma could not be computed because of the small number of mice examined in the dose range above 5000 rad. A regression analysis was performed for the tumor volume 75 days after a single dose of 4000 rad. The tumor volumes are listed in Table 4. Warfarin did not affect the regrowth of tumors after irradiation.

Table 4. Regrowth of squamous carcinoma at 75 days after 3950 rad

Mean tumor volume (mm^3) (95% confidence limits) Control	Warfarin
537 (417–685)	524 (349–766)

Although no quantitative assessment of normal tissue reactions was made in control or warfarin treated mice, there was judged to be comparable reactions in the two groups at any one radiation dose level.

DISCUSSION

The results demonstrate that for a mammary carcinoma, a methylcholanthrene induced fibrosarcoma and a methylcholanthrene induced squamous carcinoma in C3H mice there was no effect of warfarin on transplant take rate, growth pattern, and response to local irradiation. Warfarin was administered at a dose level sufficient to prolong prothrombin time by a factor of 2–3.

These quantitative data from three animal tumor systems do not corroborate the interpretation of clinical experience by Ries *et al.* [7]. In our experimental system a considerably lower concentration of warfarin in the drinking water was employed than in the work reported by Wood [2], Ketcham [12], and Brown [3]. In our animal colony, all food pellets are autoclaved; the consequent loss of vitamins probably explains the comparable increase in prothrombin time despite a lower dose of warfarin.

Hagmar [13] discussed the possible interference of anticoagulants in the interaction of the host with the tumor cell. Ries *et al.* explained their results with a possibly improved immunologic response against the tumor. Our experimental data with the antigenic methylcholanthrene induced fibrosarcoma do not support this explanation.

Except occasionally in bleeding mice or in mice debilitated from other causes a retardation of tumor growth was not observed. Ryan [14] and Ketcham [12], however, observed this effect at higher concentration of warfarin in a methylcholanthrene induced fibrosarcoma under the conditions of housing mice in

individual cages. In our experiments bleeding and markedly debilitated mice were frequently cannibalized.

Although our findings cannot be extrapolated to the clinical situation, there is no indication that anticoagulants increase the effectiveness of local radiation therapy.

Acknowledgements—We would like to thank Miss Claire Hunt and Mrs. Suca Elles for their secretarial assistance.

REFERENCES

1. J. S. Wood, Jr., Studies on the behaviour of V_2 cancer cells *in vivo*, in: *Prognostic Factor in Breast Cancer; Proc. First Tenovus Symp. Cardiff*, April 1967. Edited by A. P. M. Forrest and P. B. Kunkler, E. & S. Livingstone, Ltd., Edinburgh, 1968.
2. J. S. Wood, Jr. and D. W. Edlow, *In vivo* time-lapse cinemicrography of leukocytes: an inquiry concerning the *in vivo* behavior of lymphocytes. Chapter 4 in: *The Microcirculation*, 1969; A Symposium Edited by W. L. Winters, Jr. and A. M. Brest. Charles C. Thomas, Springfield, Ill.
3. J. M. Brown, A study of the mechanism by which anticoagulation with warfarin inhibits blood-borne metastases, *Cancer Res.* **33,** 1217 (1973).
4. E. G. Elias and A. Brugarolas, The role of heparin in the chemotherapy of solid tumors: preliminary clinical trial in carcinoma of the lung. *Cancer Chemother. Rep.* **56,** 783 (1972).
5. R. D. Thornes, Warfarin as maintenance therapy for cancer. *J. Irish. Coll. Physicians and Surgeons* **2,** 41 (1972).
6. V. Larsen, B. Mogensen, C. J. Amris and O. Storm, Fibrinolytic enzyme in the treatment of patients with cancer. *Danish med. Bull.* **5,** 137 (1964).
7. J. Ries, H. Ludwig and W. Appel, Antikoagulantien bei der Strahlenbehandlung weiblicher Genital-karzinome; I. Mitteilung: Plattenepithelkarzinom. *Med. Welt.* **19,** 2042 (1968).
8. H. D. Suit, R. J. Shalek and R. Wette, Radiation response of C3H mouse mammary carcinoma evaluated in terms of cellular radiation sensitivity. *Cell. Radiat. Biol.* **30,** 514 (1965).
9. M. Jurin and H. D. Suit, *In vivo* and *in vitro* studies of the influence of the immune status of C3Hf/Bu mice on the effectiveness of local irradiation of a methylcholanthrene-induced fibrosarcoma. *Cancer Res.* **32,** 2201 (1973).
10. H. D. Suit and C. Suchato, Hyperbaric oxygen and radiotherapy of a fibrosarcoma and of a squamous-cell carcinoma of C3H mice, *Radiology* **89,** 713 (1967).
11. E. B. Hranitzky, P. R. Almond, H. D. Suit and E. B. Moore, A cesium-137 irradiator for small laboratory animals. *Radiology* **107,** 641 (1973).
12. A. S. Ketcham, E. V. Sugarbaker, J. J. Ryan and S. K. Orme, Clotting factors and metastasis formation. *Amer. J. Roentgenol.* **111,** 42 (1971).
13. B. Hagmar, Tumour growth and spontaneous metastasis spread in two syngeneic systems. *Acta path. micro-biol. Scand.* A, **78,** 131 (1970).
14. J. Ryan, A. S. Ketcham and H. Wexler, Warfarin therapy as an adjunct to the surgical treatment of malignant tumors in mice. *Cancer Res.* **29,** 2191 (1969).

Europ. J. Cancer Vol. 11, pp. 751–755. Pergamon Press 1975. Printed in Great Britain

Levels of Cholesterol, 11-Hydroxycorticosteroids and Progesterone in Plasma from Postmenopausal Women with Breast Cancer

M. SMETHURST, T. K. BASU and D. C. WILLIAMS
Research Department, Marie Curie Memorial Foundation, The Chart, Oxted, Surrey, Great Britain

Abstract—*Plasma levels of cholesterol, 11-hydroxycorticosteroids and progesterone have been measured in postmenopausal women with early breast cancer. Results are compared with a control group of postmenopausal women with early cancer at other sites. Cholesterol levels were slightly elevated in the breast cancer group and mean values were significantly different compared to our control group. Concentrations of 11-hydroxycorticosteroids in the breast cancer group were significantly lower than in the control group but both means were within the normal range. Progesterone levels were raised in the breast cancer patients and the mean value was significantly different from the mean concentration in the control group. The results are discussed with reference to the source of plasma progesterone and its effect on adrenal metabolism.*

INTRODUCTION

VARIOUS studies have indicated a possible association between abnormal levels of androgen and corticosteroid metabolites in urine and plasma and the occurrence of breast cancer. Conflicting reports have appeared which suggest that for breast cancer patients the excretion of hydroxycorticosteroids is normal [1] or low [2], and the excretion of androgens is also low [1–3]. Plasma concentrations of hydroxycorticosteroids have been reported as normal [3, 4] or high [4], and plasma androgen levels also as normal [4, 5] or high [6]. It therefore appears that some of these metabolites, which are largely adrenal in origin, are present in abnormal concentrations in the plasma and urine of women with breast cancer.

Adrenal function is directly stimulated by ACTH, the secretion of which is influenced by a number of factors including steroid hormones [7–9]. Oestradiol and testosterone [10], but not progesterone [9], will also affect adrenal metabolism directly. In this paper we report the levels of the adrenal metabolite 11-hydroxycorticosteroid (11-OHCS) in plasma from postmenopausal women with early breast cancer, together with plasma concentrations of progesterone, and of cholesterol, the precursor of adrenal steroid metabolites. These estimations have also been carried out on a control group consisting of postmenopausal women with cancer at sites other than the breast.

Accepted 23 June 1975.

MATERIAL AND METHODS

The patients consisted of 10 postmenopausal women with early breast cancer and a control group of 15 postmenopausal women with early cancer at other sites (bronchus, rectum and stomach). The mean age in each group was 65 and 68 years respectively. None of the patients were under steroid therapy at the time

of the investigation or had a clinical record of any other disorder which could influence cholesterol levels. Most of the patients, however, were suffering from intermittent pain. Heparinised blood samples were obtained between 9–10 am following overnight fasting and plasma was stored at −20°C.

Plasma cholesterol was measured using the method of Kin and Golderberg [11], and plasma 11-OHCS concentrations were determined by the fluorimetric method of Mattingly [12]. Plasma progesterone levels were estimated by radio-immunoassay with antisera prepared in rabbits against progesterone-11-succinyl-bovine serum albumin (NEN Chemicals) using the assay procedure described by the manufacturer.

Plasma samples and water blanks of 1 ml were added to 0·1 ml of recovery tracer and progesterone was extracted with 2 × 10 ml petroleum ether. The ether extractions were pooled, evaporated to dryness, and chromatographed using a column of Sephadex LH-20 (1 × 4 cm). Progesterone was eluted with iso-octane : benzene : methanol, 90 : 5 : 5, and the fraction from 4–8 ml contained the majority of the progesterone. This system seperated progesterone from pregnenolone which is eluted between 7–12 ml. The fraction containing progesterone was dried down and re-dissolved in 2 ml ethanol. Samples of 0·2 ml and 0·4 ml were used for the assay of progesterone and 0·5 ml for the determination of recovery.

Assay samples were dried down, dissolved in 0·1 ml phosphate buffer pH 7·0, and incubated for 4 hr at 4°C with 0·1 ml antiserum and 40–60 pg [1,2-^{3}H] progesterone (40–60 Ci/mmole), to give a total volume of 0·3 ml. At the end of the incubation period, free progesterone was adsorbed with 1 ml of a cold dextran-coated charcoal suspension and finally removed by centrifugation. Aliquots of the resulting supernatant were taken for radioactive counting.

A standard curve from 20–2000 pg progesterone was used and 20 pg gave a displacement significantly different from the zero value. The blank value from extracting 1 ml of distilled water and taking it through the assay procedure was usually between 10–20 pg. The coefficient of variation within assays was 12% and between assays was 18%. The cross-reactivity was 12% for deoxycorticosterone, 5% for pregnenolone and 4% for corticosterone. However, deoxycorticosterone and corticosterone are largely eliminated at the extraction stage and pregnenolone removed by column chromatography. The cross-reactivity was 0·7% for 17α-hydroxyprogesterone, and was less than 0·3% for testosterone, androstenedione, estradiol, estrone, cortisol and cholesterol.

RESULTS

Plasma cholesterol levels are shown in Fig. 1. In the breast cancer group, levels ranged from 198–320 mg/ml plasma and in the control group the range was 130–330 mg/ml. Seven out of 10 cases in the breast cancer group and only 4 cases out of 14 in the control group had elevated cholesterol levels compared to the normal range in our laboratory of 150–235 mg/ml [13]. Although there is an overlap between the groups, the mean level of cholesterol in plasma from women with breast cancer was outside the normal range and differed from the mean level in the control group with the significance $P = 0{\cdot}05$–$0{\cdot}02$ (Table 1).

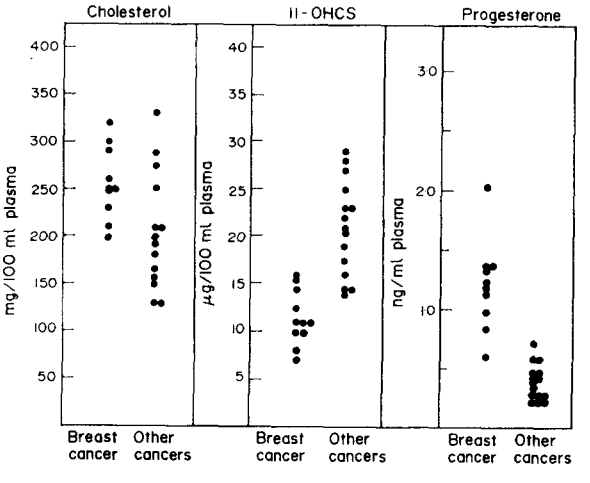


Fig. 1. Levels of cholesterol, 11-hydroxycorticosteroid and progesterone in plasma from postmenopausal women with cancer.

Levels of 11-OHCS in plasma from the breast cancer cases ranged from 7·0–16·1 μg/100 ml, but were considerably higher in the control group with a range from 14·2–29·0 μg/100 ml (Fig. 1). The mean 11-OHCS concentrations are shown in Table 1 and are significantly different ($P < 0{\cdot}001$).

Plasma progesterone levels in the control group ranged from 0·22–0·72 ng/ml, but were elevated in the breast cancer group and ranged from 0·60–2·03 ng/ml (Fig. 1). The plasma progesterone concentrations are shown in Table 1 and the mean values for each group are significantly different ($P < 0{\cdot}001$).

Table 1. *Plasma steroid levels in breast cancer cases and controls*

	Cholesterol (mg/100ml)	11-OHCS (μg/100ml)	Progesterone (ng/ml)
Breast cancer	255± 12(10)*	11.5± 0.9(11)*	1.20± 0.12(10)+
Other cancers	204± 15(14)	20.9± 1.3(15)	0.39± 0.04(15)

Each value is the mean ± S.E. Number of patients is given in parentheses. * = $P<0.05$ and + = $P<0.001$ compared to other cancers.

DISCUSSION

Although only a small number of patients have been used in this study our results clearly show that plasma 11-OHCS levels are lower in postmenopausal women with cancer compared to our control group. Reduced levels of plasma 11-OHCS in early and advanced breast cancer, irrespective of menopausal status, have also been reported in our earlier communication [14]. The choice of the control group is debatable, but we considered that a group of postmenopausal women with cancer, but free from liver and endocrine disorders, served as a better comparison to breast cancer patients than healthy women not subjected to the same stress of a debilitating disease. With the exception of 2 values, the plasma 11-OHCS levels in each group all fall within the range of 6·5–26·3 μg/ml quoted for hospital patients without endocrine, liver or renal disease [12]. Most of the patients were suffering from intermittent pain, and so far as could be determined, all were receiving similar hospital diets. Patients receiving any kind of steroid therapy were not included in this study. It is, therefore, unlikely that the marked difference in the plasma 11-OHCS levels observed in the two groups was due to factors such as drugs, pain or diet.

There are few reports of 11-OHCS levels in plasma from breast cancer patients. Arguelles *et al.* [3] found that there was no significant difference in the 11-OHCS levels in plasma from postmenopausal women with breast cancer and women with no endocrine or neoplastic disease. Both values were within the normal range. Brownsey *et al.* [15] compared the mean log concentrations of 11-OHCS in plasma from women with benign breast disease, and primary and advanced breast cancer and also found them to be similar.

The discrepancy between our results and those reported by Arguelles *et al.* [3] has no obvious explanation, but it is unclear whether plasma samples were taken at a regular time to allow for the diurnal variation in 11-OHCS levels. In fact, an alteration in the circadian rhythm in either the breast cancer or control groups in our experiment would be a possible explanation for the differences that we observed.

The small difference in cholesterol levels with this limited number of patients could have various explanations. However, in view of the low levels of 11-OHCS in the breast cancer patients, the raised cholesterol concentrations might in part result from a decrease in the utilisation of this precursor by the adrenal gland. An abnormality in cholesterol metabolism is also supported by the elevated levels of non-esterified cholesterol found in the urine of women with inoperable breast cancer [16].

The plasma progesterone level in normal postmenopausal women is reported as 0·30 ng/ml [17] and as 0·33 ng/ml in ovariectomised women [18]. In our preliminary communication we reported similar concentrations in plasma from postmenopausal women with cancer at sites other than the breast, and raised levels in the breast cancer group [19]. In premenopausal women, plasma progesterone may arise by secretion from the adrenal gland and ovary and also by the extra-glandular conversion from pregnenolone. In the early follicular phase when plasma progesterone levels are low, a significant contribution is made from all three sources [20]. It is uncertain whether the same is true for the origin of plasma progesterone in postmenopausal women. The source of the increased plasma progesterone that we have observed in postmenopausal women with breast cancer is also unknown, and is under investigation.

The metabolism of cholesterol to androgens and corticosteroids by the adrenal gland is influenced both directly and indirectly by a number of factors including steroid hormones. In the rat, oestradiol enhances adrenal corticosteroid secretion [7, 10] but most reports

indicate that testosterone and progesterone have the opposite effect and cause a decrease in adrenal weight and corticosteroid production [8, 9]. The low level of 11-OHCS in the plasma from the breast cancer patients and the low level of plasma dehydroepiandrosterone sulphate reported in women with advanced breast cancer [15] might result from a decrease in adrenal metabolism. This may be influenced by the increased levels of plasma progesterone which could arise from sources other than the adrenal gland. Alternatively, if the increased plasma progesterone was adrenal in origin, then this would indicate a further aberration in adrenal metabolism.

Acknowledgements—We thank Dr. D. Donaldson of Redhill General Hospital for the provision of plasma samples, also Mr. K. Alexander and Mr. D. Patel for their technical assistance in this study.

REFERENCES

1. E. H. D. Cameron, K. Griffiths, E. N. Gleave, H. J. Stewart, A. P. M. Forrest and H. Campbell, Benign and malignant breast disease in South Wales: a study of urinary steroids. *Brit. med. J.* **4,** 768 (1970).
2. A. P. Wade, J. C. Davies, M. C. K. Tweedie, C. A. Clarke and B. Haggart, The discriminant function in early carcinoma of the breast. *Lancet* **1,** 853 (1969).
3. A. E. Arguelles, C. Hoffman, U. L. Poggie, M. Chekherdemian, C. Saborida and O. Blanchard, Endocrine profiles and breast cancer. *Lancet* **1,** 165 (1973).
4. N. Deshpande, J. L. Hayward and R. D. Bulbrook, Plasma 17-hydroxycorticosteroids and 17-oxosteroids in patients with breast cancer and in normal women. *J. Endocrin.* **32,** 167 (1965).
5. J. R. Garnham, R. D. Bulbrook and D. Y. Wang, Conjugated and unconjugated dehydroepiandrosterone, aetiocholanolone and androsterone in the peripheral plasma of patients with cancer of the breast, ovary, uterus and prostate. *Europ. J. Cancer* **5,** 239 (1969).
6. H. Benard, J. S. Bourdin, R. Saracino and A. Seeman, Les 17-cétostéroides plasmatiques libre et totaux dans les cancers génitaux. *Cancer (Philad.)* **4,** 141 (1968).
7. M. D. Coyne and J. I. Kitay, Effect of ovariectomy on pituitary secretion of ACTH. *Endocrinology* **85,** 1097 (1969).
8. M. D. Coyne and J. I. Kitay, Effect of orchiectomy on pituitary secretion of of ACTH. *Endocrinology* **89,** 1024 (1972).
9. W. I. Rodier and J. I. Kitay, The influence of progesterone on adrenocortical function in the rat. *Proc. Soc. exp. Biol. (N.Y.)* **146,** 376 (1974).
10. H. D. Colby and J. I. Kitay, Effects of gonadal hormones on adrenocortical secretion of 5α-reduced metabolites of corticosterone in the rat. *Endocrinology* **91,** 1523 (1972).
11. E. Kin and M. Golderberg, Serum cholesterol assay using a stable Liebermann–Burchard reagent. *Clin. Chem.* **15,** 1171 (1969).
12. D. J. Mattingly, A simple fluorimetric method for the estimation of free 11-hydroxycorticosteroids in human plasma. *Clin. Path.* **15,** 374 (1962).
13. T. K. Basu, R. W. Raven and D. C. Williams, Some metabolic functions of liver in patients with advanced non-hepatic cancer. *Oncology* **29,** 417 (1974).
14. T. K. Basu, D. Donaldson and D. C. Williams, Plasma 11-hydroxycorticosteroids and cholesterol in patients bearing early and advanced breast carcinomas. *IRCS Med. Sci.* **3,** 42 (1975).
15. B. Brownsey, E. H. D. Cameron, K. Griffiths, E. N. Gleave, A. P. M. Forrest and H. Campbell, Plasma dehydroepiandrosterone sulphate levels in patients with benign and malignant breast disease. *Europ. J. Cancer* **8,** 131 (1972).
16. H. F. Acevedo, E. A. Campbell, J. C. Frich, P. J. Dugan, E. L. Saier and L. P. Merkow, Urinary cholesterol VI. Its excretion in women with inoperable inflamatory carcinoma of the breast. *Cancer (Philad.)* **34,** 1727 (1974).
17. M. F. Pichon and E. Milgrom, Competitive protein binding assay of progesterone without chromatography. *Steroids* **21,** 335 (1973).

18. T. YASHIMI and M. B. LIPSETT, The measurement of plasma progesterone. *Steroids* **11,** 527 (1968).
19. M. SMETHURST, K. ALEXANDER and D. C. WILLIAMS, Plasma progesterone levels in postmenopausal women with breast cancer. *IRCS Med. Sci.* **3,** 89 (1975).
20. B. LITTLE and R. B. BILLIAR, In: *Progress in Endocrinology* (Edited by C. GUAL), p. 871 Amsterdam, (1969).

Europ. J. Cancer Vol. 11, pp. 757–762. Pergamon Press 1975. Printed in Great Britain

A Search for Antibodies against Human Sarcoma Cells in Patients' Sera by Indirect Immunofluorescence on Fixed Cells

I. LAPREVOTTE, J. C. CHUAT, A. M. L'HIRONDEL, C. BERNARD, J. PERIES and M. BOIRON

Institut de Recherches sur les Leucémies, Laboratoire d'Hématologie Expérimentale, Hôpital Saint-Louis, 2, place du Docteur-Fournier, 75010 Paris, France

Abstract—*Fourteen human sarcomas (osteosarcomas, soft tissue and reticulum cell sarcomas), and 3 control tumours, were examined by the indirect immunofluorescence technique, using a battery of 19 sera selected from patients affected by a variety of sarcomas. The cells utilized in immunofluorescence were obtained either from fresh tumours or from cells grown in vitro, and a variety of procedures were used for fixation of the antigen preparations and for the tests. The 427 tests performed were totally negative. The reasons for this discrepancy, with the positive results obtained by various investigators, are discussed.*

INTRODUCTION

MORTON *et al.* were pioneers in providing experimental results suggesting a viral aetiology of human sarcomas [1–8]. In view of the implications of these findings, it was deemed of interest to initiate an exhaustive study of sarcomas of several histological types, in which cytology, investigation of cellular proliferation potential *in vitro*, caryologic studies, electron microscopy, virology and immunology were combined. The purpose of the present paper is to report the results of the immunological part of this investigation, where the techniques used for the processing of biopsy specimens and the fixation of tissue culture cell lines derived from the biopsies varied so that they cover a wide range of reactivity patterns.

MATERIAL AND METHODS

Tumours

The 14 tumours studied and their histological type are listed in Table 1. Control tumours consisted of 2 benign tumours (an aneurysmal cyst, a haemangiopericytoma) and 1 osteolytic tumour which occurred during the course of an agnogenic myeloid metaplasia.

Table 1. Histological type of the sarcomas studied by immunofluorescence

Histological type	Number of sarcomas of each type
Angiosarcoma	1
Fibrosarcoma	2
Fibromyxosarcoma	1
Liposarcoma	1
Myxosarcoma	1
Osteosarcoma	1
Reticulum cell sarcoma	2
Rhabdomyosarcoma	1
Synoviosarcoma	1
Sarcomas with ill-defined histological type.	3
Total	14

Antigen preparation techniques

The various techniques utilized for the

•Accepted 24 June 1975.

Table 2. Techniques utilized for the preparation of the antigens

Techniques	Number of tested samples.	Sarcomas tested	
.Imprints with biopsy or surgical samples [9].	15	.Fibrosarcoma .Liposarcoma .Myxosarcoma .Reticulum cell sarcoma.	.Rhabdomyosarcoma .Synoviosarcoma .Sarcoma with ill-defined histological type.
.Cell smears with tumour cells following trypsinization or mechanical disruption.	8	.Fibrosarcoma .Liposarcoma .Reticulum cell sarcoma.	.Rhabdomyosarcoma .Synoviosarcoma .Sarcoma with ill-defined histological type.
.Cell smears with tissue culture grown cells. 3rd to 22nd passage.	18	.Fibrosarcoma .Fibromyxosarcoma .Osteosarcoma	.Synoviosarcoma .2 Sarcomas with ill-defined histological type.
.Cells grown on slides in Leighton tubes. 1st to 24th passage.	8	.Myxosarcoma	.Osteosarcoma
.Cocultivation with calf skin cells.	4	.Angiosarcoma	.Osteosarcoma
Total	53		

preparation of the antigens are summarized in Table 2.

Fixation techniques intentionally varied, ranging from no fixation at all to fixation in acetone for 10 min, a classical fixative in immunofluorescence with viral antigens, fixation in ethanol at −60°C for 30 min, and finally fixation in acetone for 1 min, a mild method known to cause very little damage to cell membrane structures. Snap-freezing in isopentane at −160°C (liquid nitrogen) was also used. The antigens were examined either immediately or, in a few cases, following storage at −20°C, −80°C or −180°C.

Immunofluorescence techniques

The indirect method was used in all cases, by means of a Bellco Glass Immunofluorescence Kit (Bellco Glass, Inc, Vineland, New Jersey, U.S.A.). Various treatment times were tested. The antigen preparations were in most cases reacted with properly diluted test serum at 37°C for 45 min in a damp container, washed for 10 min with 2 changes of Dulbecco's PBS, and reacted with an adequate dilution of anti-human globulin goat globulin, fluorescein isothiocyanate conjugated (FITC) (Hyland Laboratories, Los Angeles, U.S.A., batch No. 2048 E001A1). In some cases, an anti IgG conjugate was used (batch No. 2201 H004A1). The preparations were then washed again as above, dipped in bidistilled water and mounted in buffered glycerol (pH 7·2). As regards the specificity of the FITC-globulin, a control reaction where the test serum was replaced by PBS served to rule out any intracytoplasmic fluorescence due to a reaction of FITC-globulin with globulins secreted by cells present in the antigen preparations. In fact, one of the tumours contained quite a number of plasma cells. The FITC-globulins were tested to determine that dilution which gave a negligible background staining and still reacted optimally with Ig G and Ig M from anti-Epstein–Barr virus (EBV) sera of known titer [10]. Two slightly different dilutions were used to minimize the risk of missing a weak reaction for the sake of a low background staining. The preparations were examined immediately in u.v. light (Osram Lamp HBO-200) with a Leitz Ortholux microscope supplied with a BG 12/3 mm primary filter and a black background condensor. The readings were performed in each case by two investigators.

Sera tested

Nineteen patients' sera were selected as test sera. A list of the malignancies affecting these patients is given in Table 3. Table 4 shows the kinds of sera against which the different histo-

Table 3. Origin of the test sera used

Tumour type.	Number of sera
Osteosarcoma	9
Reticulum cell sarcoma	2
Rhabdomyosarcoma	1
Synoviosarcoma	2
Angiosarcoma	1
Fibrosarcoma	1
Liposarcoma	1
Myxosarcoma	1
Soft tissue sarcoma	1
Total	19

of tests performed in each case are shown in Table 5.

RESULTS AND DISCUSSION

No specific fluorescence was ever observed in any of the reactions performed. A rare slightly brighter cell seen occasionally could not be taken into account. No difference resulted from the intentional and planified use of various methods for the fixation of antigen preparation, except perhaps that background staining was more important with isopentane fixation.

Table 4. Kind of test sera used against the sarcomas of different histological types

Origin of the test sera.	Histological types of the sarcomas tested.									
	Angio-sarcoma *	Fibro-sarcoma	Fibromyxo-sarcoma	Lipo-sarcoma *	Myxo-sarcoma *	Osteo-sarcoma *	Reticulum cell sarcoma *	Rhabdomyo-sarcoma	Synovio-sarcoma *	Sarcoma with ill-defined histological type. *
Osteosarcoma	+	+	+	+	+	+	+	+	+	+
Reticulum cell sarcoma	-	-	-	+	-	-	+	-	+	+
Rhabdomyo-sarcoma	+	+	+	-	+	+	+	-	+	+
Synoviosarcoma	+	+	+	+	+	+	+	-	+	+
Angiosarcoma	+	-	-	-	-	-	-	-	-	+
Fibrosarcoma	-	-	-	-	+	+	+	-	+	-
Liposarcoma	+	+	-	+	-	+	+	-	-	+
Myxosarcoma	-	-	-	-	+	+	+	-	+	+
Soft tissue sarcoma	+	+	+	-	+	+	+	-	+	+

\+ tested - non tested * 1 sarcoma reacted with the autologous serum

logical types of the sarcomas were tested. In 10 out of the 17 tumours examined, including 7 out of the 14 sarcomas, the tumour cells reacted upon the autologous serum. In addition to the test sera, control sera from the 3 non-sarcoma patients mentioned above and sera from healthy donors were included in the tests. All the sera were used without prior inactivation. In 10 tests, the tumour cells also reacted with the serum of a rat immunized against the murine oncornavirus group-specific antigen. The tests included the respective control sera and FITC-globulin [11]. Six tumours of different histological type were examined: 1 angiosarcoma, 1 fibromyxosarcoma, 1 fibrosarcoma, 1 myxosarcoma, 1 osteosarcoma, 1 synoviosarcoma.

The test (and parallel control) serum dilutions routinely used were chosen from similar investigations [10]. These tests and the number

Thus, our data are in conflict with that reported by other investigators, although, in some cases, identical techniques were used. The reasons for these differences are not clear but, at this point, a brief survey of the results obtained in the field may not be superfluous. These consist, in most cases, in positive reactions observed essentially by the indirect immunofluorescence technique, using the serum of a sarcoma-bearing patient (osteosarcoma or soft tissue sarcoma), and cells from the corresponding tumour [12–16]. Cross reactions between the patient's serum and tumours from other donors are usually obtained. The reaction seems to be specific in that either the positive sera do not react with tumours of a different histological type and with normal tissue [3, 12–14, 16] or, if they do react in some cases with such tumours, sarcoma cells only are capable of blocking the antibodies [17, a study

Table 5. *Test serum dilutions used* (†)

Diluent	Dilution	Number of tests ‡	Number of tests with the sarcomas listed in Table 1										Total number of tests, only with the sarcomas.
			Angio-sarcoma	Fibro-sarcoma	Fibromyxo-sarcoma	Lipo-sarcoma	Myxo-sarcoma	Osteo-sarcoma	Reticulum cell sarcoma	Rhabdomyo-sarcoma	Synovio-sarcoma	Sarcomas witht ill-defined histologi-cal type.	
Phosphate-buffered saline	1 : 2	7	6	0	0	0	0	0	0	0	0	1	7
	1 : 5	87	0	14	0	16	0	5	27	0	9	8	79
	1 : 10	219	7	8	7	9	21	35	16	3	52	24	182
	1 : 50	6	0	0	0	0	0	0	0	0	0	6	6
	1 : 100	69	0	0	0	0	0	15	0	5	9	13	42
Guinea pig complement diluted 1 : 30	1 : 2	30	0	0	0	0	0	0	0	0	0	18	18
—	Undiluted	9	0	8	0	0	0	0	0	0	0	1	9
Total		427	13	30	7	25	21	55	43	8	70	71	343

† With regard to sera listed in Table 3, sera of the 3 non sarcoma patients and rat sera.
‡ Total numbers comprizing tests with control tumours: total = 427.

using cytotoxic tests]. However, these positive results obtained by immunofluorescence are not isolated, but are part of a broader body of knowledge accumulated by applying many different techniques to tumours of widely differing histological types, namely melanosarcoma or breast carcinoma [6, 18–20] as well as osteosarcoma and soft tissue sarcoma. A host of techniques were thus employed, viz electron microscopy, focus formation, immunodiffusion, complement fixation, immune adherence, cytotoxic test [1–3, 7, 8, 12, 17, 21–23]. The localization of the antigens detected by these techniques is obviously diverse, ranging from cytoplasma (immunofluorescence with fixed cells) to cell membrane (cytotoxic tests, immune adherence). Immunodiffusion and complement-fixation tests were performed using sarcoma cell extracts, thereby obscuring the localisation of the detected antigens.

Finally, additional evidence for a viral aetiology of human sarcoma stemmed from the successful induction of osteosarcomas in hamsters inoculated with extracts of human osteosarcomas [24] and from positive hybridization between DNA complementary to Rauscher mouse leukaemia virus RNA and human sarcoma polysomal fraction [25]. Some authors were successful in observing the appearance of a so-called sarcoma antigen in normal cells following inoculation with filtrates of human sarcoma cells grown *in vitro* [12], and of a leukaemia in mice following inoculation with cell-free extracts from an osteosarcoma cell line [5].

Yet, in this rather impressive body of evidence, some findings were inconsistent from the very beginning. For instance, although some authors found positive reactions with both tumour cells from fresh tumours and cells grown *in vitro* [3–5, 15], others obtained only negative or, at the best, ambiguous results with fresh tumours [12–14]. On the contrary, they did observe positive reactions using cultured cells, albeit not constantly, but rather in some of the serial passages *in vitro*. The respective proportion of positive sera in the patients, in their relatives and in the population at large gave rise to totally different estimates in the different reports. In some, high percentages of positive are restricted to the patients and to their close relatives, contrasting with a much lower reactivity of sera from healthy donors [3–5, 8, 13–16]. In other reports, the proportion of positive sera is high in all groups [12, 17].

The reasons of the existing discrepancies, both between the present results and those previously reported by others and taken as a whole, and between the latter considered individually, are difficult to explain, the more so since the antigens detected are probably diverse and the techniques employed are not readily amenable to objective comparison. As far as the present study is concerned, every care was taken to rule out a technical failure by varying the technical procedures reported by others. In addition, the procedure and reagents employed were confronted to undoubtedly positive and routinely reproducible results obtained in this laboratory with various viral antigens, such as murine oncornaviruses [11] and EBV [10]. However, in this line, the problems are all the more difficult to clarify

since the exact nature of the antigen(s) involved is not known. Of course, the obtention of positive and seemingly specific reactions is strongly in favour of a viral implication in human sarcoma, and several workers have dedicated themselves to eliminating the role of passenger micro-organisms, such as mycoplasmas, in the positive reactions observed between the tumours and the patients' and healthy donors' sera. Such micro-organisms failed to be detected in a systematic study [12]. In cases where these were actually isolated from tissue cultures, which is held by most authors as a common occurrence, *in vitro* as well as *in vivo*, for that matter [26, 27, 28], no reaction was obtained between the positive sera and the mycoplasmas [2]. Other workers have ruled out the role of auto-antibodies or antibodies to HL-A antigens [13].

In reality, no definitive conclusion can be drawn from all the evidence reported so far, and even if a virus were unquestionably isolated, its aetiological role in the disease would not be assessed by the same token. An instance in point is Burkitt's lymphoma. To all these difficulties is superimposed the question of whether the cells grown *in vitro* do represent the tumour cells originally present in the tumour. No argument can be derived from the very success of their cultivation, as the surviving cells, even in the face of chromosomal alteration, might, in some cases, and might not, in others, be tumour cells. Furthermore, normal and tumour cells might coexist. A parallel situation can be found in myelofibrosis, where the exact nature of the fibroblasts recovered from the bone marrow of afflicted patients has given rise to similar problems. In such cases, a coexistence of "normal" and "malignant" fibroblasts has even been suggested [29, 30].

REFERENCES

1. F. R. Eilber and D. L. Morton, Demonstration in sarcoma patients of antitumour antibodies which fix only human complement. *Nature (Lond,)* **225,** 1137 (1970).
2. F. R. Eilber and D. L. Morton, Sarcoma-specific antigens. Detection by complement fixation with serum from sarcoma patients. *J. nat. Cancer Inst.* **44,** 651 (1970).
3. D. L. Morton, W. T. Hall and R. A. Malmgren, Human liposarcomas: Tissue cultures containing foci of transformed cells with viral particles. *Science* **165,** 813 (1969).
4. D. L. Morton and R. A. Malmgren, Human osteosarcomas. Immunologic evidence suggesting an associated infectious agent. *Science* **162,** 1279 (1968).
5. D. L. Morton, R. A. Malmgren, W. T. Hall and G. Schidlovsky, Immunologic and virus studies with human sarcomas. *Surgery* **66,** 152 (1969).
6. D. L. Morton, R. A. Malmgren, E. C. Holmes and A. S. Ketcham, Demonstration of antibodies against human malignant melanoma by immunofluorescence. *Surgery* **64,** 233 (1968).
7. W. D. Winters and D. L. Morton, Detection of human antisarcoma antibodies by immunodiffusion. *Proc. Am. Ass. Cancer Res.* **14,** 55, (1973).
8. W. C. Wood and D. L. Morton, Host immune response to a common cell-surface antigen in human sarcomas. *New Eng. J. Med.* **284,** 569 (1971).
9. T. K. Burnham, T. R. Neblett and G. Fine, The immunofluorescent tumor imprint technic. I. Description and evaluation. *Amer. J. Clin. Path.* **45,** 714 (1966).
10. J. C. Chuat, F. Lasquellec and M. Boiron, Etude séro-épidémiologique du virus Herpès EB en France: Fréquence et distribution selon l'âge des anticorps dans la région parisienne. *Path. Biol.* **18,** 713 (1970).
11. J. C. Chuat, F. Lasquellec, A. M. L'Hirondel and M. Boiron, Studies on murine sarcoma virus. II. Detection of group-specific antigens by immunofluorescence. *Int. J. Cancer* **7,** 101 (1971).
12. G. Giraldo, E. Beth, Y. Hirshaut, T. Aoki, L. J. Old, E. A. Boyse and H. C. Chopra, Human sarcomas in culture (foci of altered cells and a common antigen; induction of foci and antigen in human fibroblast cultures by filtrates). *J. exp. Med.* **133,** 454 (1971).
13. M. Moore and L. A. Hughes, Circulating antibodies in human connective tissue malignancy. *Brit. J. Cancer* **28,** suppl. I., 175 (1973).
14. M. Moore, P. J. Witherow, C. H. G. Price and S. A. Clough, Detection by immunofluorescence of intracytoplasmic antigens in cell lines derived from human sarcomas. *Int. J. Cancer* **12,** 428 (1973).

15. E. S. Priori, J. R. Wilbur and L. Dmochowski, Immunofluorescence tests on sera of patients with osteogenic sarcoma. *J. nat. Cancer Inst.* **46,** 1299 (1971).
16. C. A. Reilly, D. J. Pritchard, B. O. Biskis and M. P. Finkel, Immunologic evidence suggesting a viral etiology of human osteosarcoma. *Cancer (Philad.)* **30,** 603 (1972).
17. E. T. Bloom, Further definition by cytotoxicity tests of cell surface antigens of human sarcomas in culture. *Cancer Res.* **32,** 960 (1972).
18. P. G. Elliott, B. Thurlow, P. R. G. Needham and M. G. Lewis, The specificity of the cytoplasmic antigen in human malignant melanoma. *Europ. J. Cancer* **9,** 607 (1973).
19. E. S. Priori, G. Seman, L. Dmochowski, H. S. Gallager and D. E. Anderson, Immunofluorescence studies on sera of patients with breast carcinoma. *Cancer (Philad.)* **28,** 1462 (1971).
20. G. W. Wood and R. F. Barth, Immunofluorescent studies of human malignant melanoma. *Proc. Am. Ass. Cancer Res.* **14,** 22 (1973).
21. G. Della Porta, G. Fossati and S. Canevari, Complement-dependent cytotoxicity of serum of patients with malignant melanoma. *Proc. Amer. Ass. Cancer Res.* **14,** 33 (1973).
22. K. Irie and R. F. Irie, Surface antigens of cultured human sarcomas detected by immune-adherence test. *Proc. Amer. Ass. Cancer Res.* **14,** 119 (1973).
23. R. W. Smith, An antigenic marker for human melanocytes, *Proc. Amer. Ass. Cancer Res.* **14,** 105 (1973).
24. M. P. Finkel, B. O. Biskis and C. Farrell, Osteosarcomas appearing in Syrian Hamsters after treatment with extracts of human osteosarcomas. *Proc. nat. Acad. Sci. (Wash.)* **60,** 1223 (1968).
25. D. Kufe, R. Hehlmann and S. Spiegelman, Human sarcomas contain RNA related to the RNA of a mouse leukemia virus. *Science* **175,** 182 (1972).
26. L. Dmochowski, H. G. Taylor, C. E. Grey, D. A. Dreyer, J. A. Sykes, P. L. Langford, T. Rogers, C. C. Shullenberger and C. D. Howe, Viruses and mycoplasma (PPLO) in human leukemia. *Cancer (Philad.)* **18,** 1345 (1965).
27. J. T. Grace, J. S. Horoszewicz, T. B. Stim, E. A. Mirand and C. James, Mycoplasma (PPLO) and human leukemia and lymphoma. *Cancer (Philad.)* **18,** 1369 (1965).
28. S. J. Millian and I. Spigland, Antibodies for mycoplasmas in normal individuals and patients with neoplasias. *Cancer (Philad.)* **19,** 1820 (1966).
29. J. Hentel and K. Hirschhorn, The origin of some bone marrow fibroblasts, *Blood* **38,** 81 (1971).
30. A. K. Maniatis, S. Amsel, W. J. Mitus and N. Coleman, Chromosome pattern of bone marrow fibroblasts in patients with chronic granulocytic leukaemia. *Nature (Lond.)* **222,** 1278 (1969).

Europ. J. Cancer Vol. 11. pp. 763–764. Pergamon Press 1975. Printed in Great Britain

Erratum

G. ATASSI, M. DUARTE-KARIM and H. J. TAGNON. Comparison of adriamycin with DNA–adriamycin complex in chemotherapy of experimental tumors and metastases. *Europ. J. Cancer*, **11**, 309–316 (1975).

Due to a publisher's error Table 4 was printed incorrectly. The correct Table appears below.

Table 4. Comparison of the effect of two different forms of DNA–adriamycin complex and adriamycin administered chronically on the life span of DBA/2 mice bearing L_{1210T} leukemia

Dose (mg/kg)	DNA–adriamycin T		DNA–adriamycin N		Adriamycin	
	ILS*	SD30†	ILS	SD30	ILS	SD30
8	157	3/15	56	2/10	43	0/15
6	200	2/15	50	0/10	128	1/15

DBA/2 male mice (18–20 g) were inoculated i.v. on Day 0 with L^{1210} leukemic cells from stock DBA/2 mice bearing ascite L_{1210}. Thirty leukemic mice were used as a control.
Treatment was given i.v. on days 1, 4, 8, 12 and 17 after tumor inoculation.
*Increase in Life Span in %.
†Number of survivors after 30 days/total number of mice.

Recent Journal Contents (1975)

International Journal of Cancer — *October, 1975*

D. Chassoux and J.-C. Salomon: Therapeutic effect of intratumoral injection of BCG and other substances in rats and mice.

A. L. Boyd and T. W. Orme: Transformation of mouse cells after infection with ultraviolet irradiation-inactivated herpes simplex virus type 2.

K. Nelson, S. B. Pollack and K. E. Hellström: Specific anti-tumor responses of cultured immune spleen cells. III. Further characterization of cells which synthesize factors with blocking and antiserum dependent cellular cytotoxic (ADC) activities.

R. Montesano, L. Saint Vincent, C. Drevon and L. Tomatis: Production of epithelial and mesenchymal tumours with rat liver cells transformed *in vitro*.

C. Merenda, B. Sordat, J. P. Mach and S. Carrel: Human endometrial carcinomas serially transplanted in nude mice and established in continuous cell lines.

J. Berkelhammer, M. J. Mastrangelo, J. F. Laucius, A. J. Bodurtha and R. T. Prehn: Sequential *in vitro* reactivity of lymphocytes from melanoma patients receiving immunotherapy compared with the reactivity of lymphocytes from healthy donors.

Y. Kodera and M. A. Bean: Antibody-dependent cell-mediated cytotoxicity for human monolayer target cells bearing blood group and transplantation antigens and for melanoma cells.

M. Zöller, M. R. Price and R. W. Baldwin: Cell-mediated cytotoxicity to chemically-induced rat tumours.

A. E. Bray, P. G. Holt, L. M. Roberts and D. Keast: Early onset of serum blocking in a murine melanoma model.

P. A. Jones, J. S. Rhim, H. Isaacs Jr. and R. M. McAllister: The relationship between tumorigenicity, growth in agar and fibrinolytic activity in a line of human osteosarcoma cells.

T. Odeka: Strain-dependent expression of endogenous mouse-tropic leukemia viruses in chemically induced leukemias of mice.

J. K. Youn, D. Le François, G. Hue, M. Santillana and G. Barski: Activation of 'eclipsed' lymphoid cells from advanced tumor-bearing mice through adoptive transfer to sublethally irradiated syngeneic hosts.

W. J. Halliday, A. E. Maluish, J. H. Little and N. C. Davis: Leucocyte adherence inhibition and specific immunoreactivity in malignant melanoma.

E. Huberman, H. Bartsch and L. Sachs: Mutation induction in Chinese hamster V79 cells by two vinyl chloride metabolites, chloroethylene oxide and 2-chloroacetaldehyde.

M. R. Osborne, M. H. Thompson, H. W. S. King and P. Brookes: Retention of tritium during the binding of tritiated benzo(a)pyrene to DNA.

I. Berczi and A. H. Sehon: Rejection of tumor cells *in vitro*: A T-cell mediated reaction.

D. Silber, E. Checińska, J. Rabczyński and M. Kochman: Isozyme pattern of fructose diphosphate aldolase during hepatocarcinogenesis induced by 2-acetylaminofluorene in rat liver.

G. Steele, Jr., H. O. Sjögren, O. Lannerstad and I. Stadenberg: A long-term ^{51}Chromium assay for *in vitro* cell-mediated tumor immunity. Correlation with simultaneously performed microplate assays using lymph-node cells from tumor-bearing rats and lymphocytes from human tumor patients on cultured solid tumor target cells.

British Journal of Cancer *October, 1975*

Richard Peto, F. J. C. Roe, P. N. Lee and L. Levy: Cancer and ageing in mice and men.

C. W. Welsch, G. Louks, D. Fox and C. Brooks: Enhancement by prolactin of carcinogen-induced mammary cancerigenesis in the male rat.

J. E. Enstrom: Colorectal cancer and consumption of beef and fat.

R. L. Tumsak and R. T. Cook: The persistence of unmetabolized ^{3}H-7, 12-dimethylbenz(a) anthracene in regenerating rat liver.

N. J. F. Dodd and J. Giron-Conland: Electron spin resonance study of changes during the development of a mouse myeloid leukaemia: II. The ascorbyl radical.

D. Crowther, M. E. J. Beard, C. J. T. Bateman and R. L. Sewell: Factors influencing prognosis in adults with acute mylogenous leukaemia.

M. Tuffrey, P. Crewe, L. Dawson, J. Holliday and R. D. Barnes: Murine leukaemia virus expression in the AKR following thymectomy.

T. M. Saba and T. G. Antikatzides: Humoral mediated macrophage response during tumor growth.

D. Cohen, I. Yron, M. Haber, E. Robinson and D. W. Weiss: Effect of distal and intralesional treatment with the MER fraction of tubercle bacilli on the survival of mice carrying mammary tumour isografts.

P. R. Twentyman and N. M. Bleehen: Studies of potentially lethal damage in EMT6 mouse tumour cells treated with bleomycin either *in vitro* or *in vivo*.

L. Ivarsson and C. M. Rudenstam: Heparin, dextran 1000 and metastasis formation after i.v. tumour cell injection in dextran non-sensitive rats.

T. Ipp, G. M. Macnab, E. W. Geddes and M. C. Kew: Serum immunoglobulin levels in primary liver cancer: relationship to underlying cirrhosis and hepatitis-B (surface) antigenaemia.

R. H. Whitehead and L. E. Hughes: Tissue culture studies of malignant effusions and their usefulness as a source of target cells in cytotoxicity tests.

Book Reviews

B. W. Fox: A review of cyclophosphamide.

L. G. Skinner: IARC monographs on the evaluation of carcinogenic risk of chemicals to man.

B. I. Lord: The cell cycle in malignancy and immunity.

C. W. Gilbert: Carcinogenesis and radiation risk: a biomathematical reconnaissance.

Europ. J. Cancer Vol. 11. pp. 763–764. Pergamon Press 1975. Printed in Great Britain

Erratum

G. ATASSI, M. DUARTE-KARIM and H. J. TAGNON. Comparison of adriamycin with DNA–adriamycin complex in chemotherapy of experimental tumors and metastases. *Europ. J. Cancer*, **11**, 309–316 (1975).

Due to a publisher's error Table 4 was printed incorrectly. The correct Table appears below.

Table 4. Comparison of the effect of two different forms of DNA–adriamycin complex and adriamycin administered chronically on the life span of DBA/2 mice bearing L_{1210T} leukemia

Dose (mg/kg)	DNA–adriamycin T		DNA–adriamycin N		Adriamycin	
	ILS*	SD30†	ILS	SD30	ILS	SD30
8	157	3/15	56	2/10	43	0/15
6	200	2/15	50	0/10	128	1/15

DBA/2 male mice (18–20 g) were inoculated i.v. on Day 0 with L^{1210} leukemic cells from stock DBA/2 mice bearing ascite L_{1210}. Thirty leukemic mice were used as a control.
Treatment was given i.v. on days 1, 4, 8, 12 and 17 after tumor inoculation.
*Increase in Life Span in %.
†Number of survivors after 30 days/total number of mice.

Recent Journal Contents (1975)

International Journal of Cancer — *October, 1975*

D. Chassoux and J.-C. Salomon: Therapeutic effect of intratumoral injection of BCG and other substances in rats and mice.

A. L. Boyd and T. W. Orme: Transformation of mouse cells after infection with ultraviolet irradiation-inactivated herpes simplex virus type 2.

K. Nelson, S. B. Pollack and K. E. Hellström: Specific anti-tumor responses of cultured immune spleen cells. III. Further characterization of cells which synthesize factors with blocking and antiserum dependent cellular cytotoxic (ADC) activities.

R. Montesano, L. Saint Vincent, C. Drevon and L. Tomatis: Production of epithelial and mesenchymal tumours with rat liver cells transformed *in vitro*.

C. Merenda, B. Sordat, J. P. Mach and S. Carrel: Human endometrial carcinomas serially transplanted in nude mice and established in continuous cell lines.

J. Berkelhammer, M. J. Mastrangelo, J. F. Laucius, A. J. Bodurtha and R. T. Prehn: Sequential *in vitro* reactivity of lymphocytes from melanoma patients receiving immunotherapy compared with the reactivity of lymphocytes from healthy donors.

Y. Kodera and M. A. Bean: Antibody-dependent cell-mediated cytotoxicity for human monolayer target cells bearing blood group and transplantation antigens and for melanoma cells.

M. Zöller, M. R. Price and R. W. Baldwin: Cell-mediated cytotoxicity to chemically-induced rat tumours.

Europ. J. Cancer Vol. 11, pp. 765–770. Pergamon Press 1975. Printed in Great Britain

Solubilization of an Antigen Associated with Certain Bronchial Tumours*

D. VIZA,†‡ M. LOUVIER,† J. PHILLIPS,†§ Cl. BOUCHEIX,¶ and R. A. GUERIN¶

†*Laboratoire d'Immunobiologie, Faculté de Médecine Pitié-Salpêtrière,* 105 *boulevard de l'Hôpital,* 75634 *Paris Cedex* 13 and

¶*Centre de Traitement des Tumeurs, Hôpital de la Salpêtrière,* 87 *boulevard de l'Hôpital,* 75634 *Paris Cedex* 13

Abstract—*Epidermoid and anaplasic lung tumours have been solubilized and the soluble extracts used to raise antisera in rabbits and also in a goat. After suitable absorption, the antisera were found to be reactive in cross-over electrophoresis only with some lung tumour extracts and sera from several patients with epidermoid and anaplasic lung tumours. No reactivity was observed with sera from patients having other malignancies, including patients with lung metastases of various primary tumours, or to sera trom patients with benign lung disease. It was therefore concluded that at least one sumour associated antigen, called B*1*, was present in some lung tumours and in patients' fera.*

INTRODUCTION

FEW STUDIES have been devoted to specific neoantigens of bronchial tumours. The detection of viral antigens in alveolar tumours has been reported [1] as well as delayed hypersensitivity to soluble membrane extracts from malignant lung cells in patients with lung carcinomas [2]. However, proof of the existence of specific tumour associated antigens has not as yet been established and all data pertaining to such antigens are important for future biochemical identification and biological understanding of tumour specificities.

Using methods of extraction and solubilization already employed for the partial purification of leukaemia [3, 4] and malignant melanoma [5, 6] antigens, we undertook the characterization of antigens from bronchial tumours. The soluble product, after partial purification by column chromatography, was used for the production of antisera by immunization of rabbits and goats. After absorption, these antisera were shown active when tested in cross-over electrophoresis against extracts of lung tumours and also against the sera of certain patients with bronchial cancer, in contrast with an absence of reactivity against sera from patients with other types of cancers and with sera of non-cancerous individuals. It was thus concluded that lung tumours present specific antigens on their cell surfaces and that these can be detected in the serum of certain patients.

These antigens may be interesting for a number of reasons. The detection of antigen in patients' sera could have a considerable importance for diagnosis, immunological classification, and perhaps even the immunological treatment of patients, as it has been suggested in the case of leukaemias [4, 7]. Furthermore, it offers a rationale for active specific immunotherapy using tumour cells previously selected, and may also open new perspectives for immunotherapy, passive or adoptive, the latter

Accepted 20 June 1975.

*This work was supported by grants from the INSERM (ATP 11.74.32), the FRMF, the GEFLUC, the Lady Tata Foundation, the ARBE, the Eli Lilly and Roche Laboratories.

‡Research investigator with the INSERM.

§Fellow of the Lady Tata Memorial Foundation.

by use of transfer factor [8] or immune RNA [9].

MATERIAL AND METHODS

Bronchial tumours were taken at the time of total or partial pneumectomy and separated as much as was possible from the surrounding bronchial mesenchyme. All tissues were stored at −20°C prior to extraction, which was carried out according to techniques described elsewhere [10]. Very briefly, after gentle homogenization followed by three washes in slightly hypotonic (0·8%) saline, the cells were removed by centrifugation at 60,000 *g* for 90 min whilst the supernatant was ultracentrifuged to recover the membrane fragments. These, resuspended in distilled water constitute what has been called by Davies a "crude eluate", which is a particulate antigenic preparation needing to be submitted to enzymatic papain digestion in order to obtain solubilization. After papain digestion, the insoluble residues were removed by further ultracentrifugation at 90,000 *g* for 180 min, while the product thus solubilized was purified by Sephadex G-75 and G-200 column chromatography. An example of the elution profiles is shown in Fig. 1.

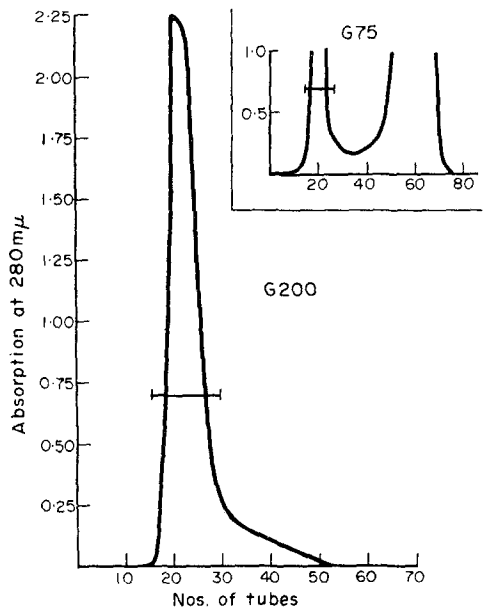


Fig. 1. Elution profiles (280 mμ) of soluble antigen LEM. The pool of the first peak obtained from filtration on Sephadex G-75 (inset) was subsequently passed on Sephadex G-200. The contents of tubes 16-30 were dialysed against double distilled water and freeze-dried to produce the final product.

Soluble material was injected into rabbits and goats by both intravenous and subcutaneous routes, the latter with Freund's complete adjuvent, at bi-weekly intervals using 1 mg of soluble material per injection. The antisera collected 15 days after the 5th injection were absorbed with a pool of normal human plasma (NHP) polymerized with gluteraldehyde [11]. Antisera were absorbed twice against two different pools made of 4–5 NHP and thereafter tested against a panel of NHP pools. Absorption was continued until no reactivity against NHP was observed. Three antisera were thus made; 2 against the same individual soluble extract (LEM) in rabbits (EC, ED) and goat (GAL), and 1 against a tumour eluate (αW).

Immune complexes were prepared for subsequent immunizations as follows: a patient's serum which reacted with an absorbed goat antiserum was depleted of its non-tumour antigens by triple absorption with insolubilized anti-NHP-serum. This serum was then complexed with the insolubilized anti-lung xeno-antiserum. Soluble antigen at 1 mg/ml was similarly treated for the preparation of complexes. All complexes were constituted volume to volume, i.e. 1 ml of insolubilized antiserum for 1 ml of antigen or patient's serum. They were washed three times in PBS solution in order to eliminate unbound material. The antigen insoluble complex was used for rabbit immunization at the equivalent of 0·1 mg bound soluble extract per injection representing 1/10 of the amount of soluble antigen previously used alone. Similarly, the equivalent of 0·1 ml of patient's serum was used in the insoluble antigen/antibody complex form for injection of rabbits. Rabbit antisera were collected 15 days after the first immunization.

The antisera were tested after absorption by double diffusion (DD) and cross-over electrophoresis (COE) [12] in agar gel against soluble spleen extracts and also extracts of leukaemic or melanoma cells. Cross-over electrophoresis was used for testing patients' sera. Soluble extracts were used at 8 mg/ml. Both soluble extracts and patients' sera were used at 20λ and 11λ per test for DD and COE respectively.

RESULTS

The absorbed antisera failed to react with soluble spleen extracts from non-cancer patients, and leukaemic and melanoma cell extracts. However, when the same antisera were tested against soluble extracts and eluates of lung tumours, a reaction was observed in certain cases (Table 1). The antigen associated with certain bronchial tumour extracts responsible for this reaction has been called "antigenic specificity B1".

Table 1. *Reaction of antisera GAL, EC and ED with tumour eluates and soluble extracts. LEM is the eluate from which the soluble material used for the preparation of GAL, EC and ED antisera was obtained*

Lung tumour				
Code			Histology	Reaction
LEM		(soluble)	Epidermoid	+
TAL	3	(eluate)	Not available	−
TAL	4	(eluate)	Epidermoid	−
TAL	5	(eluate)	Not available	−
TAL	6	(eluate)	Epidermoid	+
TAL	7	(eluate)	Lung metastases of nasopharyngeal cancer	−
TAL	8	(eluate)	Tumour pool (3 Epidermoid tumours)	+
TAL	16	(soluble)	Epidermoid	+
TAL	21	(eluate)	Epidermoid	+
TAL	23	(eluate)	Epidermoid	−

It is interesting to note that the absorbed goat antiserum GAL, prepared by immunization with a soluble extract of an epidermoid lung tumour reacted against this tumour extract, but no reactivity was observed against the soluble extract of the apparently healthy lung tissue mesenchyme of the same patient which was removed at partial pneumectomy.

The antisera were tested in COE against sera from patients with bronchial tumours, leukaemia, or tumours of other histological origins. From 234 such sera tested, 70 were from lung cancer patients. Only 12 sera from the latter group were found positive (Table 2). These reactions were mostly minimal with a few exceptions showing stronger reactivity (Fig. 2). Sera from patients with non-cancerous diseases of the lung (bronchitis and tuberculosis) and also sera from patients with epidermoid tumours of the buccal cavity were found negative.

The presence of oncofoetal specificities in bronchial tumours was investigated. The antiserum GAL was tested against soluble embryonic extracts from whole embryos of 3–6 months of gestation, whilst lung tumour extracts and patients' sera were also tested against antisera prepared against soluble embryonic extracts. These antiembryonic antisera were absorbed with solubilized spleen extracts and freeze-dried NHP. In both cases, no reaction was observed, neither in DD nor in COE.

Antisera obtained by injection of immune complexes of insolubilized antiserum and serum antigen or soluble membrane extract were proven reactive against the patient's serum carrying the circulating antigen B1. Absorptions were nevertheless necessary as the antiserum obtained was oligospecific, but not monospecific. Preliminary data show that if the oligospecific antiserum thus obtained is used to produce a new complex for immunization and this procedure is repeated, the fourth antiserum is apparently monospecific, as assessed by the COE technique.

DISCUSSION

The solubilization of surface material from spleen cells, leukaemic cells and melanoma cells has allowed us to recover transplantation antigens (HL-A) [13], leukaemic associated antigens (LAA) [3–4], a leukaemo-embryonic antigen [14], and melanoma associated antigens [5–6]. The procedure followed here for the extraction of lung tumour associated antigens was similar. However, the G200 separation did not produce the usual profile of excluded and included fractions [13]; practically all the material was found in the excluded fraction containing the high molecular weight molecules (HMW). This HMW fraction was therefore used since it was already known that it also contains the tumour specificities in the case of leukaemia.

The use of insoluble complexes for immunization presents a triple advantage for these experiments: (a) obtaining a complex which carries only a few, if any, antigens apart from the tumour one, thus producing an oligospecific antiserum; (b) obtaining an antiserum after the first or second injection of the complex; (c) the amount of antigen necessary for these immunizations is less than 1/25th of the amount of antigen usually required, an appreci-

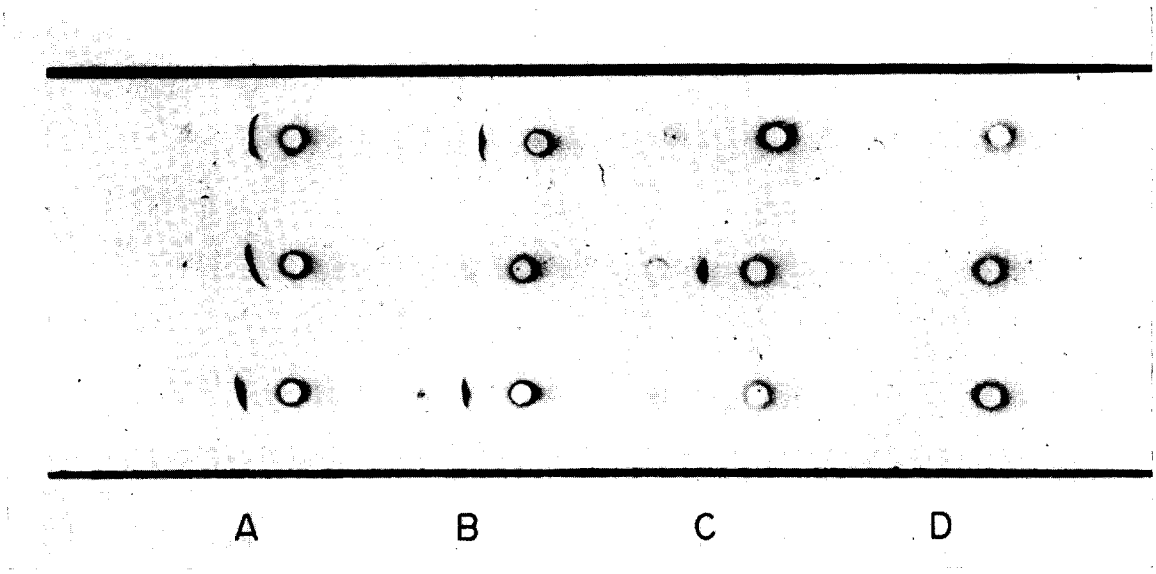


Fig. 2. Example of patterns of reactions obtained in COE with antilung antiserum and patients' sera. Rows A and B present typical "strong" reactions, whereas Row D (top) and Row B (middle) show "minimal" reactions which are not very clear on the photograph.

(to face p. 768)

Table 2. Reactions of antiserum GAL with sera from lung tumour patients, other cancer patients and healthy volunteers when tested in cross-over electrophoresis. It is worth noting that LEM patient's serum was among the six positive sera from patients with epidermoid tumours

Antiserum 3 GAL (goat anti lung serum) and antisera EC and ED (rabbit anti lung sera)

Diagnosis	Number of sera tested	Number positive
1. Lung cancer		
(A) Epidermoid	42	6
(B) Anaplasic		
(1) Small cells	10	2
(2) Large cells	5	0
(C) Others		
(1) Mucoid	2	1
(2) Cylindroma	1	0
(3) Spinocellular	1	0
(4) Mesothelioma	1	1
(5) Adenocarcinoma (apparently primary)	1	1
(D) Unknown	7	1
Total:	70	12
2. Other cancers		
Adenocarcinoma		
Lung metastases, primary unknown	1	0
Lung metastases of breast cancer	4	0
Lung metastases of colon cancer	1	0
Lung metastases of ovary cancer	1	0
Lung metastases of pancreas cancer	1	0
Brain	10	0
Breast	5	0
Colon	1	0
Digestive tract	4	0
Epidermoid carcinomas of the buccal cavity	12	0
Glandular carcinoma	1	0
Glioblastoma	2	0
Kidney	4	0
Leukaemia	49	0
Lymphoma	3	0
Lymphosarcoma	2	0
Melanoma	19	0
Ovary	2	0
Pancreas	3	0
Reticulum cell sarcoma	3	0
Uterus	2	0
Total:	130	0
3. Non-cancerous		
Tuberculosis	11	0
Bronchitis	6	0
Healthy volunteers	23	0
Human plasma pools	6	0
Total:	46	0

able economy considering the scarcity of soluble product.

The antigenic specificity B1 associated with certain bronchial tumours, as detected by xenoantisera, was found in lung cancer patients' sera but not in sera of patients having other types of malignancy which argues in favour of its specific association with bronchial cancer. This is consistent with the findings of Mohr *et al.* [15]. However, the percentage of patients thus detected (12 out of 70, Table 2) is low (17%). Several hypotheses could be put forward to explain this, above all the relative insensitivity of the tests used. The existence of an antigenic polymorphism, and correspondingly the existence of more than one antigenic variety in bronchial cancer, the B1 we are describing being one among several which are mutually exclusive, might be one reason; conversely, the presence of antigens which are masked in certain tumours, or a low differential shedding of the tumour specificities of others are further plausible explanations, not mutually exclusive, of the failure to detect circulating antigens in a larger percentage of patients.

There was no correlation between the histological types of the tumour and the presence of the B1 specificity in the patient's serum. Epidermoid and anaplasic tumours both reacted with equal frequency against the antiserum. However, the 12 patients with epidermoid tumours of the buccal cavity tested were all negative, thus suggesting that B1 antigen is strictly associated with bronchial carcinoma. Other histological types viz. alveolar cell carcinoma were not available for testing at the time of preparation of this report.

Recent observations have reported positive skin tests in patients with bronchial cancers, using partially purified soluble tumour extracts, thus suggesting the existence of specific bronchial tumour antigens [2]. The absence of reactivity in some of the tumour extracts used in these tests is consistent with our findings and with our hypothesis of antigenic masking and/or the existence of several specificities. Others [16] applying similar techniques to bronchial carcinoma tissues have reported results corroborating the observations reported here, pleading for the existence of an antigenic polymorphism.

It should be noted that the specificity B1 is distinct from the carcino-embryonic antigen (CEA) [17], as well as the leukaemo-embryonic [14], and the melanoma [5, 6] specificities we have described. The lack of reactivity of specific antisera corresponding to these antigens, when they were tested against the lung tumour extracts, and the absence of reactivity of the anti-B1 antisera when tested against the leukaemia, melanoma, and embryonic extracts rules out the possibility of cross-reactivity.

The circulating tumour antigen offers obvious applications for differential diagnosis as well as for the preparation of specific antisera by immunization with patients' sera, previously absorbed by anti-NHP antisera insolubilized with glutaraldehyde. This could constitute another way of exploring the presence of unknown antigenic specificities in unreactive patients' sera.

Specific tumour antigens will certainly play a considerable role in the diagnosis and treatment of the cancer disease and perhaps the understanding of its pathogenesis. The knowledge of the antigenic determinants of these tumours could, for example, open the way to a rational specific active immunotherapy. In effect, when a specific active immunotherapy in bronchial tumours is envisaged, the specificity described here should be taken into consideration and the patient who is found positive with the previous serodiagnosis for B1 antigen should be immunized with an antigenic preparation containing this specificity.

Acknowledgement—We would like to thank Professor Toty for providing us with tumour material. The excellent technical assistance of Mrs. Avril Collard is gratefully acknowledged.

REFERENCES

1. R. E. Nordquist, Specific antigens in human alveolar cell carcinoma. *Cancer Res.* **33,** 1790 (1973).
2. A. Hollinshead, T. H. M. Stewart and R. B. Herberman, Delayed-hypersensitivity reactions to soluble membrane antigens of human malignant lung cells. *J. nat. Can. Inst.* **52,** 327 (1974).
3. D. Viza, D. A. L. Davies and R. Harris, Solubilisation and partial purification of human leukaemic specific antigens. *Nature (Lond.)* **227,** 1249 (1970).
4. R. Harris, D. Viza, R. Todd, J. Phillips, R. Sugar, R. F. Jennison, Greta Marriot and M. H. Gleeson, The detection of human leukaemia associated antigens in leukaemic serum and normal embryos. *Nature (Lond.)* **233,** 556 (1971).

5. D. Viza and J. Phillips, Extraction and solubilization of cell surface antigens from malignant melanomas. *Rev. Inst. Pasteur (Lyon)* **4,** 339 (1971).
6. D. Viza and J. Phillips, Identification of an antigen associated with melanoma. *Int. J. Cancer,* **16,** 312 (1975).
7. D. Viza, Immunological aspects of human leukaemia. *Ser. Haematol.* **5,** 87 (1972).
8. D. Viza, J.-M. Goust, R. Moulias, L. K. Trejdosiewicz, Avril Collard and Nicole Müller-Bérat, *In vitro* production of transfer factor by lymphoblastoid cell lines. *Transplant. Proc. VII,* suppl. 1, 329 (1975).
9. Y. H. Pilch and K. P. Ramming, Transfer of tumour immunity with Ribonucleic acid. *Cancer (Philad.)* **26,** 630 (1970).
10. D. A. L. Davies, Soluble mouse H-2 isoantigens. *Transplantation* **5,** 31 (1967).
11. S. Avrameas and T. Ternynck, The cross linking of proteins with glutaraldehyde and its use for the preparation of immunoabsorbents. *Immunochemistry* **6,** 53 (1969).
12. B. J. Culliford, Precipitin reactions in forensic problems. *Nature (Lond.)* **201,** 1092 (1964).
13. D. A. L. Davies, A. J. Manstone, D. Viza J. Colombani and J. Dausset, Human transplantation antigens: the HL-A (HU-1) system and its homology with the mouse H-2 system. *Transplantation* **6,** 571 (1968).
14. D. Viza. Human leukemic and leukemoembryonic antigens. In *Embryonic and fetal antigens in cancer.* Editors N. J. Anderson and J. H. Coggin, Jr. Springfield, Va.: USAEC Report Conf-710527, 1971, Vol. 1, pp. 369-377.
15. J. A. Mohr, R. E. Nordquist, R. R. Everett, R. E. Coalson and J. J. Coalson, Alveolar cell carcinoma-like antigen and antibodies in patients with alveolar cell carcinoma and other cancers. *Cancer Res.* **34,** 1904 (1974).
16. P. Lo Gerfo, F. P. Herter, J. Braun and H. J. Hansen, Tumour associated antigen with pulmonary neoplasms. *Ann. Surg.* **175,** 495 (1972).
17. P. Gold and S. O. Freeman, Specific carcinoembryonic antigens of the human digestive system. *J. exp. Med.* **88,** 142 (1965).

Europ. J. Cancer Vol. 11, pp. 771–782. Pergamon Press 1975. Printed in Great Britain

Methotrexate Inhibition of CCRF-CEM Cultures of Human Lymphoblasts*

DEWAYNE ROBERTS and ELLEN V. WARMATH
Laboratories of Pharmacology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101, USA

Abstract—*The biochemical basis for methotrexate (MTX) inhibition of human lymphoblasts was examined with the CCRF-CEM cell line. Methotrexate inhibition of growth by cultures of this human lymphoblast cell line was prevented by a combination of hypoxanthine, thymidine and deoxycytidine and was reversed competitively by folinic acid. Within 5 min MTX inhibited the incorporation of isotopically-labeled deoxyuridine, hypoxanthine and glycine into DNA, although thymidine incorporation continued normally for more than 90 min before becoming inhibited. In media supplemented with thymidine plus deoxycytidine, 2- to 5-fold more MTX was required for 50% inhibition of growth than in regular media. In media supplemented with hypoxanthine, or thymidine, deoxycytidine and 5-fluorodeoxyuridine, folinic acid competitively reversed MTX inhibition. At 0·02 and 0·01 μM MTX, inhibition of dihydrofolate reductase (DHFR) required 6–16 hr to develop, and deoxyuridine incorporation into DNA was not inhibited. In media containing dialyzed serum 0·08 μM MTX inhibited DHFR within 60 min and deoxyuridine incorporation within 85 min. After preincubation with 0·08 μM MTX for 3 hr, cells recovered traces of DHFR in 20 min. Simultaneously, deoxyuridine incorporation into DNA started to recover and attained control rate in 60 min with less than 10% recovery of DHFR activity. These data indicate: (a) CCRF-CEM cells have 10- to 20-fold more DHFR than required for cell division. (b) Growth inhibition of cultures resulted from thymidine deficiency. (c) MTX did not selectively inhibit either thymidine or purine biosynthesis. Consideration of these factors suggests that MTX inhibition of cell division resulted from the inhibition of DHFR.*

INTRODUCTION

MTX is an analog of folic acid and an inhibitor of cell division. Folate and tetrahydrofolate derivatives reverse the inhibition of microbial cultures and cell cultures by MTX, block the drug's intoxication of rodents and humans, and interfere with the drug's oncolytic activity [1–4]. Growth inhibition of cell cultures by MTX is reversed by products of tetrahydrofolate-dependent reactions. Thymidine and a purine for nucleic acid synthesis and, with some cell cultures, glycine for protein synthesis, are required to reverse MTX inhibition [5, 6]. A human lymphoblast cell line, CCRF-CEM, also requires deoxycytidine to maintain growth in the presence of thymidine [7].

Although the oncolytic activity of MTX is generally attributed to an inhibition of DHFR, at higher drug concentrations, thymidylate synthetase and a number of other enzymes are inhibited also [1, 8–11]. DHFR catalyzes the reduction of folate or dihydrofolate to tetrahydrofolate, and inhibition of DHFR by MTX blocks the synthesis of tetrahydrofolate, which is required for synthesis of precursors of DNA, RNA and protein. Interest in secondary sites of inhibition by MTX has been stimulated by the general lack of correlation between the drug's oncolytic activity and the inhibition of DHFR [12].

Condit and Mead observed that folinic acid accumulated in the liver after folate was administered to mice. This accumulation was prevented by MTX [13], however, the drug did

Accepted 26 May 1975.

*Supported by Research Grants CA-11148, CA-12732, and CA-08480 from the National Cancer Institute, NIH, and by ALSAC.

not block conversion of dihydrofolate to folinic acid. These studies were recently confirmed and extended with new methodology [14]. Prolongation by MTX of the lives of tumor-inoculated mice is correlated with greater MTX uptake by ascites tumor cells but not with the level of DHFR activity in these cells [15, 16]. With DHFR activity inhibited in mouse ascites tumors by MTX administration, only partial inhibition of deoxyuridine incorporation into DNA is observed *in vitro* with tumor cells [17]. The further addition of MTX to these murine ascites tumor cells increased the inhibition of deoxyuridine incorporation by a sensitive cell line, L1210, and an insensitive tumor line, P329, but not of a MTX-resistant variant of L1210. MTX inhibition of DHFR from leukemia cells is observed for both MTX-sensitive and insensitive human leukemias [12].

The early studies of human leukemia by Winzler *et al.* indicated that low concentrations of MTX more effectively inhibit formate incorporation into thymine of DNA than into purines of RNA. Therefore, a differential sensitivity of various one-carbon pathways to MTX has been considered [18]. A series of studies relates MTX action to an inhibition of thymidylate synthetase [1], to an elevation of thymidylate synthetase activity and recovery from drug action [19, 20], or to an elevation of thymidylate synthetase activity with resistance [21, 22]. However, a pharmacologically important relation between DHFR and MTX is indicated by: (a) the stoichiometric inhibition of DHFR at pH 6·1 and the very significant, although slightly less effective, inhibition of the enzyme at higher pH [23–25]; (b) *in vivo* titration of enzyme activity to which drug retention is attributed [25]; and (c) the correlation of MTX resistance with elevation of DHFR activity in association with more rapid recovery of enzyme activity after MTX inhibition [2, 26, 27].

Discrepancies appear to exist between the inhibition of DHFR by MTX and the drug's oncolytic effectiveness [19]. Although acute lymphocytic leukemia is more responsive to MTX than acute myelogenous leukemia, DHFR activity of lymphoblasts and myeloblasts was inhibited equally by MTX [28]. After treatment with MTX, the recovery of DHFR activity in leukocytes from patients with leukemia was not correlated with the sensitivity of their disease category to the drug [12, 28, 29]. However, a more pronounced inhibition of deoxyuridine incorporation was observed after MTX administration to patients with acute lymphocytic leukemia [12].

The studies that follow with a human lymphoblast cell line examine the biochemical basis for discrepancies between inhibition of DHFR, DNA synthesis and cell division by MTX.

MATERIAL AND METHODS

Materials

Thymidine-(^{3}H-methyl), deoxyuridine-6-^{3}H, hypoxanthine-8-^{3}H, and glycine-1-^{14}C were obtained from Schwarz-Mann, Orange, N.J. Folic-G-^{3}H acid was obtained from Amersham/Searle Corp., Arlington Heights, Ill., and was purified by chromatography on Whatman No. 1 paper with 0·01 M sodium phosphate buffer, pH 7·0. Eagle's minimal essential medium with Earle's salts and L-glutamine for spinner cultures was obtained from Grand Island Biological Company, Grand Island, N.Y. Fetal calf serum was purchased from Grand Island Biological Company, or from Flow Laboratories, Inc., Rockville, Md. Folinic acid, calcium leucovorin for injection, was a product of Lederle Laboratories. MTX was a gift of the Lederle Laboratories, and a single batch was used for these studies. Nonlabeled thymidine, deoxyuridine, hypoxanthine and glycine were obtained from Calbiochem, La Jolla, California.

Cell line and culture conditions

The CCRF-CEM cell line in these studies was derived from the blood of a patient with acute leukemia that developed from lymphosarcoma [30]. This lymphoblast line was maintained in Eagle's minimal essential medium with Earle's salt solution lacking $CaCl_2$ and supplemented with 10 times the phosphates, with L-glutamine and with 10% fetal calf serum. The medium contained methionine and choline as potential sources of one-carbon units as well as folic acid. Presumably, glycine as well as low concentrations of purines, thymidine and reduced folates were present in the serum complement. The culture cannot be maintained indefinitely with media containing dialyzed fetal calf serum. In the presence of MTX, the cultures would grow normally if the medium was supplemented with 100 μM hypoxanthine, 10 μM thymidine, and 100 μM deoxycytidine. Deoxycytidine prevented the initial inhibition of growth by thymidine which would otherwise have delayed growth of cultures by approximately 18 hr. The cells grew unattached to the flask, or in suspension as spin cultures.

Isotopic studies

Incorporation of thymidine-(^{3}H-methyl) and deoxyuridine-6-^{3}H into DNA was assayed by collecting the cells on a glass fiber filter and repeatedly washing them with cold 0·9%NaCl solution followed by cold 5% trichloroacetic acid. The damp filter was dropped into a counting vial containing 10 ml of liquid scintillation medium. After standing for approximately 20 min, the contents were vigorously mixed and counted by liquid scintillation technique.

Hypoxanthine-8-^{3}H was primarily incorporated into RNA. A 2-hr hydrolysis at 37° in 1 N NaOH was adequate to remove the RNA when the remaining TCA-insoluble material was collected on a glass filter. After repeated washing with 0·9% NaCl solution and 5% TCA, the precipitate with DNA was assayed by liquid scintillation technique to measure incorporation into DNA.

Assay of glycine-1-^{14}C incorporation into adenine of the nucleic acids required separation of the RNA and DNA followed by separation of the purine and pyrimidine bases by two-dimensional paper chromatography. The cells from a 100-ml culture were collected by centrifugation at 500 ***g*** for 5 min, and the cell pellet was washed three times with 40 ml 0·9% NaCl solution and five times with 5 ml of 5% TCA. The precipitate was resuspended in 1 ml of 1 N KOH and incubated for 2 hr at 37°. After the suspension was chilled in an ice bath, 1 ml of 1 N HCl-15% TCA was added, and the precipitate, which contained DNA, was collected by centrifugation for 5 min at 500 ***g***.

The ribotides from RNA in the supernatant fluid were hydrolyzed to release the purines. One-half ml of 10% $HClO_4$ was added to the supernatant fluid, and the precipitate of $KClO_4$ was packed by centrifugation. The supernatant fluid was removed, and 260 μl of 12 N HCl was added to the fluid. The purine nucleotides were hydrolyzed by heating at 100° for 1 hr to release the bases. Adenine was isolated by the same technique used for DNA.

The precipitate with DNA was washed three times with 5 ml of 5% TCA, then resuspended in 0·35 ml of 1 N HCl and heated for 1 hr in a boiling water bath. The remaining insoluble material was collected by centrifugation and discarded. The supernatant fluid was evaporated to dryness at 60–65°. The residue was dissolved in water and placed along a 1-cm front on Whatman No. 1 paper and chromatographed, descending, with saturated aqueous butanol plus 0·01 volume of 15 N NH_4OH [31]. The adenine was located by co-chromatography with a standard in another region of the chromatogram. After the strip with adenine was cut from the original chromatogram, the adenine was rechromatographed in a second dimension with isopropanol–HCl–water [31] The adenine was eluted with water from the second chromatogram, and the eluate was evaporated to dryness. The adenine was redissolved in water and identified by the ratio of absorbancy at 260 and 280 mμ, after correction for background absorbancy with a corresponding region from a blank chromatogram. Radioactivity was measured by liquid scintillation technique.

Assay of DHFR

The isotopic assay with folate-^{3}H was used to assay DHFR [32]. The cells from 40-ml cultures were collected by centrifugation for 5 min at 500 ***g***. The cell pellets were resuspended with 20 ml of 0·9% NaCl and recollected by centrifugation, after which the cells were washed with 10 or 20 ml and then with 3 ml of 0·9% NaCl solution. The final cell pellet was resuspended in 0·2 or 0·5 ml of 0·01 M Tris, pH 7·5, and then disrupted by sonic oscillation. An aliquot of homogenate was removed for assay of protein [33], and the remainder was centrifuged for 25 min at 20,000 ***g***. The supernatant fluid was frozen at −20° until thawed for assay of DHFR activity.

RESULTS

Recovery of CCRF-CEM cultures from MTX inhibition

CCRF-CEM cultures were tested for loss of viability after 4, 8, 12 and 24 hr of inhibition by 10 μM MTX. Viability was assayed by the increase in cell number after the medium was supplemented with 100 μM hypoxanthine, 10 μM thymidine, and 100 μM deoxycytidine (Fig. 1). When these metabolites were added to the media, cultures rapidly resumed cell division after exposure to MTX for 4, 8 or 12 hr. After 24 hr of exposure to MTX, a delay followed the addition of metabolites before growth resumed. These results indicate that after prolonged exposure to MTX a large proportion of the cells in CCRF-CEM cultures were capable of cell division when the medium was supplemented with thymidine, hypoxanthine and deoxycytidine.

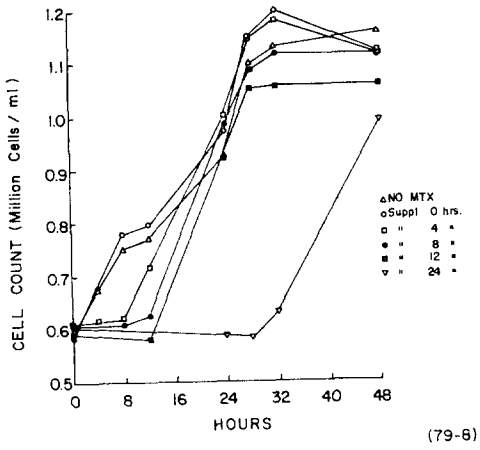


Fig. 1. Reversal of inhibition of CEM cultures by MTX. Spin cultures were initiated with 0·60 million CEM cells/ml in medium supplemented with 10 μM MTX. At 0, 4, 8, 12 or 24 hr, 100 μM hypoxanthine, 100 μM deoxycytidine, and 10 μM thymidine were added to one culture.

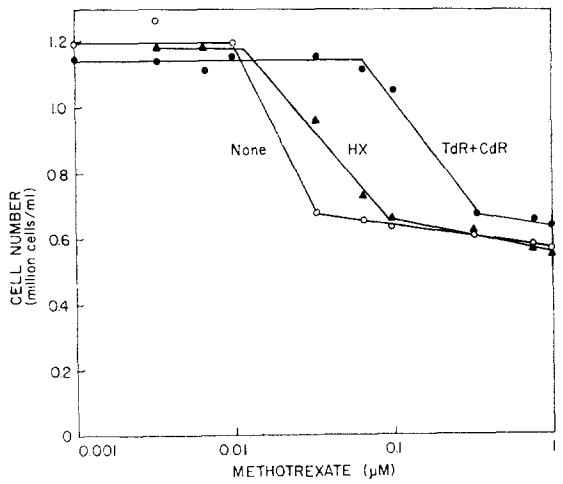


Fig. 2. Inhibition by MTX of CEM cultures supplemented with hypoxanthine or with thymidine and deoxycytidine. Three groups of cultures were inoculated with 0.60 million CEM cells/ml. Duplicate cultures in each group received from 0·001 μM to 1 μM MTX. One group of cultures was supplemented with 100 μM deoxycytidine and 10 μM thymidine (TdR+CdR). The second group was supplemented with 10 μM hypoxanthine (HX). Another group was diluted with water. Growth was measured at 19 hr and at 43 hr. The 43-hr growth is presented to show that growth was not prevented by an initial lag.

Methotrexate sensitivity of CCRF-CEM cultures supplemented with hypoxanthine or thymidine

By adding (a) MTX and hypoxanthine or (b) MTX, thymidine and deoxycytidine to media, the drug's specific effect relative to the missing metabolite, thymidine or hypoxanthine respectively, was monitored by changes in growth rate of cultures as the drug concentration varied. Hypoxanthine supplementation permitted the study of MTX effect on thymidine metabolism. Conversely, when the medium was supplemented with thymidine plus deoxycytidine, MTX interference with purine metabolism could be determined.

Other investigators have observed a decreased sensitivity to MTX when the medium was supplemented with either thymidine or a purine. In these earlier studies, supplementation with purine reduced MTX inhibition more effectively than supplementation with thymidine [2]. The CCRF-CEM cell culture was less sensitive to MTX when the medium was supplemented with thymidine plus deoxycytidine than with hypoxanthine (Fig. 2). Twice as much MTX was required for 50% reduction of growth when hypoxanthine was added to the medium. With

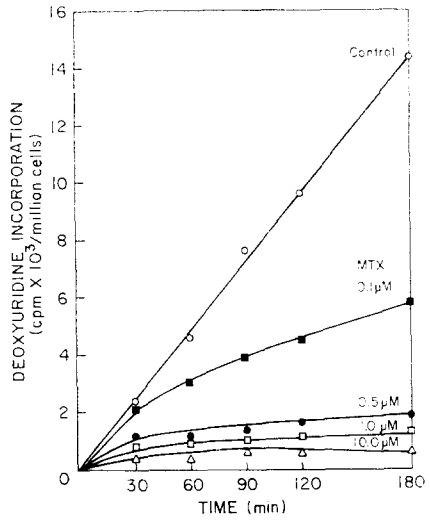


Fig. 3. Inhibition by MTX of 6-H³-deoxyuridine incorporation into DNA. Deoxyuridine, 1·13 μCi (21·8 Ci/mmole), was added at time 0 to 2-ml aliquots of a CEM culture. The cells with deoxyuridine were incubated at 37° for various periods of time. These aliquots were supplemented with 0·1 to 10 μM MTX or with water. At termination of incubation, 2 ml of cold 0·9% NaCl was added, and the cells immediately flushed onto a glass fiber filter where they were washed with cold 0·9% NaCl followed by cold 5% trichloroacetic acid before counting by liquid scintillation.

thymidine plus deoxycytidine supplementation, approximately 8-fold more MTX was required for 50% inhibition of growth than in unsupplemented medium. This study was repeated three times, and although the quantitative relation varied in the studies, a similar qualitative relation was observed between hypoxanthine, thymidine and MTX. In contrast to other studies, hypoxanthine supplementation did not enhance MTX toxicity of CCRF-CEM cultures. These results indicate that growth of CCRF-CEM cultures was less sensitive to purine deficiency than to thymidine deficiency.

Sensitivity of purine and thymidine biosynthesis de novo *to MTX*

Purine biosynthesis was measured by glycine incorporation into adenine of RNA or DNA. This incorporation requires N^5, N^{10}-methenyltetrahydrofolate and N^{10}-formyltetrahydrofolate for biosynthesis of purine nucleotides [9]. Deoxyuridine incorporation into DNA requires N^5, N^{10}-methylenetetrahydrofolate for the conversion of deoxyuridine to thymidine 5′-monophosphate prior to incorporation into DNA [9].

In less than 30 min, 0·5 to 10 μM MTX completely inhibited deoxyuridine incorporation into DNA (Fig. 3). Extrapolation from the drug inhibited incorporation to the control rate indicates that the tetrahydrofolate cofactor pool was probably depleted in 5–15 min. Three hr after the addition of 0·1 μM MTX, deoxyuridine incorporation was incompletely inhibited.

Glycine incorporation into adenine of DNA appeared as sensitive to MTX inhibition as deoxyuridine incorporation, but because of the low specific activity of 1-^{14}C-glycine, incorporation was not measured earlier than 1 hr after its simultaneous administration with 10 μM thymidine, 100 μM deoxycytidine, and 0·05 to 0·5 μM MTX to cultures. Equivalent inhibition of glycine incorporation into both RNA and DNA was observed as expected if the drug were blocking *de novo* synthesis of purines (Table 1). By comparison with glycine incorporation in the controls, minus MTX, the pronounced inhibition of glycine incorporation, approximately 85% during the 1-hr incubation with 0·5 μM MTX, is compatible with a depletion of the tetrahydrofolate pool within 5 to 10 min after drug addition. Comparison of the effect of MTX on deoxyuridine and on glycine incorporation suggests that within the limits of experimental error, thymidine and purine synthesis were equally sensitive to the drug. If the synthesis of DNA continues beyond 5–15 min, then thymidine or purine pools must be adequate to compensate for the block of *de novo* synthesis.

Table 1. *Methotrexate inhibition of* 1-^{14}*C-glycine incorporation into adenine of nucleic acid*

Methotrexate (μM)	RNA	DNA
Expt I		
0	122.4 121.4	36.8 30.5
0.02	148.8 132.1	53.5 44.2
0.05	61.7	32.2 21.5
0.10	49.5 42.8	10.8 11.5
0.20	29.4 27.4	6.4 5.4
Expt II		
0	91.2 ± 7.3 (4)	22.5 ± 0.7 (4)
0.05	83.8 76.1	20.4 20.3
0.10	57.4 53.1	12.1 11.9
0.20	34.8 30.1	8.7 8.7
0.50	13.0 14.1	3.5 4.8

CCRF-CEM cultures were supplemented with 10 μM thymidine and 100 μM deoxycytidine. After addition of 0.5 μc glycine-1-^{14}C per ml, specific activity of 44 mc/mmole, the cultures were incubated at 37° for 60 min with methotrexate. The glycine incorporated into adenine of RNA and DNA was assayed as described in Materials and Methods.

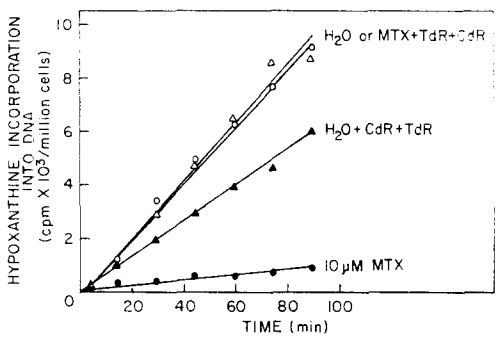


Fig. 4. *Requirements for* 8-^{3}*H-hypoxanthine incorporation into DNA by CEM cultures in the presence of* 10 μ*M MTX. A series of* 2*-ml aliquots from a CEM culture with* 0·645 *million cells/ml were incubated for various intervals with* 0·72 μ*Ci* 8-^{3}*H-hypoxanthine,* 23·5 *Ci/mmole, at* 37°*C. One half of the aliquots were supplemented with* 10 μ*M MTX. One group of MTX-supplemented cultures and one control group were also supplemented with* 10 μ*M thymidine (TdR) plus* 100 μ*M deoxycytidine (CdR). Incubation was terminated by the addition of cold* 0·9% *NaCl and centrifugation at* 500 *g for* 5 *min at* 4°. *The cells were washed once with* 0·9% *NaCl before adding* 1 *ml of* 1 *N NaOH and incubating for* 2 *hr at* 37°. *After chilling the tubes in an ice bath,* 1 *ml of* 1 *N HCl-*15% *trichloroacetic acid was added, and the acid-insoluble residue was collected on glass fiber filters and washed with* 5% *trichloroacetic acid. Hypoxanthine incorporation into DNA was assayed by liquid scintillation counting.*

Maintenance of physiologically active pools of deoxynucleoside triphosphates

Thymidine and hypoxanthine can be incorporated into DNA only as long as adequate pools of all four deoxynucleoside triphosphates are present in the cells. When the medium is supplemented with hypoxanthine, depletion of thymidine triphosphate will terminate incor-

poration into DNA. Supplementation of the medium with thymidine should prevent termination of hypoxanthine incorporation.

Within 5 min after the addition of 10 μM MTX, hypoxanthine-8-^{3}H incorporation was severely inhibited, although traces of hypoxanthine continued to be incorporated throughout the 90-min study (Fig. 4). As expected, if hypoxanthine incorporation were limited by a lack of thymidine triphosphate, supplementation of the medium with thymidine and deoxycytidine would restore hypoxanthine incorporation to the rate for unsupplemented cultures. However, addition of 10 μM thymidine and 100 μM deoxycytidine to control cultures

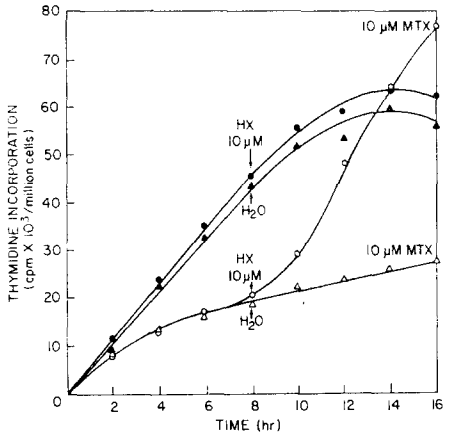


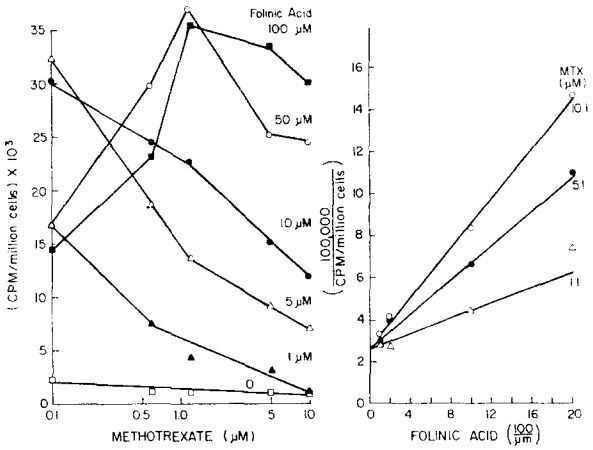
Fig. 5. Thymidine incorporation into DNA with prolonged incubation in the presence of 10 μM MTX. Sixty-ml cultures, 0·76 million cells/ml, were incubated with 10 μM thymidine, 80 μCi/μmole, at 37° in a 5% CO_2-air environment in the presence or absence of 10 μM MTX. At 2-hr intervals for 16 hr triplicate 1-ml aliquots of each culture were removed and assayed for thymidine incorporation into DNA as described in Fig. 3. Eight hr after initiation of the study, 10 μM hypoxanthine (HX) was added to one MTX-treated and to one control culture.

reduced hypoxanthine-8^{3}H incorporation into DNA. The pools of tetrahydrofolate cofactors and of thymidine triphosphate were inadequate to maintain DNA synthesis at control rates for more than 5 min.

Time to the onset of a purine deficiency was examined after the addition of tritiated thymidine, deoxycytidine, and 10 μM MTX (Fig. 5). Two hr after these additions, thymidine incorporation into DNA was reduced approximately 30%. Thymidine incorporation was further reduced by MTX as the experiment continued, although thymidine utilization by the controls was linear for more than 8 hr and continued for 12–14 hr. Hypoxanthine supplementation after 8 hr did not alter thymidine utilization by the controls. However, in the MTX-treated cultures, hypoxanthine stimulated thymidine incorporation, and the subsequent rate of incorporation exceeded the control rate. In other studies, inhibition of thymidine incorporation developed simultaneously from 90 min after the addition of 0·5 and 10 μM MTX to cultures. This study indicates that treatment with MTX does lead to a delayed purine deficiency and to the inhibition of thymidine incorporation into DNA. However, a pronounced purine deficiency did not develop unless thymidine and deoxycytidine were added with MTX to the cultures.*

Folinic acid reversal of MTX inhibition

Since CCRF-CEM cultures will not grow indefinitely in medium which is supplemented



A *B*

Figs. 6A and 6B. Folinic acid reversal of MTX inhibition of deoxyuridine incorporation into DNA. Cultures were preincubated for 18 hr with 0·1 μM MTX to reduce the size of thymidine, purine and tetrahydrofolate pools. The cultures were then supplemented with hypoxanthine, 100 μM. Two-ml aliquots were removed and incubated at 37° for 1 hr with various concentrations of folinic acid and MTX. The effect of MTX and folinic acid on incorporation of deoxyuridine into DNA is shown in Fig. 10A. The concentration of deoxyuridine-6-^{3}H was 1 μM with 1 μCi/ml. Figure 10B presents the double reciprocal plot for response to selected concentrations of MTX and folinic acid.

with dialyzed fetal calf serum, cultures were preincubated for 18 hr with 0·1 μM MTX to reduce the size of intracellular and extracellular tetrahydrofolate pools. These cultures were used to study the relation between folinic acid and MTX with respect to the incorporation of deoxyuridine-6-^{3}H into DNA. The medium was supplemented with 100 μM hypoxanthine to circumvent the inhibition of purine biosynthesis by MTX.

After 18 hr preincubation with 0·1 μM

*Unpublished observation.

MTX, deoxyuridine incorporation was approximately 13% of the maximum rate observed with folinic acid supplementation (Fig. 6A). Although deoxyuridine incorporation was stimulated by folinic acid, the higher concentrations of folinic acid were also inhibitory. This inhibition was prevented by increasing the concentration of MTX. The maximum rate of deoxyuridine incorporation was observed with 50 or 100 μM folinic acid plus 1 μM MTX.

As the concentration of MTX was increased, inhibition of deoxyuridine incorporation became more pronounced, and folinic acid less effectively reversed the inhibition. The reciprocal plot of folinic acid concentration vs the reciprocal of deoxyuridine incorporation at various concentrations of MTX indicated a competitive relation between the concentration of folinic acid and MTX (Fig. 6B). With the biosynthesis of thymidine 5′-monophosphate as the growth-limiting reaction in the presence of MTX, a competitive relation was observed between folinic acid and MTX, rather than product replacement.

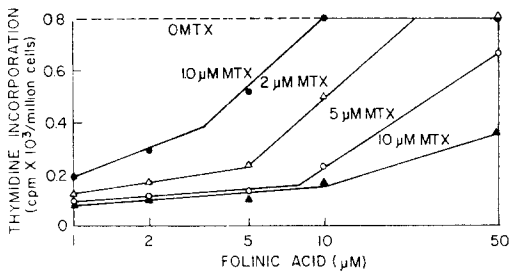
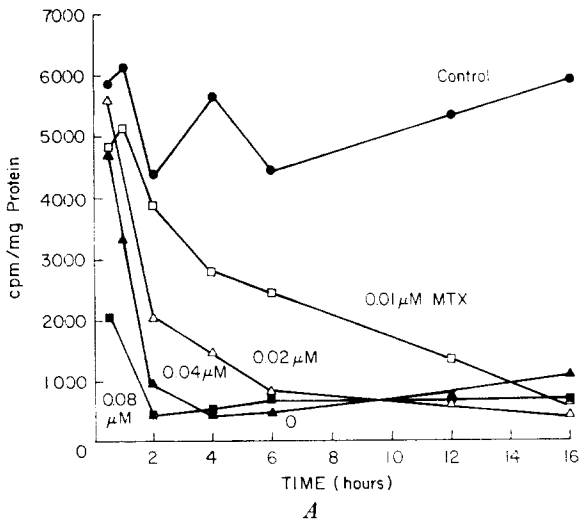
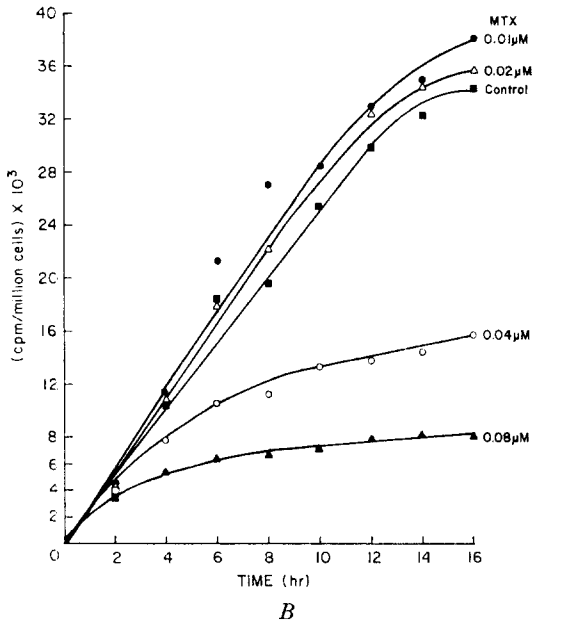


Fig. 7. Relation between MTX and folinic acid on the growth of CEM cultures supplemented with 10 μM thymidine, 100 μM deoxycytidine, and 0·1 μM 5-fluorodeoxyuridine. The cultures were initiated with 0·488 million CEM cells/ml. Growth of the cultures was measured 42 hr later.

Relation between folinic acid and MTX in the presence of 5-fluorodeoxyuridine

The relation between folinic acid and MTX on growth of CCRF-CEM cultures was also examined with conditions where the biosynthesis of purines was rate-limiting. In order to block thymidylate synthetase and to reduce the possibility that an abortive oxidation of N^5, N^{10}-methylenetetrahydrofolate was occurring, 0·1 μM 5-fluorodeoxyuridine was added to inhibit the enzyme. Inhibition of cell division by 5-fluorodeoxyuridine was reversed by supplementing the medium with 10 μM thymidine plus 100 μM deoxycytidine. With these concentrations of pyrimidines, the biosynthesis of purines was the remaining growth-limiting reaction inhibited by MTX (Fig. 7). As the concentration of folinic acid was increased, inhibition of cell division by MTX was reduced and, conversely, increasing the concentration of





Figs. 8A and 8B. The effect of MTX, 0·01 to 0·08 μM, on DHFR activity and on deoxyuridine incorporation into DNA of CCRF-CEM cultures. At 2-hr intervals aliquots were assayed for DHFR activity, Fig. 8A. Deoxyuridine, 1 μM and 1 μCi/ml. was added at initiation of the study, Fig. 8B. The study was initiated with 0·57 million cells/ml.

MTX reduced the effectiveness of folinic acid. Approximately the same relation was observed at 18 hr as at 42 hr. A plot of the reciprocal for folinic acid concentration versus the reciprocal of the increase in cell number indicated a competitive relation between folinic acid and MTX.

Correlation between MTX inhibition of DHFR and of deoxyuridine incorporation

These data indicate that methylation of deoxyuridine 5′-monophosphate and *de novo* synthesis of purines are of approximately equivalent sensitivity to MTX. This is the expected response if MTX were inhibiting a single site such as DHFR. The results in Fig. 8A indicate that DHFR activity was inhibited in less than 2 hr by 0·08 μM MTX. Deoxyuridine incorporation into DNA was partially inhibited at 2 hr, and inhibition increased with time (Fig. 8B). With 0·04 μM MTX, there was a greater delay in the inhibition of DHFR and deoxyuridine incorporation. At 0·02 and 0·01 μM MTX, 6 and 16 hr exposure respectively, were required to reduce DHFR activity below the sensitivity

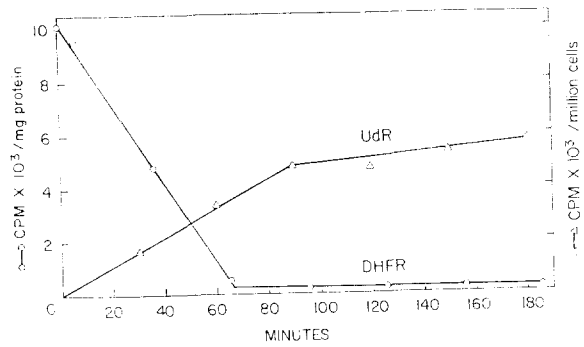
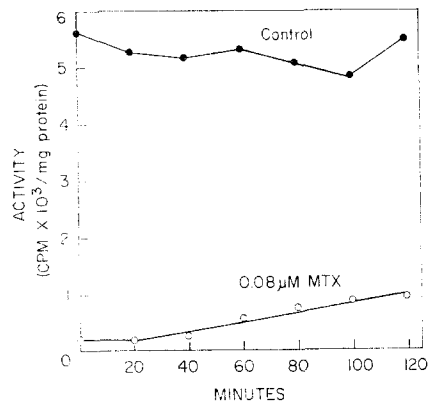


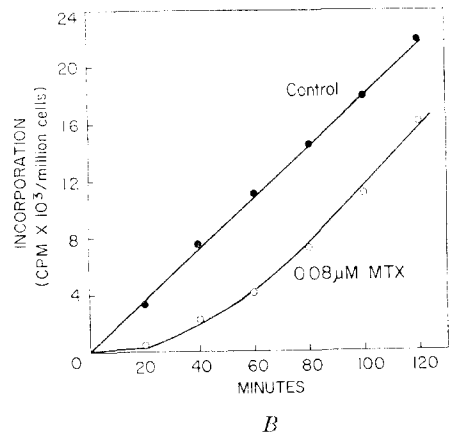
Fig. 9. Development of the inhibition of dihydrofolate reductase activity and deoxyuridine incorporation by 0·08 μM MTX. The culture contained 0·54 million cells/ml and 1 μM deoxyuridine, 1 μCi/ml.

of the assay, and at these drug levels MTX did not inhibit deoxyuridine incorporation.

The absence of an inhibition of deoxyuridine incorporation at a time when DHFR activity cannot be measured after addition of 0·01 and 0·02 μM MTX, indicates a possible dissociation of MTX effect on these two parameters (Fig. 8B). However, this dissociation could indicate that undetected levels of DHFR were adequate to support deoxyuridine incorporation into DNA by whole cells.

The effect of 0·08 μM MTX on the inhibition of DHFR and deoxyuridine incorporation was studied in greater detail (Fig. 9). The inhibition of DHFR preceded by approximately 25 min an inhibition of deoxyuridine incorporation. With 10 μM MTX, less than 5 min were required to establish an inhibition of deoxyuridine or hypoxanthine incorporation (Figs. 3 and 4). Therefore, only a fraction of the delay between inhibition of DHFR and deoxyuridine incorporation can be attributed to time required for depletion of "tetrahydrofolate" or thymidine nucleotide pools. However, two additional factors could contribute to the discrepancy: (1) DHFR is assayed at pH 6·1, and additional drug trapped intracellularly, but not enzyme-bound, during isolation of the cells may bind to the enzyme after sonic disruption and before assay, or (2) The kinetics of enzyme–drug





Figs. 10A and 10B. Recovery of dihydrofolate reductase activity and of deoxyuridine incorporation after inhibition by MTX. CCRF-CEM cultures were preincubated for 3 hr with 0·08 μM MTX. The cells were collected by centrifugation at 500 g and resuspended, 0·55 million cells/ml, in the original volume of fresh media containing 10% dialyzed fetal calf serum. The recovery of DHFR activity is presented in A and deoxyuridine incorporation in B. Deoxyuridine concentration was 1 μM with 1 μCi/ml.

binding may change in cells when the level of free DHFR is reduced [25]. If unbound drug were present intracellularly, time would be required for efflux of the drug and if most of the drug bound to DHFR is retained, then recovery of DHFR activity will result from synthesis of enzyme [7].

Recovery from MTX inhibition of DHFR activity and incorporation of deoxyuridine

MTX, 0·08 μM, was added to CCRF-CEM cultures, and after 3 hr the cells were collected by centrifugation and resuspended in fresh medium containing dialyzed fetal calf serum. DHFR activity was detected between 20 and 40 min after resuspension in MTX-free medium (Fig. 10A). Two hr after resuspension of the cells, DHFR activity was approximately one-sixth the level in control cultures. The recovery of deoxyuridine incorporation into DNA occurred between 20 and 40 min after resuspension of the cells, and by 60 min the rate of deoxyuridine incorporation paralleled the control cultures (Fig. 10B). At 60 min DHFR activity was less than 10% of the level in control cultures. A similar pattern for recovery of DHFR activity and for restoration of deoxyuridine incorporation was observed also with cells after 18 hr of preincubation with 0·08 μM MTX.

DISCUSSION

MTX inhibition of growth by CCRF-CEM cultures was reversed by supplementing the media with products of reactions that require tetrahydrofolate cofactors. Thymidine plus deoxycytidine was more effective than hypoxanthine in preventing the inhibition of growth by low concentrations of MTX, and CCRF-CEM cultures could resume cell division with little or no delay after inhibition by the drug for up to 24 hr. Supplementation with deoxycytidine was required to prevent an initial inhibition by thymidine.

Thymidine plus deoxycytidine was more effective than hypoxanthine in preventing inhibition by MTX of growth. However, methylation of deoxyuridine with subsequent incorporation into DNA and incorporation of glycine into adenine nucleotides of RNA or DNA were inhibited equally by MTX. The greater efficacy of thymidine relative to hypoxanthine in preventing the inhibition of growth is attributed to larger pools of purines than of thymidine. Within 5 min after the addition of 10 μM MTX, a thymidine deficiency limited the incorporation of hypoxanthine into DNA, and this inhibition correlated with the rapid inhibition of deoxyuridine incorporation into DNA by this concentration of drug. The incorporation of thymidine into DNA, which requires purine deoxynucleoside triphosphates, continued normally for 90 min and, furthermore, in other confirmatory studies, MTX was unable to cause a pronounced purine deficiency unless the medium was supplemented with thymidine and deoxycytidine.

Folinic acid reversed MTX's inhibition of deoxyuridine incorporation into DNA. Although the larger concentrations of folinic acid inhibited deoxyuridine incorporation, a competitive relationship was observed between MTX and folinic acid at selected concentration ratios. Folinic acid also reversed the MTX-induced inhibition of purine biosynthesis. These studies indicated that MTX was inhibiting folate metabolism in CCRF-CEM cells. The competitive relation between MTX and folinic acid is attributed to competition for uptake by the cells [34–36]. Competition between MTX and folinic acid at some locus specifically related to either thymidine or purine biosynthesis appears unlikely since the competition was demonstrated in media that was supplemented with either thymidine or hypoxanthine. In addition, the intracellular concentration of free drug for the studies presented in Fig. 9, 10A and 10B was probably too low for significant inhibition of thymidylate synthetase or any other enzyme inhibited by the drug [9, 20].

The most likely locus for MTX inhibition of folate metabolism was DHFR. Inhibition of DHFR would lead to a depletion of tetrahydrofolate cofactors in cells synthesizing thymidine 5′-monophosphate, and a depletion of these cofactors would simultaneously inhibit *de novo* synthesis of thymidine and purine nucleotides [9]. If MTX inhibited the growth of cultures as a result of an inhibition of DHFR, then a dose- and time-dependent relation should exist between the inhibition of DHFR activity and deoxyuridine incorporation into DNA.

Interpretation of the data from Fig. 8, 9 and 10 on the relation between inhibition of DHFR and deoxyuridine incorporation requires consideration of several factors. The intracellular concentration of unbound MTX is generally assumed to be vanishingly small if uninhibited DHFR activity exists in the cell. Furthermore, the rate of inhibition of DHFR activity is assumed to approximate the rate of MTX uptake until the enzyme is nearly saturated [25]. However, the formation of the drug-enzyme complex is reversible [25], and the affinity between MTX and DHFR can be modified by thymidylate synthetase [37]. *In vitro*, affinity between DHFR and MTX is lower at physiological pH than at pH 6·1 [38], and as the level of free enzyme is reduced, the

kinetics of inhibition shift from "stoichiometric" toward a more classical, equilibrium type reaction [25]. This is generally observed as persistence of low levels of activity on titration of DHFR by inhibiting with MTX [25, 38]. In addition, dihydrofolate, the *in vivo* substrate, competes more effectively with MTX than folate [38]. In designing the present studies, the stoichiometric relationship between the drug and the enzyme was emphasized by selecting an assay with folate as substrate at pH 6·1. The apparent discrepancy between the inhibition of DHFR activity by 0·01 μM and 0·02 μM MTX and the absence of an inhibition of deoxyuridine incorporation into DNA by these concentrations is attributed to a persistence of DHFR activity in the intact cell. Observation of a similar discrepancy between the inhibition of DHFR activity and deoxyuridine incorporation into DNA with L1210 cells led to the present studies with a human lymphoblast culture [17]. Goldman and Sirotnak and Donsbach have extended the original observation and observed respectively that the intracellular concentration of MTX for L-cells and L1210 cells must be slightly in excess of the stoichiometric equivalent of intracellular DHFR before a pronounced inhibition of deoxyuridine incorporation occurs [39, 40].

The kinetics for inhibition of DHFR and deoxyuridine incorporation into DNA as well as recovery from drug inhibition were therefore examined in greater detail (Fig. 9 and 10). An approximately 25-min lag was observed between inhibition of DHFR activity and of deoxyuridine incorporation into DNA by 0·08 μM MTX. By extrapolation from the data in Fig. 9, continued uptake of MTX after the disappearance of DHFR activity and prior to inhibition of deoxyuridine incorporation would only permit the intracellular accumulation of MTX equivalent to 30% of the initial DHFR activity. This lag is attributed to the shift in kinetics of drug inhibition as DHFR activity becomes vanishingly small, and utilization of pre-existing pools of tetrahydrofolate cofactors and thymidine nucleotides. However, the slight excess of drug expected to accumulate intracellularly in the 25 min between the disappearance of DHFR and the inhibition of deoxyuridine incorporation is considered to be less than the concentration required to effectively inhibit thymidylate synthetase activity in homogenates of CCRF-CEM cells [20].

Time required for efflux of MTX, slight dissociation of the drug–enzyme complex, possibly synthesis of new enzyme and restoration of tetrahydrofolate cofactor and thymidine triphosphate pools contribute to the 20-min lag before the initial recovery of deoxyuridine incorporation into DNA and DHFR activity (Fig. 10). The complete recovery of the normal rate for deoxyuridine incorporation into DNA prior to full recovery of DHFR activity suggests that CCRF-CEM cells normally contain at least 10–20 times more enzyme activity than required to maintain growth of the culture.

The present information is compatible with a single locus for MTX inhibition of CCRF-CEM cultures. We suggest that inhibition of DHFR by MTX is responsible for the inhibition of growth by CCRF-CEM cultures.

REFERENCES

1. J. Borsa and G. F. Whitmore, Studies relating to the mode of action of methotrexate. III. Inhibition of thymidylate synthetase in tissue culture cells and in cell-free systems. *Molec. Pharmacol.* **5,** 318 (1969).
2. M. T. Hakala, S. F. Zakrzewski and C. A. Nichol, Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. *J. biol. Chem.* **236,** 952 (1961).
3. G. H. Hitchings, G. B. Elion and S. Singer, *Chemistry and Biology of Pteridines.* p. 290, Churchill, London (1954).
4. J. A. R. Mead, J. M. Venditti, A. W. Schrecker, A. Goldin and J. C. Keresztesy, The effect of methotrexate in mice. *Biochem. Pharmacol.* **12,** 371 (1963).
5. M. T. Hakala and E. Taylor, The ability of purine and thymine derivatives and of glycine to support the growth of mammalian cells in culture. *J. biol. Chem.* **234,** 126 (1959).
6. R. R. Rueckert and G. C. Mueller, Studies on unbalanced growth in tissue culture. I. Induction and consequences of thymidine deficiency. *Cancer Res.* **20,** 1584 (1960).
7. D. O. Schachtschabel, H. Lazarus, S. Farber and G. E. Foley, Sensitivity of cultured human lymphoblasts (CCRF-CEM cells) to inhibition by thymidine. *Exp. cell. Res.* **43,** 512 (1966).

8. J. R. Bertino, The mechanism of action of the folate antagonists in man. *Cancer Res.* **23.** 1286 (1963).
9. R. L. Blakley, *The Biochemistry of Folic Acid and Related Pteridines*, American Elsevier, New York (1969).
10. A. J. Wahba and M. Friedkin, Direct spectrophotometric evidence for the oxidation of tetrahydrofolate during the enzymatic synthesis of thymidylate. *J. biol. Chem.* **236,** PC11 (1961).
11. W. C. Werkheiser, The biochemical, cellular, and pharmacological action and effects of the folic acid antagonists. *Cancer Res.* **23,** 1277 (1963).
12. D. Roberts, In search of a key. *Nat. Cancer Inst. Monogr.* **34,** 118 (1971).
13. P. T. Condit and J. A. R. Mead, Further observations on the site of action of methotrexate. *Biochem. Pharmacol.* **12,** 94 (1963).
14. L. C. Misra, A. S. Parmar and J. A. R. Mead, A method for assessing dihydrofolate reductase inhibition *in vivo*. *Anal Biochem.* **48,** 515 (1972).
15. D. Kessel, T. C. Hall, D. Roberts and I. Wodinsky, Uptake as a determinant of methotrexate response in mouse leukemias. *Science* **150,** 752 (1965).
16. D. Roberts, I. Wodinsky and T. C. Hall, Studies on folic reductase. III. Level of enzyme activity and response to methotrexate of transplantable mouse tumors. *Cancer Res.* **25,** 1899 (1965).
17. D. Roberts and I. Wodinsky, On the poor correlation between the inhibition by methotrexate of dihydrofolate reductase and of deoxynucleoside incorporation in DNA. *Cancer Res.* **28,** 1955 (1968).
18. R. J. Winzler, A. D. Williams and W. R. Best, Metabolism of human leukocytes *in vitro*. I. Effects of A-methopterin on formate-C^{14} incorporation. *Cancer Res.* **17,** 108 (1957).
19. D. Roberts, T. C. Hall and D. Rosenthal, Coordinated changes in biochemical patterns: The effect of cytosine arabinoside and methotrexate on leukocytes from patients with acute granulocytic leukemia. *Cancer Res.* **29,** 571 (1969).
20. D. Roberts and E. V. Loehr, Elevation of thymidylate synthetase activity in CCRF-CEM cells. *Cancer Res.* **31,** 1181 (1971).
21. R. B. Dunlap, N. G. L. Harding and F. M. Huennekens, Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*. *Biochemistry* **10,** 88 (1971).
22. R. Raunio and M. T. Hakala, Tetrahydrofolate-dependent enzymes in sarcoma 180 cells sensitive and resistant to amethopterin. *Biochem. Pharmacol.* **17,** 1744 (1968).
23. J. R. Bertino, A. Cashmore, M. Fink, P. Calabresi and E. Lefkowitz, The "induction" of leukocyte and erythrocyte dihydrofolate reductase by methotrexate. II. Clinical and pharmacological studies. *Clin. Pharmacol. Ther.* **6,** 763 (1965).
24. D. Roberts and T. C. Hall, The reduction of folate and of dihydrofolate by homogenates of leukocytes from patients with leukemia or with myeloid metaplasia. *Cancer Res.* **27,** 994 (1967).
25. W. C. Werkheiser, Specific binding of 4-amino folic acid analogues to folic acid reductase. *J. biol. Chem.* **236,** 888 (1961).
26. G. A. Fischer, Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells. *Biochem. Pharmacol.* **7,** 75 (1961).
27. D. K. Misra, S. R. Humphreys, M. Friedkin, A. Goldin and E. J. Crawford, Increased dihydrofolate reductase activity as a possible basis of drug resistance in leukemia. *Nature (Lond.)* **189,** 39 (1961).
28. D. Roberts and T. C. Hall, Enzyme activities and deoxynucleoside utilization of leukemic leukocytes in relation to drug therapy and resistance. *Cancer Res.* **29,** 166 (1969).
29. D. Roberts and T. C. Hall, Dihydrofolate reductase activity and deoxynucleoside incorporation into DNA of human leukocytes: relation to methotrexate administration. *Cancer (Philad.)* **20,** 905 (1967).
30. G. E. Foley, H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone and R. E. McCarthy, Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer (Philad.)* **18,** 522 (1965).
31. G. R. Wyatt, Separation of nucleic acid components by chromatography on filter paper. In *The Nucleic Acids*, Vol. 1, p. 243, Academic Press, New York (1955). Ed. Edwin Chargapp and J. N. Davidson.

32. D. Roberts, An isotopic assay for dihydrofolate reductase. *Biochemistry* **5,** 3549 (1966).
33. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurements with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
34. I. D. Goldman, N. S. Lichtenstein and V. T. Oliverio, Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. *J. biol. Chem.* **243,** 5007 (1968).
35. T. C. Hall, D. Roberts and D. H. Kessel, Methotrexate and folic reductase in human cancer. *Eur. J. Cancer* **2,** 135 (1966).
36. M. T. Hakala, On the nature of permeability of sarcoma-180 cells to amethopterin *in vitro*. *Biochim. Biophys. Acta* (*Amst.*) **102,** 210 (1965).
37. M. Kawai and B. L. Hillcoat, Interaction of thymidylate synthetase and dihydrofolate reductase enzymes *in vitro* and *in vivo*. *Cancer Res.* **34,** 1619 (1974).
38. J. R. Bertino, B. A. Booth, A. L. Bieber, A. Cashmore and A. C. Sartorelli, Studies on the inhibition of dihydrofolate reductase by the folate antagonists. *J. biol. Chem.* **239,** 479 (1964).
39. I. D. Goldman, The mechanism of action of methotrexate. I. Interaction with a low-affinity intracellular site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell mouse fibroblasts. *Mol. Pharmacol.* **10,** 257 (1974).
40. F. M. Sirotnak and R. C. Donsbach, The intracellular concentration dependence of antifolate inhibition of DNA synthesis in L1210 leukemia cells. *Cancer Res.* **34,** 3332 (1974).

Europ. J. Cancer Vol. 11, pp. 783–786. Pergamon Press 1975. Printed in Great Britain

Carcinoembryonic Antigen (CEA) and Related Antigens in Blood Cells and Hematopoietic Tissues*

M. BORDES, S. KNOBEL and F. MARTIN

Laboratory of Experimental Medicine, INSERM U-45, Faculté de Médecine, Dijon, (France).

Abstract—*Indirect immunofluorescence was used to detect and localize carcinoembryonic antigen (CEA) and non-specific cross-reacting antigen (NCA) in blood cells and hematopoietic tissues. CEA was never found, but NCA could easily be identified in the cytoplasm of blood polymorphonuclear cells, bone marrow myelocytes, and monocytic cells (probably macrophages) infiltrating the spleen and lymph nodes. In normal colon and colonic adenocarcinoma, CEA was present in the walls of epithelial cells, and in round, isolated polymorphonuclear or mononuclear cells, infiltrating the submucosa and the stroma of the tumor.*

INTRODUCTION

CARCINOEMBRYONIC antigen (CEA) was first defined as a cancer- and organ-specific antigen, restricted to cancerous and fetal digestive tissues [1]. This specificity was later questioned, and CEA was detected in various epithelial tissues and secretions [2, 3]. CEA, or an immunologically related antigen, has recently been demonstrated on the walls of normal erythrocytes [4]. A glycoprotein, sharing a common antigenic site with CEA, was independently substantiated by several groups, and called non-specific cross-reacting antigen(NCA) [5], normal glycoprotein (NGP) [6] and CEX [7]. Exchange of reagents has proven the immunologic identity of NCA, NGP and CEX [7]. This antigen has been found, in association with CEA, in normal and cancerous epithelial tissues [8], but was also present in normal spleen [5], an organ which does not contain epithelial tissue. The present work takes into consideration the location of CEA and NCA in blood cells and hematopoietic tissues.

Accepted 1 April 1975.

*This work was supported by research grants CA-401 and 74.1.080.04 from the Institut National pour la Santé et la Recherche Médicale.

MATERIAL AND METHODS

Antigens and antisera

Purified CEA was prepared in this laboratory, according to previously reported procedure [9]. Anti-CEA serum was prepared by repeated immunization of rabbit with purified CEA. This serum, referred to as 42/f, has both anti-CEA and anti-NCA reactivities. It was made specific for CEA by absorption of perchloric acid extract from normal human spleen (50 mg/ml). This absorbed serum will be referred to as 42/f2.

Blood and tissue specimens

Samples of blood have been obtained from 20 healthy donors of various blood group status. Morphologically normal, sternal bone marrow samplings came from 5 patients with mild anaemia, of non-malignant origin. Smears of blood and bone marrow were immediately fixed for 20 min in 95% ethanol. The other tissues were fixed and embedded according to Sainte-Marie [10]. The non-cancerous specimens were 2 spleens, surgically removed after a traumatic rupture, 2 cervical lymph nodes (biopsies), with mild subacute lymphadenitis,

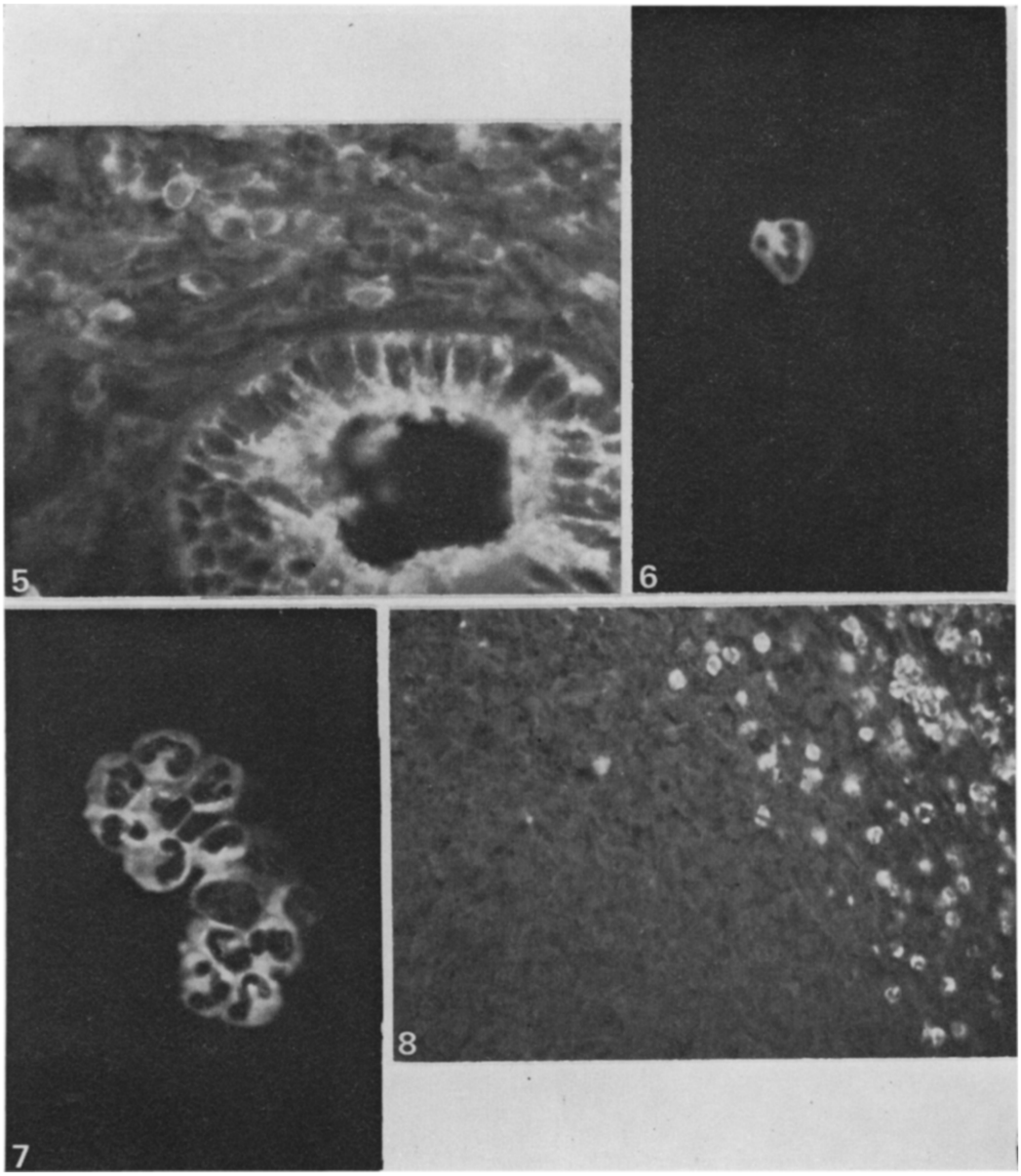


Fig. 5. *Colonic adenocarcinoma stained by anti-NGP antiserum (indirect immunofluorescence). The membrane of tumor cells (bottom, right), but also round cells infiltrating the stroma (arrow) are stained.* (× 550).

Fig. 6. *Circulating blood polymorphonuclear cell stained by anti-NGP antiserum (indirect immunofluorescence).* (× 550).

Fig. 7. *A clone of cells of the granulopoietic line stained by anti-NGP antiserum (indirect immunofluorescence) on a bone marrow smear.* (× 550).

Fig. 8. *Section of normal spleen, stained by anti-NGP antiserum. The lymphocytes of the germinal center are negative, but the round cells (polymorphonuclear and/or macrophages) around the center are stained.* (× 200).

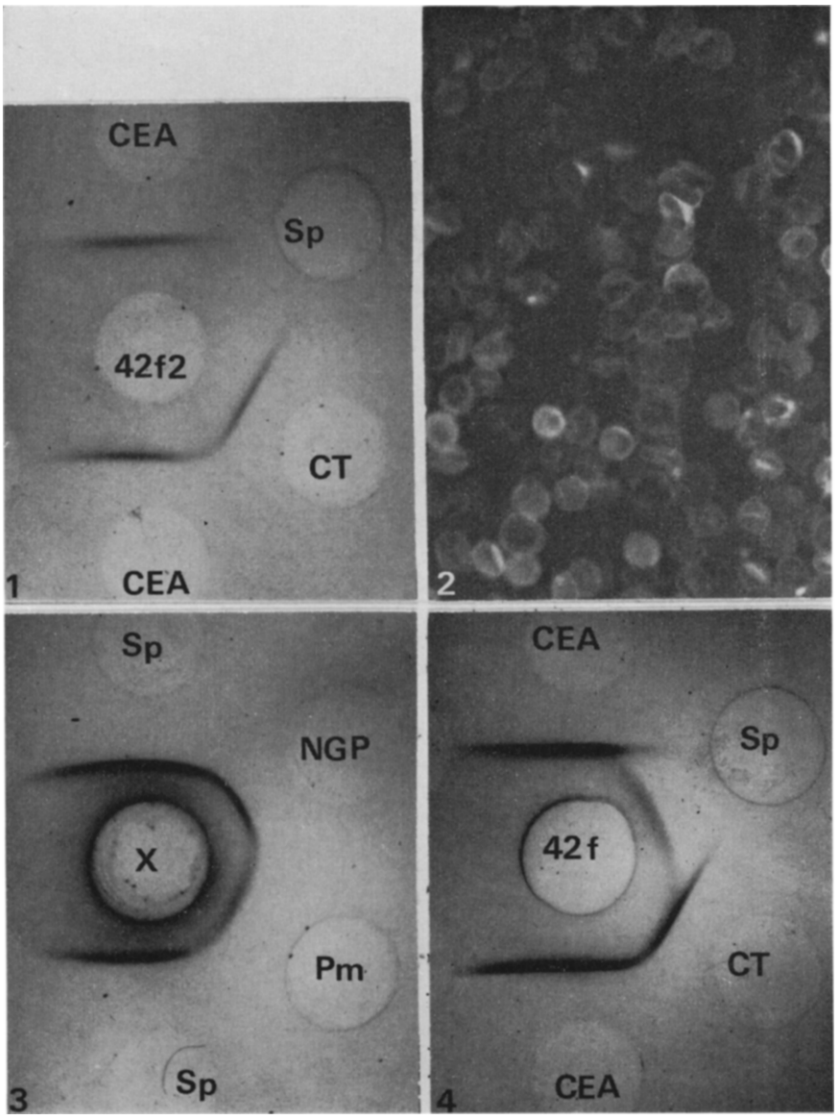


Fig. 1. *Reaction of anti-CEA antiserum* (42*f*2, *central well*) *with purified CEA and perchloric acid extract of colonic carcinoma* (*CT*); *the serum does not react with perchloric acid extract of normal spleen* (*Sp*).

Fig. 2. *Membrane immunofluorescence of blood group A erythrocytes with anti-CEA serum, before absorption with A red blood cells. This fluorescence is completely extinguished by absorption of the serum with blood group A erythrocytes.* (× 550).

Fig. 3. *Reaction of anti-CEX antiserum* (*X*, *central well*) *with perchloric acid extracts of normal spleen* (*Sp*), *and polymorphonuclear cells* (*Pm*) *and purified NGP.*

Fig. 4. *Reaction of an anti-CEA antiserum non absorbed for NCA* (42*f*, *central well*) *with purified CEA, perchloric acid extracts of colonic carcinoma* (*CT*) *and normal spleen* (*Sp*).

(to face p. 784)

and a morphologically normal lung obtained at autopsy. Three colonic adenocarcinomas, and their adjacent, non-cancerous mucosa (surgical material), were also used in this work.

Peritoneal macrophages

These were prepared from the ascitic fluid of a patient with alcoholic liver cirrhosis. Five ml were disseminated into tissue culture flasks (Falcon), poured off after 2 hr, and replaced by Ham's FIO medium, supplemented by 20 % fetal calf serum. The adhering cells were mostly macrophages, as proven by phagocytosis of iron carbonyl. After 3 days of culture, they were fixed by absolute ethanol and tested for immunofluorescence.

Immunofluorescence staining

The indirect immunofluorescence technique, used to stain smears and sections, has been previously reported [11]. 42/f, 42/f2, anti-CEX, anti-NGP and anti-NCA sera were respectively diluted as follows 1/80, 1/80, 1/20, 1/20 and 1/100 according to preliminary results. In the second step, the fluorescein isothiocyanate-conjugated reagents were anti-goat globulin rabbit antiserum (Institut Pasteur, Paris) diluted 1/10 for anti-CEX serum and anti-rabbit globulin sheep antiserum (Institut Pasteur, Paris) diluted 1/10, for the other sera. Special precautions were taken to avoid non-specific reactions. All the smears and sections were treated by sodium nitrite–acetic acid in accordance with Burtin and Sabine [12]. Fluorescein labeled sera were absorbed by acetonic extracts of normal human spleen (150 mg/ml). Normal rabbit serum was systematically used as a negative control. Observations were made with a Leitz Orthoplan microscope with a vertical illuminator.

Membrane immunofluorescence

Erythrocytes from blood collected on sodium oxalate were washed 3 times in phosphate-buffered saline (PBS), incubated for 20 min at room temperature with an equal volume of antiserum, diluted as mentioned above. The red blood cells were washed 3 times in PBS, then incubated in the same volume of fluorescein-labelled serum diluted 1/10. After 3 further washings, they were mounted on slides and examined for membrane immunofluorescence

Immunodiffusion

Double immunodiffusion was carried out according to a modification of Ouchterlony's technique [2].

RESULTS

Normal rabbit serum

On immunofluorescence, normal rabbit serum, used as control, does not stain ethanol-fixed smears or tissue sections of hematopoietic tissues or colonic adenocarcinoma.

Anti-CEA serum

On immunodiffusion (Fig. 1), anti-CEA serum 42/f 2 gives a precipitin line with perchloric extract of colonic tumor or purified CEA, but does not react with perchloric extracts of spleen. The immunofluorescence staining of colonic tumors and non cancerous colonic mucosa by this serum has been described elsewhere [11]. When tested on fixed blood or bone marrow smears, serum 42/f2 stains the membrane of erythrocytes and erythroblasts from patients of blood group A or AB, but not of O or B.

Membrane immunofluorescence is still more clearly evidenced on unfixed, suspended erythrocytes (Fig. 2). This anti-blood group A activity of serum 42/f2 can be completely removed by incubating the serum 5 times with an equal volume of washed blood group A1 red cells. The absorbed serum no longer reacts with erythrocytes or erythroblasts, but maintained reactivity with purified CEA (immunodiffusion), colonic tumors and non-cancerous colon (immunofluorescence). Sections of spleen and lymph nodes are not stained by serum 42/f2.

Anti-NCA sera

Double immunodiffusion shows that anti-NCA, anti-CEX and anti-NGP sera, and unabsorbed serum 42/f have a common reactivity against perchloric extract of normal human spleen (Fig. 3, 4). On immunofluorescence, these sera stain the membrane of colo-rectal cancer cells and non-cancerous colon epithelial cells, as previously reported by Burtin *et al.* [8]. However non-cancerous, round cells, infiltrating the stroma of the tumors, are also strongly positive: bright-green granular fluorescence is localized to the cytoplasm bringing out, in negative, the nucleus which is polylobed or kidney-shaped (Fig. 5). On blood smears, erythrocytes, platelets and lymphocytes are negative, but the sera stain the cytoplasm of polymorphs, and probably, also, of monocytes (Fig. 4). In bone marrow, normoblasts and megakaryocytes are negative, but the cytoplasm of the cells of the granulopoietic line is stained (Fig. 7) Although. the accurate identification of the cells is difficult, staining seems to increase with the

degree of maturation: polymorphonuclear cells and metamyelocytes are strongly positive, the reactivity of myelocytes and promyelocytes is weaker, and myeloblasts are probably negative. On spleen and lymph node sections, anti-NCA and related antisera react with round cells, usually mononuclear, infiltrating the lymph node sinusoids, marginal zone and red pulp of the spleen, but not in germinal centers (Fig. 8). The positive cells have the shape and location of macrophagic reticulo-endothelial cells. Similar positive cells can be found in the lung, invading the alveolar lumen and the septa. The peritoneal macrophages cultivated from ascitic fluid are also stained by anti-NCA antisera. Absroption experiments demonstrate the common immunologic reactivity of antigen present in cancerous or non-cancerous colonic epithelial cells, and macrophagic or polymorphonuclear cells. After absorption with perchloric extract of normal spleen, or buffy coat of a patient with chronic myeloid leukemia, or purified NGP, anti-NCA and related sera no longer stain intestinal carcinoma cells, or epithelial cells of non-cancerous colon.

Polymorphonuclear and macrophagic cells are not stained when normal rabbit serum is substituted for anti-NCA serum on ethanol-fixed smears or tissue sections. On the other hand, a bright membrane immunofluorescence was observed when living polymorphs, in suspension, were treated by normal rabbit serum; this non-specific reaction prevents the use of this method for NCA location on the membrane of living, polymorphonuclear cells or macrophages.

DISCUSSION

The presence of anti-blood group A reactivity in anti-CEA serum before absorption with blood group A erythrocytes is not surprising, since the purified CEA samples used to immunize rabbits have a blood group A antigenic activity. From the work of Holburn *et al.* [13], this activity seems to be due to antigenic determinants on the CEA molecule, rather than contamination by blood group A glycoprotein. After absorption of anti blood group A antibodies, anti-CEA serum does not react with blood cells or hematopoietic tissues. This lack of reactivity of anti-CEA serum with erythrocytes is at variance with data of Nery *et al.* [4], who report partial cross-reactivity between CEA and a material extracted from red blood cell membrane. Disagreement between Nery's and present data could be explained in various ways. The CEA-like antigen could not be recognized by anti-CEA serum used in this work, or could have been buried too deeply in the erythrocyte membrane. On the other hand, membrane immunofluorescence could be quenched by hemoglobin.

Antisera directed against the antigen cross-reacting with CEA (called NCA, NGP or CEX) strongly stain the cytoplasm of polymorphonuclear cells, myelocytes and mononuclear cells, which probably are macrophages. In addition to this localization, NCA is present in cancerous and non-cancerous epithelial cells of the digestive tract. Staining of the digestive epithelial cells could be related to a residual anti-CEA reactivity of the antisera against CEX, NCA or NGP used in this work. This seems unlikely as these sera stain the adjacent cellular membranes, a localization previously reported by Burtin *et al.* [8] as specific for NCA, whereas anti-CEA serum only stains the apical pole of the cancerous cells. Furthermore, the absorption experiments performed in this work demonstrate that polymorphonuclear NCA and epithelial NCA have a common immunologic reactivity.
Localization in leukocytes of an apparently tumor-associated antigen has been reported previously by Dufour *et al* [14] in a 2-acetylaminofluorene-induced rat hepatoma. Similar findings were made with dimethylhydrazine-induced rat colonic carcinoma [15]. In both cases rabbit antisera raised against tumor extracts, and made specific by absorption with normal tissues extracts, did not stain tumor cells, but the polymorphonuclear cells present in the tumor, hematopoietic tissues, and normal blood. However, contrary to the above-mentioned rat antigen(s), NCA is also present in normal and cancerous epithelial cells. This advances the possibility that the antigen, detected in macrophages and polymorphonuclear cells, could be absorbed by pinocytosis, instead of being locally synthesized. While this hypothesis could be true with regard to the tumour, it can not explain the localization of NCA in myelocytes and metamyelocytes in the bone marrow of patients without cancer.

The immunofluorescence staining of colon carcinoma by anti-NCA serum demonstrates the marked infiltration of the tumor stroma by polymorphonuclear and macrophagic cells. Whether these cells are only clearing necrotic tissues, or playing an active role in the host defense against cancer is yet to be determined.

Acknowledgements—This work could not have been done without the reagents generously given by Dr. Burtin (Villejuif), Dr. Darcy (London) and Dr. Mach (Lausanne). The authors wish to express their thanks to Pr. Cabanne, Centre G.F. Leclerc, Dijon, for his material help, and Mr. Thoret, for his excellent technical assistance.

REFERENCES

1. P. Gold and S. O. Freedman, Specific carcinoembryonic antigens of the human digestive system. *J. exp. Med.* **122,** 467 (1965).
2. F. Martin and M. S. Martin, Demonstration of antigens related to colonic cancer in the human digestive system. *Int. J. Cancer* **6,** 352 (1970).
3. G. Pusztaszeri and J. P. Mach, Carcinoembryonic antigen (CEA) in non digestive cancerous and normal tissues. *Immunochemistry* **10,** 197 (1973).
4. R. Nery, H. Bullman and A. L. Barsoum, Carcinoembryonic antigens of erythrocyte membranes. *Nature (Lond.)* **246,** 44 (1973).
5. S. von Kleist, G. Chavanel and P. Burtin, Identification of a normal antigen that cross-reacts with the carcinoembryonic antigen. *Proc. natl. Acad. Sci. (Wash.)* **69,** 2492 (1972).
6. J. P. Mach and G. Pusztaszeri, Carcinoembryonic antigen (CEA): demonstration of a partial identity between CEA and a normal glycoprotein. *Immunochemistry* **9,** 1031 (1972).
7. D. A. Darcy, C. Turberville and R. James, Immunological study of carcinoembryonic antigen (CEA) and a related glycoprotein. *Brit. J. Cancer* **28,** 147 (1973).
8. P. Burtin, S. von Kleist, M. C. Sabine and M. King, Immunohistological localization of carcinoembryonic antigen and non-specific cross-reacting antigen in gastrointestinal normal and tumoral tissues. *Cancer Res.* **33,** 3299 (1973).
9. F. Martin and M. S. Martin, Radioimmunoassay of carcinoembryonic antigen in extracts of human colon and stomach. *Int. J. Cancer* **9,** 641 (1972).
10. G. Sainte-Marie, Paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10,** 250 (1962).
11. M. Bordes, R. Michiels and F. Martin, Detection by immunofluorescence of carcinoembryonic antigen in colonic carcinoma, other malignant or benign tumours, and non-cancerous tissues. *Digestion* **9,** 106 (1973).
12. P. Burtin and M. C. Sabine, Inhibition of the non-specific fixation of fluorescent globulins. *Rev. europ. Étud. clin. biol.* **17,** 76 (1972).
13. A. M. Holburn, J. P. Mach, D. Macdonald and M. Newlands, Studies of the association of the A, B and Lewis blood group antigens with carcinoembryonic antigen (CEA). *Immunology* **26,** 831 (1974).
14. D. Dufour, S. Lemieux, A. Tremblay and R. Estrada, Immunochemical studies of extramedullary hematopoiesis and of the participation of leukocytes in carcinogenesis. In *Embryonic and Fetal Antigens in Cancer.* (Edited by N. G. Anderson and J. H. Coggin) Vol. 2, p. 199. Oak Ridge National Laboratory, Oak Ridge, Tennessee (1972).
15. F. Martin, M. S. Martin, M. Bordes and S. Knobel, Antigens associated with intestinal carcinomas chemically induced in rats. *Int. J. Cancer* **15,** 144 (1975).

Europ. J. Cancer Vol. 11, pp. 787–793. Pergamon Press 1975. Printed in Great Britain

Cytotoxic Sulphonamides Designed for Selective Deposition in Malignant Tissue

G. ABEL*, T. A. CONNORS*, P. GODDARD*, H. HOELLINGER†
NGUYEN-HOANG-NAM†, L. PICHAT‡, W. C. J. ROSS*, D. E. V. WILMAN*

**Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London SW3 6JB, England*
†INSERM—Unité de Recherches de Toxicologie Expérimentale, Hôpital F. Widal—200 rue du Faubourg Saint-Denis, 75010 Paris, France
‡Service des Molécules Marquées, C.E.N.-Saclay, B.P. No. 2, 91190 Gif-sur-Yvette, France

Abstract—*The tumour and liver concentrations of a range of sulphonamides of different pKa values have been measured. Although a number of them concentrated to a much greater extent in the mouse plasma cell tumour than in liver, no correlation was observed between this property and pKa value. A series of dimethyltriazenes were prepared from the sulphonamides and tested for their anti-tumour activity. Two dimethyltriazenes derived from sulphamerazine and sulphapyridine are highly selective anti-tumour agents but no evidence was obtained that this effect was due to their selective concentration in tumours. In tests against the TLX5 lymphoma, a phenyltriazene was shown to be dose-schedule dependent, daily administration of the compound being more effective than single doses. The biological half-life of the phenyltriazenes was shown to be of importance in determining their anti-tumour effects, agents with longer half-lives being more active than agents of shorter half-lives.*

INTRODUCTION

AFTER injection, it is not usual for small molecular weight materials to attain a high concentration in animal tumours compared to liver and other normal tissues. However, such a selective concentration of a chemical in a tumour has been reported for the two sulphonamides, sulphapyrazine and sulphadiazine. Following repeated administration, deposits of sulphapyrazine were observed in the Walker carcinoma, and analysis of various organs of the rat at this time showed that four times as much of the sulphonamide had deposited in the tumour than in the liver [1]. Sulphadiazine was similarly shown to have a high affinity for the Yoshida sarcoma in rats, the tumour to liver ratio being 2:1 two hr after injection [2].

In an extension of this work it was shown that sulphadiazine and three-related sulphapyrimidines concentrated selectively in a number of mouse transplanted tumours [3]. The high accumulation of these sulphonamides in malignant tissue has been claimed to be due to the more acid pH of cancer cells [2]. Sulphonamides in solution at alkaline pH may precipitate at acid pH because of the extreme insolubility of the unionised form. By selecting a derivative of the correct pKa value, more of the agent will precipitate in malignant cells than in normal cells because of the higher acidity of the former. The anti-bacterial sulphonamides have no anti-cancer activity in their own right, but in attempts to exploit their affinity for tumour cells, a derivative of sulphadiazine was synthesised containing a cytotoxic nitrogen mustard group [2]. However, although this compound had the same pKa value as sulphadiazine, it did not concentrate selectively in the Walker carcinoma as predicted. Furthermore, it was found in tissue distribution studies, using concentrations of sulphadiazine low enough for all of the unionised form to be in solution, that there was still a differential between the concentration of the sulphonamide in tumour and liver. Had this difference been due to the precipitation of the

Accepted 23 June 1975.

unionised form in the more acid tumour cells, then at such low concentrations this effect should no longer be present.

Thus, while certain sulphonamides definitely accumulate in transplanted tumours, the mechanism by which this occurs is not clear. The tissue distribution of a wide range of sulphonamides has now been studied and attempts have been made to correlate their chemical and physico-chemical properties with their ability to concentrate selectively in the PC6 mouse tumour, in order to guide the synthesis of related cytotoxic agents which might similarly accumulate selectively in tumour cells.

MATERIALS AND METHODS

Sulphamerazine and sulphamethizole were a generous gift from May and Baker, sulphanilylglycine and sulphapyridine from Roche, and sulphamethoxazole from Burroughs Wellcome. A large batch of sulphamethoxazole, used for the preparation of one of the triazenes, was a gift from Roche. The remainder of the sulphonamides were obtained from commercial sources.

The 3,3-dimethyltriazene derivatives were prepared from the parent sulphonamides by the following method. 0·05 Mole of the sulphonamide suspended in 125 ml N hydrochloric acid and 200 ml water at 0°C was diazotised by the addition of 0·05 mole sodium nitrite in 15 ml water. After a further 0·5 hr at 0°C excess nitrite was decomposed with sulphamic acid. Following the addition of 0·2 mole dimethylamine in 80 ml water, the triazene either precipitated immediately or after 0·5 hr, 50% aqueous acetic acid was added cautiously until precipitation occurred. The products were crystallised and identified as shown in Table 1.

^{14}C and ^{3}H labelled materials were prepared at Service des Molécules Marquées, C.E.N.-Saclay, France, by adapting the above procedure to radioactive synthesis.

Sulphadiazine triazene (pyrimidine-2-^{14}C) (5 mCi/mmole) was synthesised from sulphadiazine (pyrimidine-2-^{14}C) [4] on a 0·3 mmole scale.

Sulphadiazine triazene (methyl-^{3}H) (200 mCi/mmole) labelled in the side chain was prepared from dimethylamine-^{3}H as starting radioactive material, on a 0·5 mmole scale. The method [5] for the preparation of dimethylamine-^{14}C hydrobromide was used to obtain dimethylamine-^{3}H. Thus, methylbromide-^{3}H (0·86 mmole, 172 mCi) was frozen into a tube containing N-methyl *p*-toluenesulphonamide (1·0 mmole), methanol (2 ml) and N sodium hydroxide (2 ml). The tube was sealed under vacuum and stirred at 70°C for 15 hr. The mixture was concentrated, diluted with water and the solution extracted continuously with ether. The crude (N,N-dimethyl-^{3}H) *p*-toluenesulphonamide was purified by preparative thin-layer chromatography (yield = 0·5 mmole, 110 mCi). Dimethylamine-^{3}H was released from the dimethylamide-^{3}H by the action of 48% hydrobromic acid (3 ml) for 2 hr at 100°C and the contents were treated with N sodium hydroxide (3·5 ml). Dimethylamine-^{3}H was transferred in a high vacuum system into sulphadiazine (1·0 mmole) diazonium chloride and allowed to react as previously.

The crude labelled sulphadiazine triazenes were purified by column chromatography on Sephadex G-10 using water followed by N/10 ammonia solution as eluants. The radiochemical

Table 1

Compound	M.Pt	Crystal form	Solvent	Analysis								U.V. Spectral Data			
				Required				Found							
				C	H	N	S	C	H	N	S	λ_{max}	ϵ	λ_{max}	ϵ
10-279†	235°	prisms		47.0	4.6	27.4	10.5	46.8	4.6	27.6	10.7	220	21,000	323	21,500
10-342	248°C (decomp)	needles	ethanol	48.7	5.0	26.2	10.0	49.0	5.1	26.2	10.2	221	15,300	320	19,900
10-344	192°C (decomp)	plates	ethanol	42.4	4.2	22.5	20.6	42.7	4.3	22.6	19.9	227	9,000	323	20,600
10-345	219°C (decomp)	needles	ethanol	51.4	5.0	22.9	10.5	51.4	5.0	22.9	9.9	225	12,200	323	20,000
10-353	185°C (decomp)	needles	ethanol	46.6	4.9	22.6	10.4	46.8	4.8	22.6	10.2	219	8,200	321	17,300

† R.C.S. Audette, T.A. Connors, H.C. Mandel, K. Merai and W.C.J. Ross: Biochem.Pharmacol. 22, 1855-64 (1973).

purity > 98% was checked by thin-layer radiochromatography and the chemical purity was determined by u.v. spectrophotometry.

The tissue distribution of the sulphonamides and the labelled triazenes were measured in tissue extracts as previously described, by the Bratton–Marshall technique or by scintillation counting [3].

The solubilities of the sulphonamides and the triazenes were obtained by adding them to 10 ml. Sorenson's phosphate buffer containing Tween 80 at pH 7·5 and 37° and shaking on a rotary shaker to give a saturated solution. The suspensions were filtered through a Millex 25 disposable filter, pore size 0·22 μm. The optical density of the solutions were read at 37° at the appropriate λ_{max} value.

Partition coefficients were measured by dissolving the triazenes and sulphonamides in dimethylsulphoxide and adding to Sorenson's pH 7·5 phosphate buffer at room temperature, so that the final concentration of dimethylsulphoxide was 1%. The sulphonamides were prepared at a concentration of 10 μg/ml and the triazenes at 5 μg/ml. 6 ml aliquots of these solutions were partitioned against 3 ml of *n*-octanol by mixing on a rotary mixer at room temperature for 3·5 hr. The optical density of the aqueous phase was measured before and after partitioning and the value po calculated from the formula $Po = 2(A - A^1)/A^1$ where A = initial absorbance of the aqueous solution and A^1 = absorbance of aqueous phase after shaking with octanol.

The plasma cell tumours were obtained by subcutaneous transplantation of tumour fragments by trocar into female Balb/C$^-$ mice. The tumours were used for distribution studies, 24–28 days after transplantation when they were between two and three grams in weight.

In the anti-tumour tests against the TLX/5 lymphoma, about 10^5 ascites cells were injected subcutaneously in the inguinal region of CBA/LAC female mice and the animals treated intraperitoneally with the drugs either as suspension in arachis oil or 10% dimethylacetamide or acetone in oil. The agents under test were administered three days after tumour transplantation at a series of two-fold spaced doses ranging from lethal to non-effective. Anti-tumour effectiveness was measured by the increase in life-span of treated groups compared to a control group.

The "biological half-lives" of 1-(*p*-carbamoylphenyl)-3,3-dimethyl triazene and 1-(*p*-carbamoylphenyl)-3-methyl-triazene were obtained by injecting these agents intraperitoneally at their maximum tolerated dose into a group of mice. At the same time as the injection and at intervals thereafter, groups of five animals were injected subcutaneously with 10^5 TLX/5 lymphoma cells in the inguinal region. From the extension in survival time of the animals compared with animals injected with tumour cells only at the same time, the period during which the triazenes were present in the animals at significantly active dose levels could be estimated.

RESULTS AND DISCUSSION

Figure 1 shows the relationship between the pKa of a number of sulphonamides and their distribution between the PC6 tumour and liver of Balb/C$^-$ mice. Sulphadiazine (compound f) has the optimum pKa for selective precipitation in the tumour (assuming intracellular pH values of around 6·5 for the tumour and 7·5 for normal cells) and its concentration in the tumour is almost twice as high as in the liver. However, sulphamethoxazole (compound d) is concentrated to an even greater extent while two sulphonamides with inappropriate pKa's, sulphanilylglycine (compound a) and sulphanilamide (compound j) are also taken up selectively by the tumour. No close correlation therefore exists between pKa and accumulation of the sulphonamide in malignant cells.

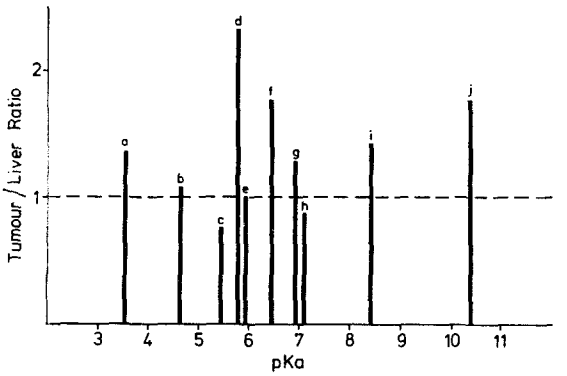
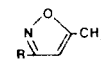


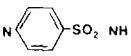
Fig. 1. Tumour: liver concentration ratios of a range of sulphonamides of varying pKa values

(*a*)	*sulphanilylglycine*	(*f*)	*sulphadiazine*
(*b*)	*sulphisoxazole*	(*g*)	*sulphamerazine*
(*c*)	*sulphamethizole*	(*h*)	*sulphathiazole*
(*d*)	*sulphamethoxazole*	(*i*)	*sulphapyridine*
(*e*)	*sulphaphenazole*	(*j*)	*sulphanilamide*

After an injection of 500 mg/kg of the sulphonamides, and assuming uniform distribution, their optimum concentration in tissues could be as high as 0·5 μg/mg wet weight tissue = 2·5 μg/mg dry weight. Some of the sulphonamides have tumour concentrations of this value. (Table 2), showing they are readily

Table 2

Compound	pKa	Tumour[x] Conc.	T/L[†] Ratio	Solubility*	Po[≠]	Compound	pKa	Tumour[x] Conc.	T/L[†] Ratio	Solubility*	Po[≠]
$R.CH_2\,COOH$ Sulphanilylglycine	3·52	0·77	1·36	18·7	0·026	 Sulphamerazine	6·93	1·05	1·27	1·12	0·75
 Sulphisoxazole	4·62	2·41	1·07	13·1	0·134	 Sulphathiazole	7·1	1·14	0·87	1·62	0·37
 Sulphamethizole	5·45	0·92	0·74	15·0	0·077	 Sulphapyridine	8·43	0·73	1·43	0·06	7·89
 Sulphamethoxazole	5·81	1·67	2·31	12·5	0						
 Sulphaphenazole	5·91	1·33	1·02	2·36	1·29	R–H Sulphanilamide	10·43	1·64	1·77	13·9	0·13
 Sulphadiazine	6·48	0·95	1·78	1·01	0·055						

R = H_2N–  –SO_2NH

x = ug/mgm dry weight after i.p. injection of 500mg/kg in DMSO

† = Ratio of concentrations between liver and tumour

* = Solubility in 0·1M phosphate buffer at pH 7·5 and 37°C in mg/ml.

≠ = Partition coefficient between phosphate buffer and n-octanol

taken up by cancer cells. However, these sulphonamides which attain the highest concentration in the tumour are not necessarily those which show the greatest difference in concentration between tumour and liver. Thus sulphisoxazole is taken up very readily by the tumour, but also by the liver since the ratio of concentrations in the two tissues is about one. Conversely, sulphadiazine is not concentrated in the tumour to the same extent as sulphisoxazole but there is a large difference between tumour and liver concentrations. Judged by these criteria, the optimum sulphonamide to use in the design of cytotoxic agents would presumably be sulphamethoxazole, which is readily taken up by the tumour and which shows a large difference in concentration between tumour and liver.

A series of dimethyltriazenes were prepared from five of the sulphonamides shown in Fig. 1, and their activity against the TLX/5 lymphoma compared with the activity of 3,3-dimethyl-1-phenyltriazene. From Fig. 2 it can be seen that the majority of the triazenes can extend the life span of the animals bearing TLX/5 lymphoma by about 60% at the optimum dose. A significant anti-tumour effect is usually observed at three two-fold spaced dose levels. The triazenes prepared from sulphamethoxazole, sulphadiazine and sulphathiazole are no more selective in their anti-tumour activity

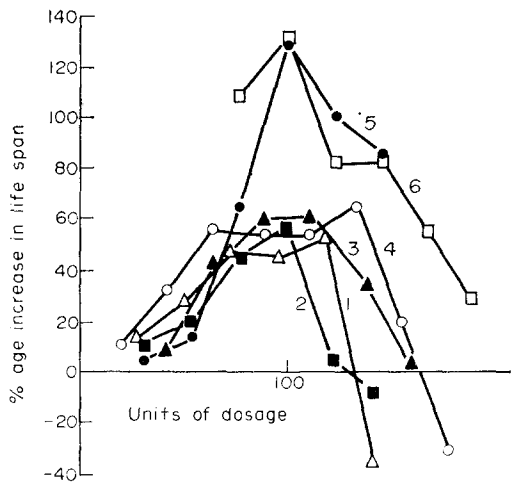


Fig. 2. Effects of various sulphonamide triazenes on the growth of the TLX/5 lymphoma in CBA/LAC mice

(1) *phenyltriazene optimal dose* 32 *mg/kg* × 5
(2) *sulphathiazole triazene* 10-344 *optimal dose* 160 *mg/kg* × 5
(3) *sulphamethoxazole triazene* 10-353 *optimal dose* 75 *mg/kg* × 5
(4) *sulphadiazene triazene* 10-279 *optimal dose* 50 *mg/kg* × 5
(5) *sulphapyridine triazene* 10-345 *optimal dose* 200 *mg/kg* × 5
(6) *sulphamerazine triazene* 10-342 *optimal dose* 40 *mg/kg* × 5

The optimal dose is the dose level that gives the highest %I.L.S.

than 3,3-dimethyl-1-phenyltriazene although both sulphamethoxazole and sulphadiazine

have a high tumour/liver concentration (Table 2). However, two triazenes, those prepared from sulphamerazine and sulphapyridine are very active on the TLX/5 tumour and at their optimum doses can increase the life span of tumour-bearing animals by 130%. They are also active at, at least, four two-fold spaced dose levels. As expected, the substitution of the aromatic amino group by a dimethyltriazeno group decreases water solubility and increases the octanol/water partition coefficient (Table 3). The two highly active triazenes are also the two least water soluble derivatives.

Table 3

Compound	Solubility (μg/ml)	P_O	Compound	Solubility (μg/ml)	P_O
Sulphadiazine	1,010	0.055	Sulphadiazine triazene	68.0	0.87
Sulphamerazine	1,120	0.75	Sulphamerazine triazene	10.1	6.07
Sulphathiazole	1,620	0.37	Sulphathiazole triazene	49.2	11.00
Sulphapyridine	60	7.89	Sulphapyridine triazene	15.4	30.64
Sulphamethoxazole	12,500	0	Sulphamethoxazole triazene	412	2.25

Figure 3 A and B shows the radioactivity of tumour and liver homogenates after injection of ^{14}C ring labelled and ^{3}H methyl labelled sulphadiazine triazene. Although the tumour/liver ratio of sulphadiazine is 1·78, the radioactivity in the liver and tumour is about the same after injection of either of the labelled triazenes of sulphadiazine. This radioactivity may of course represent not only the triazene but also its metabolites and these must be identified and measured before the true distribution of the triazene between liver and tumour can be estimated. However, similarly to the nitrogen mustard derivative of sulphadiazine, the addition of the triazeno group to the sulphonamide may have resulted in the loss of the ability of the sulphonamide to concentrate in tumours.

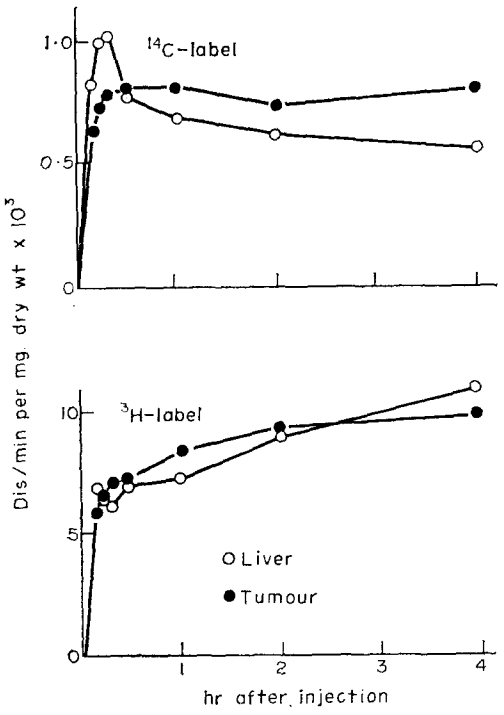
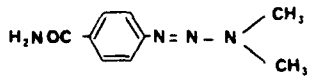


Fig. 3. Radioactivity in homogenates of tumour and liver at various times after injection of ^{14}C-labelled sulphadiazine triazene (5 mC/mmole, 6 mg/kg intraperitoneally) and ^{3}H-labelled sulphadiazine triazene (200 mC/mmole; (200 mC/mmole; 1.53 mg/kg intraperitoneally).

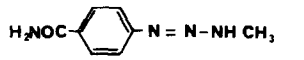
Agents which show a wide variation in their anti-tumour effects depending on the dose schedules used are usually phase specific agents acting exclusively on cells in one phase of the cell cycle. Alkylating agents are not phase specific and there is usually little difference in their anti-tumour action whether they are given as large single doses or as the same amount of drug fractionated over several days. If the triazenes act by methylating macromolecules, they should be similar to alkylating agents in as much as they would not be expected to be phase-specific and therefore not dose schedule dependent. However, both 1-(*p*-carbamoylphenyl)-3,3-dimethyl triazene and its monomethyl analogue are clearly dose schedule dependent (Table 4). The greatly improved anti-tumour effects of daily doses compared with single doses appear to be due to the non-additive toxicity of the daily doses combined with their additive anti-tumour action. Thus, five daily doses of 200 mg/kg of 1-(*p*-carbamoylphenyl)-3,3-dimethyltriazene are not a lot more toxic than a single dose of 200 mg/kg, while one injection of 400 mg/kg is lethal.

Table 4



1-(p-Carbamoylphenyl)-3,3-dimethyltriazene

Dose† mg/kg	% I.L.S.*	Dose† mg/kg	% I.L.S.*
12.5	3.9	12.5 x 5	13.7
25	17.6	25 x 5	54.9
50	17.6	50 x 5	50.9
100	23.5	100 x 5	47.9
200	21.5	200 x 5	3.9
≠ 400	− 60.8	≠ 400 x 5	− 60.8



1-(p-Carbamoylphenyl)-3-methyltriazene

Dose‡ mg/kg	% I.L.S.*	Dose‡ mg/kg	% I.L.S.*
3.75	0	3.75 x 5	4.2
7.5	0	7.5 x 5	6.3
15	4.2	15 x 5	35.4
30	10.4	30 x 5	50.6
60	18.8	60 x 5	10.4
≠ 120	− 35.4	≠ 120 x 5	− 45.8

† compounds administered intraperitoneally as a suspension in 10% acetone/oil.
‡ compounds administered intraperitoneally as a suspension in 10% DMA/oil.
* I.L.S. = increase in life span compared to controls.
≠ animals die from drug induced toxicity.

Similarly, with the monomethyltriazene, there is little difference in toxicity between a single dose of 60 mg/kg and five daily doses, whereas a single dose of 120 mg/kg is lethal. If both the toxicity and anti-tumour effects are due to methylation of macromolecules, then it is possible that normal tissues sensitive to triazenes can recover from this methylation possibly by a repair mechanism whereas the TLX/5 tumour cells cannot. In this connection, it is of interest that many macromolecules in tumour cells have an abnormally high level of methylation.

The dose schedule dependency of the triazenes may explain the superior anti-tumour properties of the triazenes prepared from sulphamerazine and sulphapyridine . These two chemicals were the two least soluble triazenes synthesised and since in all tests intraperitoneal injections were

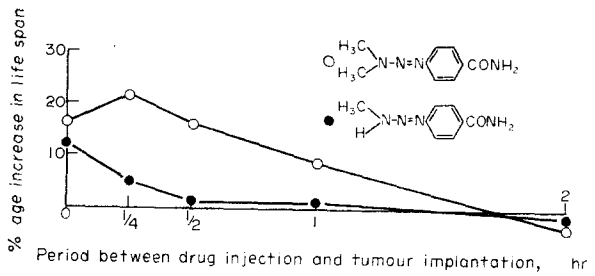


Fig. 4. "Biological half-lives" of two phenyltriazenes.

used, it may well be that they are less well-absorbed from the peritoneal cavity and thus act as a "depot" form of the drug, giving prolonged blood levels. It would be of interest to determine whether the more water-soluble triazenes would be just as active if they were given by infusion. That the biological half-life of the triazenes is an important factor in determining their anti-tumour effect is demonstrated by the results in Fig. 4. 1-(*p*-Carbamoylphenyl)3,3-dimethyl triazene has a much longer half-life than its active metabolite 1-(*p*-carbamoylphenyl)-3-methyltriazene and is a more effective anti-tumour agent. Direct administration of the active metabolite is less effective than using the dimethyltriazene, because the latter, which is demethylated by liver microsomes, serves as a slow release form of the active metabolite which is itself chemically unstable. Obviously the design of monomethyl triazenes with long biological half-lives is of interest, and this is at present under investigation.

Clinically it may well be that the results with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC) may be improved by using it at its optimum dose schedule, possibly as an infusion. Although the synthesis of tumour inhibitory triazenes based on sulphonamides can give agents with greater anti-tumour selectivity, this may not be the result of their selective deposition in tumour, but their insolubility which leads to prolonged blood levels after intraperitoneal injection. The design of monomethyltriazenes of longer chemical half-life and their use at their optimal dose schedules may lead to chemicals with improved anti-tumour properties.

REFERENCES

1. C. D. Stevens, P. M. Quinlin, M. A. Meinken and A. M. Kock, Sulfapyrazine precipitated in cancer tissue upon repeated glucose injections. *Science* **112,** 561 (1950).
2. N. Calvert, T. A. Connors and W. C. J. Ross, Aryl-2-halogenoalkylamines XXV. Derivatives of sulphanilamide designed for selective deposition in neoplastic tissue. *Europ. J. Cancer* **4,** 627 (1968).

3. G. ABEL, T. A. CONNORS, W. C. J. ROSS, NGUYEN-HOANG-NAM, H. HOELLINGER and L. PICHAT, The selective concentration of sulphadiazine and related compounds in malignant tissue. *Europ. J. Cancer* **9,** 49 (1973).
4. H. HOELLINGER, NGUYEN-HOANG-NAM and L. PICHAT, Synthèse de la sulfadiazine et de ses dérivés: la (N^4-acétylsulfanilamido)-2 pyrimidine, la (benzénesulfonamido)-2 pyrimidine et la (*p*-toluènesulfonamido)-2 pyrimidine marquées par le ^{14}C dans le noyau pyrimidine. *J. labld Compds* **9,** 161 (1973).
5. H.-H. KÜHNAU and H.-R. SCHÜTTE, Synthesen radioaktiv markierter verbindungen; Synthese von N-Phenyl-N′-dimethylharnstoff (Fenuron) – [Phenyl-^{14}C] und N-Phenyl-N′-dimethylharnstoff (Fenuron)—[$^{14}CH_3$] bzw. N-(p-Chlorphenyl)-N′-dimethylharnstoff (Monuron) – [$^{14}CH_3$]. *Z. Chem.* **9,** 231 (1969).

Europ. J. Cancer Vol. 11, pp. 795–799. Pergamon Press 1975. Printed in Great Britain

A Combination of Adriamycin, CCNU and Hydroxyurea in the Treatment of Disseminated Bronchogenic Carcinoma

PIERRE ALBERTO,* LAURENT BARRELET,† BERNARD CHAPUIS† and BERNARDO GARCIA†

Division of Oncohematology, Department of Medicine, Geneva, Switzerland

Abstract—*Twenty-eight patients with disseminated bronchogenic carcinoma were treated with an induction therapy consisting of adriamycin, CCNU and hydroxyurea. Those who achieved objective remission or tumor stabilisation were randomized to a maintenance treatment consisting either of the same as the induction treatment or a four weekly alternating sequence of the induction treatment and a four drug combination of vincristine, methotrexate, cyclophosphamide and procarbazine.*

Of the initial 28 patients, 8 had objective responses, 4 showed minor improvement, 8 remained without change, 5 progressed and 3 died within the first 3 weeks of treatment. Responses were more frequent among anaplastic (6/12) than epidermoid (1/11) tumors. The toxicity consisted of thrombopenia, leucopenia, nausea and vomiting. The myelodepressive effect was found to be slightly cumulative when treatment was repeated. The survival of responders was longer than that of non-responders. No difference was observed between the two maintenance treatments.

INTRODUCTION

In a previous study of the Swiss Group for Clinical Cancer Research [1], a combination of methotrexate, vincristine, cyclophosphamide and procarbazine proved useful in the treatment of disseminated primary lung carcinoma. A 50% remission rate was obtained when the four drugs were used simultaneously, compared with 20% for a sequential regimen. The remission rate was related to the tumor cell type, anaplastic small cell tumors being the most responsive, and epidermoid tumors the less responsive. The effect of response on survival was found to be small, even for small cell carcinoma, where the median survival of responders was 25 weeks longer than non-responders. Thus far, the overall clinical value of this polychemotherapy schedule remained unsatisfactory, in view of an insufficient effect of the treatment on survival, and also of the low response rate in squamous cell tumors.

Since this previous study, new agents have been used with some success in the treatment of disseminated lung carcinoma. Among these are adriamycin [2], nitrosoureas [3, 4, 5] and hydroxyurea [6]. A combination of these drugs could possibly contribute to improve the overall result of chemotherapy, either for the treatment of tumors resistant to and/or relapsing after another chemotherapy, or as a part of a more sophisticated program using, as soon as chemotherapy is indicated, a sequential regimen of different combinations.

With this background in mind, the goals of the present study were as follows:

(1) To determine the clinical tolerance of a combination of adriamycin (ADM), cyclohexyl-chloroethylnitroso-urea (CCNU) and hydroxyurea (HU), given simultaneously in short intensive courses.

Accepted 23 June 1975

*Chairman, Swiss Group for Clinical Cancer Research, and Head, Clinical Oncology Unit, Dpt. of Medecine, Geneva, Switzerland.

†Clinical Oncology Unit, Dpt. of Medecine, Geneva, Switzerland.

Address for reprints: Dr. Pierre Alberto, Centre d'Onco-Hématologie, Hôpital Cantonal, 24, rue Michel-du-Crest, 1205 Geneva, Switzerland.

(2) To assess the therapeutic effectiveness of this polychemotherapy in the treatment of disseminated bronchogenic cancer.
(3) To investigate the importance of the cell type on the therapeutic responsiveness to the experimental treatment.

MATERIAL AND METHODS

Between January and May 1972, 28 patients with histologically or cytologically proven disseminated or relapsing bronchogenic carcinoma were treated according to this study. Histological typing corresponds to the WHO classification proposed by Kreyberg *et al.* [7]. Table 1 summarizes the clinical characteristics of this group. Nine patients had been treated before the present study, 5 surgically (3 pneumonectomies, 1 lobectomy, 1 blank thoracotomy), 3 with chemotherapy and 1 with radiotherapy. All patients received antibiotic treatment for at least one week before the required pretherapeutic investigations were performed.

Table 1. *Clinical characteristics of* 28 *patients with bronchogenic carcinoma treated with a combination of adriamycin, CCNU and hydroxyurea*

Number of patients		28
Cell type: Epidermoid	11	
Large cell anaplastic	7	
Small cell anaplastic	5	
Adenocarcinoma	3	
Unspecified	2	
Sex (Male/Female)		26/2
Mean age (range)		59·6 (43–76)
Prior treatment: Surgery		5
Radiotherapy		1
Chemotherapy		3
Disease extension: Limited to lungs and mediastinum		19
Disseminated		9

All patients were given the same induction treatment (Fig. 1) consisting of two identical five day courses of ADM + CCNU + HU, during the first and the fifth weeks. ADM (20 mg/m^2 by rapid intravenous injection) was given on days 1 and 2. CCNU (80 mg/m^2 orally) was given in one dose on day 1. HU (1000 mg/m^2 orally in three divided doses) was given daily for 5 consecutive days. Clinical and laboratory tests were repeated weekly until the

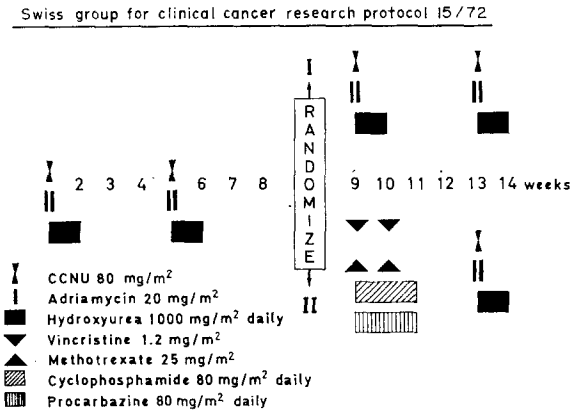


Fig. 1. *Study design for protocol* 15/72.

eighth week, at which time the induction phase was terminated and the therapeutic response evaluated. Cases with objective remission or tumor stabilization were randomized into two groups for maintenance treatment, the first receiving the same regimen as during the induction, the second a monthly alternation of the induction regimen and a four drug combination with vincristine, 1·2 mg/m^2 i.v. day 1 and 8; methotrexate 25 mg/m^2 i.v. day 1 and 8; cyclophosphamide 80 mg/m^2 orally day 1 to 14 and procarbazine, 80 mg/m^2 orally day 1–14. The therapeutic responses were evaluated at the end of the eighth week and classified in

Table 2. *Outcome of combination therapy with adriamycin, CCNU and hydroxyurea in* 28 *patients with bronchogenic carcinoma*

		Breakdown of patients by cell type				
	Number of patients	Epiderm.	Small cell	Large cell	Adeno ca.	Unspec.
Evaluable	28	11	5	7	3	2
Objective responses	8	1	3	3	0	1
Short responses (< 8 weeks)	4	2	1	0	1	0
No change	8	4	1	0	2	1
Progression	5	4	0	1	0	0
Early death	3	0	0	3	0	0

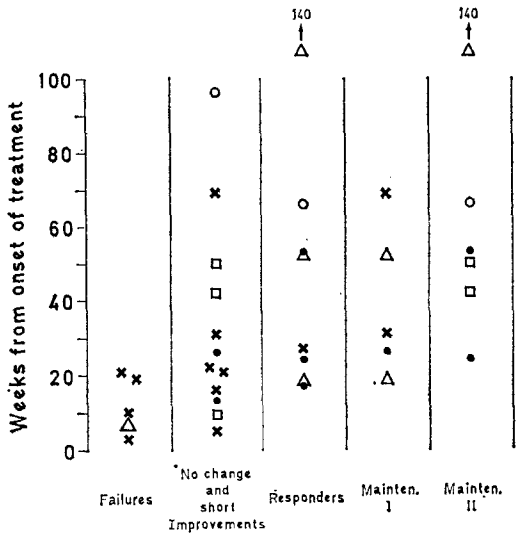


Fig. 2. Protocol 15/72. Breakdown of survival as per response category and maintenance treatment.
× *epidermoid* (11)
△ } *large cell anaplastic* (7)
● } *small cell anaplastic* (5)
□ *adenocarcinoma* (3)
○ *unspecified* (2)

5 categories: objective remission (more than 50% tumor reduction as measured by the product of two diameters, or equivalently evaluated if only one measurable diameter was available), short improvement (with tumor regrowth before the eighth week), no change (tumor stabilisation), tumor progression, and early death (within 3 weeks, related or not to toxicity). Interpretation of each therapeutic result was controlled by at least two of the authors. Whenever possible, maintenance treatment was given during the entire period of remission or stabilization. All patients were followed until death.

RESULTS

Table 2 summarizes the therapeutic responses. Of 28 patients, all were adequately treated, and 8 showed an objective remission. Five tumors were progressive, three patients died before the fourth week, two due to toxic agranulocytosis with sepsis. Eight cases were graded as no change and 4 remissions relapsed before the eighth week of treatment.

As for the influence of the cell type on the therapeutic responsiveness, 3/5 patients showed positive antitumour response for anaplastic small cell carcinoma, 3/7 for anaplastic large cell carcinoma, 1/11 for squamous cell carcinoma, and 1/2 for unspecified type of carcinoma.

Among 16 patients in remission or stabilisation at the end of induction, 5 received no maintenance treatment: two because of refusal, two because of geographical difficulties, and one because of obstructive jaundice. Eleven patients were randomized for the maintenance phase, with 5 being allocated to the induction regimen, 6 to the alternating three and four drug regimens.

Figure 2 illustrates the survival by response category and maintenance treatment.

Sixty-eight courses of ADM + CCNU + HU were administered to the 28 patients, the number of courses per patient varying from 1 to 8. The mean dosage of each of the 3 drugs actually administered in each course as compared with the theoretical protocol amounts

Table 3. Toxic complications of combination therapy with adriamycin CCNU and hydroxyurea in 28 patients with bronchogenic carcinoma

	Toxicity*								
	Haematologic†			Digestive			Alopecia		
	0	low	high	0	low	high	0	low	high
Induction treatment:									
All patients	2	18	7	14	12	1	17	10	0
Responders only	0	6	2	5	3	0	3	5	0
Maintenance treatment:									
Three drugs	0	1	4	1	4	0	2	3	0
Three drugs alternated with four drugs	0	4	2	3	3	0	3	3	0

*One patient inevaluable (death within 1 week).
†Key for haematologic toxicity:
Low: leucocytes between 1,000 and 4,000/mm³ or thrombocytes between 30,000 and 150,000/mm³.
High: leucocytes less than 1,000/mm³ or thrombocytes less than 30,000/mm³.

was 96% for ADM, 96% for CCNU (approximated to 10 mg) and 92% for HU (approximated to 250 mg). The most serious toxic manifestation was hematologic depression. Other observed side effects were digestive (nausea, vomiting, stomatitis), alopecia and benign cutaneous desquamation. Table 3 summarizes the observations of toxicity for induction and maintenance treatment. Seven patients reached WBC values of less than 1000/mm^3 or platelet values of less than 30,000/mm^3 during induction. Figures 3 and 4 relate the hematologic toxicity to the number of previously given courses of the 3 drug combination inducting

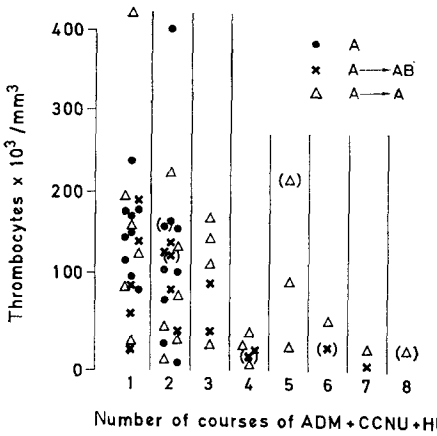


Fig. 3. Protocol 15/72. Hematologic toxicity: Nadir of thrombocyte count after each successive course of ADM+CCNU+HU. (A = ADM+HU) − (AB = ADM+CCNU = HU Alternating with VCR+MIX+CTX+PC. Figures in ( ) correspond to courses with reduced dosage.

CCNU. These values are compatible with a cumulative toxicity on platelets and to a lesser degree on leucocytes, although this never gave rise to serious marrow hypoplasia. Cardiac toxicity was never observed. The total dose of ADM ranged from 60 to 420 mg.

DISCUSSION AND CONCLUSIONS

The objectives of this study were to determine the clinical tolerance and therapeutic usefulness of a combination of ADM + CCNU + HU in primary lung carcinoma. The results obtained suggest the following answers:

(1) With the dosages used in this study, a combination of ADM + CCNU + HU is practically feasible, even for out-patients. The limiting toxicity is hematologic, with a slight tendency to be cumulative. Other side effects were principally digestive. In the dosages used, the hematologic depression was severe in many cases and was responsible for two toxic deaths following the first course of treatment. Concerning adriamycin, we never observed cardiac toxicity. Practically, it would probably have been preferable to give the full dosage of ADM in each course on day 1, thus avoiding a second i.v. injection.

(2) The combination ADM + CCNU + HU as used here is effective, with an overall remission rate of 29%. Moreover the survival was longer for responders (17–140 weeks) than for treatment failures (3–21 weeks).

Like other previously studied combination programs for lung carcinoma, the remission rate seems to be cell type dependant. Comparing

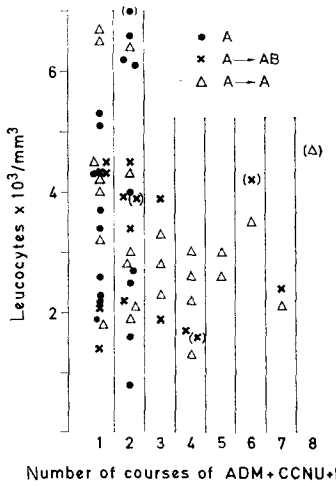


Fig. 4. Protocol 15/72. Hematologic toxicity: Nadir of leucocyte count after each successive course of ADM+CNU+HU. (A = ADM+CCNU+HU) − (AB = ADM+CCNU+HU Alternating with VCR+MIX+CIX+PC. Figures in ( ) correspond to courses with reduced dosage.

epidermoid tumors with both anaplastic types taken together, the corresponding figures are 1/11 and 6/12. This difference is significant for $p = 0{\cdot}05$ (chi square tests with Yates correction = 4·5).

In conclusion, a combination ADM + CCNU + HU as presented here could possibly be of some therapeutic value in the palliative treatment of primary lung carcinoma, particularly after failure of more commonly used drugs, as cyclophosphamide, methotrexate or vincristine. Moreover, the informations gained during this study about the tolerance of ADM, CCNU and HU in combination could be of interest in the elaboration of further chemotherapeutic programs.

Acknowledgement—The authors wish to thank Farmitalia Milano and Farmitalia Freiburg in Breisgau for the supply of Adriamycin as well as Dr. S. K. Carter, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, U.S.A. for the supply of CCNU.

REFERENCES

1. P. ALBERTO, Remission rates, survival and prognostic factors in combination chemotherapy for bronchogenic carcinoma. *Cancer Chemother. Rep.* Part 3, **4,** 199 (1973).
2. S. K. CARTER, A. DI MARCO, M. GHIONE, I. H. KRAKOFF and G. MATHÉ, Edited by *International Symposium on Adriamycin,* Springer, Berlin, Heidelberg, New York, 1972.
3. V. T. DE VITA, P. P. CARBONE, A. H. OWENS, Jr, G. L. GOLD, M. J. KRANT and J, EDMONSON, Clinical Trials with 1,3-Bis (2-chloroethyl)1-1nitrosourea, NSC-409962, *Cancer Res.* **25,** 1876 (1965).
4. H. H. HANSEN, O. S. SELAWRY, F. M. MUGGIA, M. D. WALKER, Clinical studies with 1-(2-Chloroethyl)-3-Cyclohexyl-1-Nitrosourea (NSC 79037), *Cancer Res.* **31,** 223 (1971).
5. R. M. WHITTINGTON, J. L. FAIRLY, H. MAJIMA, M. E. PATNO and R. PRENTICE, BCNU (NSC-409962) in the treatment of bronchogenic carcinoma. *Cancer Chemother. Rep.* Part 1 **56,** 739 (1972).
6. D. T. KAUNG, W. S. WALSH, S. SBAR, M. E. PATNO, Hydroxyurea (NSC-32065) in therapy for nonresectable cancer of the lung. *Cancer Chemother. Rep.* **52,** 271 (1968).
7. L. KREYBERG, A. A. LIEBOW and E. A. BEHLINGER, *Histological Typing of Lung Tumors.* World Health Organization, Geneva, 1967.

Europ. J. Cancer Vol. 11, pp. 801–807. Pergamon Press 1975. Printed in Great Britain

The Second Generation of EORTC-ICIG* Experimental Screening for Systemic Immunity Adjuvants

Its Significance for Cancer Immunotherapy A Comparison of BCG and its Hydrosoluble Extract

G. MATHE, OLGA HALLE-PANNENKO, IRENE FLORENTIN, MARTINE BRULEY-ROSSET, M. KAMEL, I. J. HIU and CHARLOTTE BOURUT

**Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, (INSERM et Association Claude-Bernard), 14–16 avenue Paul-Vaillant-Couturier, 94800-Villejuif, France.*

Abstract—*Two systemic adjuvants of immunity: fresh living BCG and one hydrosoluble extract from BCG called HIU II were submitted to a new screening battery composed of four immunological tests: the haemolytic plaque-forming cell assay, a macrophage activation test and two immunoprophylaxis assays, one using L1210 leukaemia and the other the solid Lewis tumour. This study again emphasizes the importance of the parameters of route and time in adjuvant administration. Depending on the experimental conditions, the effects of these two adjuvants were found to be either positive, since potentialization of antibody formation, macrophage activation and tumour growth inhibition could be observed, or nil, or disastrous, since BCG could enhance the Lewis tumour growth.*

The importance of these findings is discussed with reference to the possible application of non specific cancer immunotherapy in man.

INTRODUCTION

THE SYSTEMATIC search for new compounds prepared by extraction or synthesis and screened for their oncostatic properties [1], or simultaneously their oncostatic, immunostimulant, and virostatic actions [12] has been very rewarding in cancer chemotherapy.

We have shown that active immunotherapy (AI), which is the immune stimulation applied *after* the tumour is established, is able to eradicate an experimental murine neoplasia, provided the number of tumour cells produced by the graft [3, 4], or left by surgery [5] or chemotherapy [6] does not exceed 10^5. The most efficient form of AI is the combination of irradiated tumour cells and BCG [3, 4].

After our first clinical randomized trial on active immunotherapy had demonstrated its efficacy in eliminating the residual imperceptible disease left by chemoradiotherapy in patients suffering from acute lymphoid leukaemia [7, 8], many other studies were conducted in man, some with negative results [9] but most of them showing a beneficial action attributable to active immunotherapy: they included leukaemic lymphosarcoma [10], non leukaemic lymphosarcoma [11], acute myeloid leukaemia [12, 13], chronic myeloid leukaemia [14], melanoma [15, 16] and bronchial carcinoma [17].

In our trials, fresh living BCG from Pasteur

Accepted 11 April, 1975.

Institute was applied to skin scarifications [7]; in other trials it was either administered in the same way or injected with ordinary needles i.d. or s.c., or with a heaf gun [12]. In some trials, *C. parvum* or *C. granulosum* [18] or poly I-poly C [8] were combined with BCG.

There are now enough positive results to advise clinicians to use active immunotherapy in the treatment of imperceptible residual disease [19], that is to say in patients submitted to treatment(s) but presenting a poor cure expectancy. A stumbling block in its scientific use is nevertheless caused by the various responses given by different adjuvants, and even between different BCG preparations and modes of administration. Hence, it was necessary to set up an experimental investigation using laboratory animals to obtain further insight into the mechanism of action of the agents already used as immunity systemic adjuvants. For this purpose, the first battery composed of five tests was established between 1970 and 1973: the haemolytic plaque-forming cell test, the graft vs host reaction, and the prevention of three tumours: a leukaemia, the L1210; a carcinoma, the Lewis tumour; and a sarcoma, the ICIG CI_1 tumour [20]. Using this battery we compared three families of agents considered to be adjuvants: (a) preparations of BCG and extracts of BCG, (b) *Corynebacterium parvum* and preparations of *C. granulosum*, (c) polynucleotides. They were injected at the optimal dose (determined by preliminary experiments) via four routes: i.v., i.p., s.c. and i.d.

The conclusions drawn from these trials are the following: an agent can be an immunostimulator using one modality of administration or one preparation, and immunosuppressive using others; some agents can exert an antitumour effect with a given modality of administration or preparation, and enhance (solid) tumour growth with another. The agent more frequently exerting immunostimulation and an oncostatic effect is fresh Pasteur Institute BCG.

Repeating the same tests several times with the same preparations and comparing our results with those obtained by the workers who supplied the material, we found that: (a) the test giving the least reproductible result is GVH; (b) there are no great differences between the results given by the solid Lewis tumour and the solid ICIG CI_1 tumour; (c) our screening quite often gave negative results for agents which had been found to be "adjuvants" by workers using other tests. Hence we decided to suppress: (a) the GVH reaction which appears to be unsatisfactory; (b) the ICIG CI_1 prophylaxis test in order to save mice and money.

Moreover, several lines of evidence provide compelling reasons for considering macrophages as agents in tumour killing. Administration of agents which stimulate macrophage functions is associated with resistance to tumour growth [21, 22, 23], and *in vitro*, such activated macrophages can kill target cells in a non specific manner [24]. Accordingly, we have added a macrophage activation test which appears to be very sensitive.

In this paper we will describe our present (second generation) battery and, as examples, we will present the results from two agents, the fresh Pasteur Institute BCG and the HIU II soluble extract of BCG.

MATERIAL AND METHODS

Mice. (DBA/2 × C57B1/6) F1 three-month-old female mice were obtained from "Orleans La Source" Laboratory.

Material. Two substances were submitted to our screening. (a) living BCG: a fresh preparation, less than 7 days old, was supplied by the Pasteur Institute and used at the dose of 1 mg per mouse (7×10^6 viable units/mg); (b) HIU II: a soluble extract from BCG membrane was prepared in our institute by Dr. Hiu [25] and used at the dose of 0.5 mg per mouse. Mice were injected with these two agents either i.v. or s.c. The day of injection depended upon the test and was determined by preliminary experiments. The four tests chosen for this screening are summarized in Table 1.

Tests

Macrophage activation test. Peritoneal cells, obtained from adjuvant treated or from control mice by washing the peritoneal cavity, were suspended in RPMI 1640 culture medium (Gibco) supplemented with 10% foetal calf serum (FCS) (Gibco) at a concentration of 2×10^6 macrophages/ml. One ml of this suspension was seeded in each well of a disposable plastic culture plate (Falcon Disposo Trays 96 CVTC). Plates were incubated for two hr at 37°C and then washed vigorously to remove the non adherent cells. At this point, ^{51}Cr labeled L1210 tumour cells were added to give an optimal ratio of one target cell to twenty macrophages (10^5 tumour cells/ml/well). After 18 hr of incubation at 37°C in a water saturated air-CO_2 atmosphere, the amount of ^{51}Cr released in 0·8 ml of culture medium was determined in a Packard autogamma spectro-

Table 1. *Tests and methods used for the screening of immunity systemic adjuvants*

Technique	Day 0	Day of adjuvant administration	Results
(1) Macrophage activation	Peritoneal macrophages harvested	− 3, −7, or −14	Tumour cell non specific lysis (^{51}Cr release)
(2) PFC (Jerne assay)	10^9 SRBC i.p.	−14, −5, −3, 0 + 1, +2, or + 3	PFC at day +4
(3) L1210	10^3 leukaemic cells i.v.	−14 (BCG) − 3 (Hiu II)	Mortality
(4) Lewis tumour	2×10^6 tumour cells s.c.	−14 (BCG) − 3 (Hiu II)	Mortality

meter and the cytotoxicity was expressed in counts per minute (CPM) per culture. In each experimental group, the arithmetic mean of CPM from 8–12 cultures and its 95% confidence limits were calculated. Statistical comparisons between means were performed using the Student's *t*-test.

Haemolytic plaque-forming cell assay. Mice were inoculated with BCG or HIU II at different time intervals before (day −14, −5, −3), or at the same time (day 0), or after (day +1, +2, +3) i.p. administration of 10^9 sheep red blood cells (SRBC). Spleen cell suspensions were prepared from individual recipients four days after the immunization with SRBC and the number of plaque-forming cells (PFC) in the suspension was counted by the technique of localized haemolysis in agar (Jerne assay) [26]. The adjuvant effect was estimated by an index (I) calculated as follows:

$$I = \frac{\text{Mean number of PFC/spleen in adjuvant treated mice}}{\text{Mean number of PFC/spleen in control mice}}$$

Statistical comparisons between the means were performed using the Student's *t*-test.

L1210 immunoprophylaxis assay. L1210 leukaemia is maintained in ascitic form by serial passages performed every 6–8 days. Mice were inoculated i.v. with BCG at day −14 or with HIU II at day −3 before the i.v. administration of 10^3 L1210 cells per mouse. The mortality of the animals was recorded and the results were expressed as follows:

$$I = \frac{\text{Median survival time of adjuvant treated mice}}{\text{Median survival time of control mice}}$$

Lewis immunoprophylaxis assay. The Lewis tumour is a solid tumour which produces lung metastasis. Mice were inoculated i.v. or s.c. either with BCG at day −14 or with HIU II at day −3 before the s.c. administration of 2×10^6 tumour cells. The mortality was recorded and the results were expressed as above. The non-parametric test of Wilcoxon was used for the statistical evaluations.

RESULTS

Macrophage activation test

Peritoneal macrophages, harvested 3, 7 or 14 days after BCG or HIU II administration to (DBA/2 × C57Bl/6) Fl mice, were tested for their ability to exert a non specific cytotoxic action *in vitro* against L1210 tumour cells (Table 2).

A macrophage cytotoxic reaction could not be evidenced 3 days after s.c. BCG inoculation, whereas a significant increase in ^{51}Cr release from the tumour cells was observed after exposure to macrophage monolayers from s.c. BCG treated animals 7 or 14 days before.

Macrophages from mice injected i.v. with BCG 3 days previously were highly cytotoxic for the L1210 target cells. This activity dropped at day 7, but remained significantly higher than in control macrophage cultures, and it increased again at day 14.

The capacity of macrophages to mediate cytotoxicity was already demonstrable 3 days after s.c. or i.v. HIU II administration. This tumoricidal activity remained quite stable throughout the observation period when the adjuvant was given i.v. But the kinetics were slightly different after s.c. HIU II administration since the macrophage cytotoxic activity increased progressively with time.

Haemolytic plaque—forming cell assay

BCG and HIU II were injected at different times before or after SRBC immunization (Tables 3 and 4).

Table 2. In vitro *non specific cytotoxic activity against* L1210 ^{51}Cr *labeled tumour cells of peritoneal macrophages from animals treated with* BCG *or Hiu II,* 3, 7, *or* 14 *days before*

Day	BCG		Hiu II	
	i.v.	s.c.	i.v.	s.c.
− 3	830 ± 28* $P < 0.001$†	538 ± 18 N.S.	1209 ± 143 $P < 0.001$	1281 ± 44 $P < 0.001$
− 7	620 ± 24 $P < 0.001$	611 ± 32 $P < 0.001$	1286 ± 45 $P < 0.001$	1360 ± 117 $P < 0.001$
−14	939 ± 46 $P < 0.001$	609 ± 18 $P < 0.001$	1146 ± 154 $P < 0.001$	1473 ± 73 $P < 0.001$
Controls	546 ± 12		1059 ± 43	

*Mean number of CPM in 8–12 cultures of macrophages and the 95% confidence limits.
†Statistical analysis: P values greater than 0·05 were considered as non-significant (N.S.).

Table 3. Effect of Pasteur Institute BCG on PFC against SRBC

Days*	i.v.		s.c.	
	I†	Significance	I	Significance
−14	2·8	$P = 0.02$	0·75	N.S.
− 5	1·1	N.S.‡	1·1	N.S.
− 2	1·1	N.S.	1·1	N.S.
0	1·1	N.S.	1	N.S.
+ 1	1·1	N.S.	1	N.S.
+ 2	1·1	N.S.	0·75	N.S.
+ 3	1	N.S.	1	N.S.

* − before, + after the injection of SRBC at day 0.
†I = Mean number of PFC per spleen of BCG treated mice/Mean number of PFC per spleen of the controls.
‡Statistical analysis: P values under 0·05 are considered as non significant (N.S.).

Table 4. Effect of Hiu II (Soluble) on PFC against SRBC

Days*	i.v.		s.c.	
	I†	Significance	I	Significance
−14	0·75	N.S.‡	0·75	N.S.
− 5	0·75	N.S.	0·75	N.S.
− 3	1·6	$P = 0.05$	0·8	N.S.
0	0·75	N.S.	0·75	N.S.
+ 1	0·9	N.S.	0·9	N.S.
+ 2	0·75	N.S.	1	N.S.
+ 3	0·75	N.S.	0·75	N.S.

* − before, + after the injection of SRBC.
†I = Mean number of PFC per spleen of Hiu II treated mice/Mean number of PFC per spleen of controls.
‡Statistical analysis. P values under 0·05 are considered as non significant (N.S.).

Stimulation of antibody formation could not be evidenced when the two adjuvants were given by the s.c. route whatever their time of administration. When BCG was inoculated i.v., it caused a significant increase in the number of direct PFC per spleen only if given 14 days prior to antigen administration. Intravenous injection of HIU II 3 days before antigenic stimulation also caused a slight but significant amplification of the SRBC response.

L1210 immunoprophylaxis

Results presented in Table 5 show that the median survival time of L1210 inoculated mice was significantly prolonged only after the i.v. injection of BCG at day −14. HIU II given at day −3 was ineffective regardless of the route of administration.

Lewis immunoprophylaxis

Median survival time in Lewis tumour grafted animals was significantly delayed after i.v. BCG administration 14 days before tumour graft (Table 6). In contrast, when BCG was inoculated s.c., enhancement of tumour growth was observed and treated mice died before controls. HIU II, whatever the route of administration employed, did not affect the solid tumour development.

DISCUSSION

The present work had two main objectives: (a) to find a battery of tests capable of detecting systemic adjuvants of immunity of possible use in cancer active immunotherapy or immunoprophylaxis which are sufficiently diversified with regard to sensitivity and mechanisms of

Table 5. *Immunoprophylaxis of L*1210 *leukaemia*

Adjuvant	i.v.		s.c.	
	I†	Significance	I	Significance
Pasteur Institute* BCG	1·6	$P = 0{\cdot}05$	1	N.S.
Hiu II*	1	N.S.‡	1	N.S.

*BCG is injected at day −14, Hiu II at day −3.
†I = Median of survival of adjuvant treated animals/ Median of the controls.
‡Statistical analysis: P values under 0·05 are considered as non significant (N.S.).

Table 6. *Immunoprophylaxis of Lewis tumour*

Adjuvant	i.v.		s.c.	
	I†	Significance	I	Significance
Pasteur Institute* BCG	2·6	$P = 0{\cdot}01$	0·8	$P = 0{\cdot}02$
Hiu II*	1	N.S.‡	1·08	N.S.

*BCG is injected at day −14, Hiu II at day −3.
†I = Median of survival of adjuvant treated mice/ Median of the controls.
‡Statistical analysis: P values under 0·05 are considered as non significant (N.S.).

action; (b) to compare the fresh living Pasteur Institute BCG with a hydrosoluble extract of this mycobacteria.

In order to realize the first objective, four tests were selected to analyse the effects of adjuvants on various facets of the immune response. The haemolytic plaque forming cell assay was used to determine the effect of adjuvants on antibody formation which can be mediated by an influence on antigen processing by macrophages, helper T cell function, or a direct action on B lymphocytes. Using the macrophage activation test, we examined the effect of adjuvants on macrophages as effector cells in cell mediated immunity. The two operational tests of L1210 and Lewis tumour immunoprophylaxis enabled us to study the adjuvant induced alterations in the immune status of the host depending on interactions between cell mediated and humoral mechanisms of immunity.

The present study again shows that the parameters of route and time of administration can qualitatively and quantitatively modulate the action of adjuvants. In the macrophage activation test, BCG is more effective when given i.v. than s.c., whereas the hydrosoluble extract HIU II is equally active whatever the time and route of administration. The kinetics of action of these two agents on antibody formation is clearly different, since both BCG and HIU II potentiate the immune response when given i.v., but BCG only at 14 days prior to antigen administration, and HIU II only at 3 days before. The immunoprophylaxis assays show that adjuvants have the potential to affect tumour immunity in various ways. When BCG is inoculated i.v. 14 days before the tumour graft, it inhibits the growth of L1210 and Lewis tumour. In contrast, HIU II, given 3 days before the tumour graft, does not affect the growth of either tumour whatever its route of administration. These results indicate that the optimum time may not be identical for the macrophage activation test, the Jerne assay and tumour immunoprophylaxis. We may therefore be misled by a negative or unfavorable effect following adjuvant administration at a given time since another time schedule, may, on the contrary, produce a positive effect.

The macrophage activation test which was recently introduced into the battery has provided particularly interesting data permitting the early detection of the effects of adjuvants. This induction of tumoricidal activity in macrophages seems to be a general mechanism of action of immunostimulatory substances [27]. A macrophage population, selectively killing neoplasic cells, would have considerable importance and all procedures that lead to the production of cytotoxic macrophages may be essential for successful immunotherapy. Unfortunately, there does not seem to be a constant correlation between the ability of one adjuvant to induce a macrophage activation and its *in vivo* efficiency in non specific immunotherapy assays. Thus HIU II, which strongly activates macrophages, is totally ineffective against Lewis tumour growth, whereas BCG, used under experimental conditions where high tumoricidal activity of macrophages is induced, causes an enhancement of this tumour. This enhanced tumour growth after s.c. BCG administration may be due to different phenomena such as an increased level of blocking factors [28] or an immunodepression caused by lymphocytes being trapped in the draining lymph nodes or spleen [29].

In conclusion, this battery offers an interesting and economic method of screening and comparing systemic immunity adjuvants. When considering the results obtained with the two agents chosen for this study, it is interesting to note the effectiveness of the hydrosoluble

extract of BCG in the two tests, which allows us to detect two possible modes of adjuvant action, in contrast to its ineffectiveness against tumour immunity which is obviously a highly complex multifacetted process. In conclusion, when an agent is positive in two out of the four tests in this screening battery, we consider it should be the object of further study even though some operational tests prove negative.

With regard to enhanced tumour growth induced by s.c. BCG administration, our results should make clinicians cautious about injecting BCG s.c. in the case of solid tumours, though on this point also, more pharmacological studies are necessary.

REFERENCES

1. S. Carter, Some thoughts on experimental screening. *Biomedicine* **22,** 5 (1975).
2. Institut de Cancérologie et d'Immunogénétique Brochure, 1973–1974, Villejuif.
3. G. Mathé, Immunothérapie active de la leucémie L1210 appliquée après la greffe tumorale. *Rev. franc. Etud. clin. biol.* **13,** 881 (1968).
4. G. Mathé, P. Pouillart and F. Lapeyraque, Active immunotherapy of L1210 leukaemia applied after the graft of tumour cells. *Brit. J. Cancer* **23,** 814 (1969).
5. R. B. Thompson, V. Alberola and G. Mathe, Evaluation of surgery, chemotherapy and immunotherapy on Lewis lung tumour. *Europ. J. clin. Biol.* **17,** 900 (1972).
6. G. Mathe, O. Halle-Pannenko and C. Bourut, Immune manipulation by BCG administered before or after cyclophosphamide for chemo-immunotherapy of L1210 leukaemia. *Europ. J. Cancer* **10,** 661 (1974).
7. G. Mathe, J. L. Amiel, L. Schwarzenberg, M. Schneider, A. Cattan, J. R. Schlumberger, M. Hayat, F. De Vassal, Active immunotherapy for acute lymphoid leukemia. *Lancet* **1,** 697 (1969).
8. G. Mathé, J. L. Amiel, L. Schwarzenberg, M. Hayat, P. Pouillart, M. Schneider, A. Cattan, C. Jasmin, D. Belpomme, J. R. Schlumberger, F. De Vassal, M. Musset and J. L. Misset, Immunothérapie active des leucémies aiguës et des lymphosarcomes leucémiques. Bilan de 10 ans. Etude de 200 cas. *Nouv. Presse med.* **4,** 1337 (1975).
9. Medical Research Council, Treatment of acute lymphoblastic leukaemia. Comparison of immunotherapy (BCG), intermittent methotrexate and no therapy after a five month intensive cytotoxic regimen (Concord trial). *Brit. med. J.* **4,** 189 (1971).
10. G. Mathé, P. Pouillart, L. Schwarzenberg, M. Hayat, J. L. Amiel and F. De Vassal, Leukaemic conversion of non-Hodgkin's malignant lymphomas. *Brit. J. Cancer* **31,** 96 (1974).
11. J. E. Sokal C. W. Aungst and M. Synderman. Prolongation of remission in stage I and II lymphoma by BCG vaccination. *Proc. Amer. Ass. Cancer Res.* **15,** 13 (1974).
12. R. Powles, T. J. McElmain, P. Alexander, D. Crowther, G. Fairley and M. Pike, Immunotherapy of acute myeloblastic leukemia in man. In *Investigation and Stimulation of Immunity in Cancer Patients* Recent Results in Cancer Research, No. 47 (Edited by G. Mathé and R. Weiner), Vol. 1, p. 449. Springer-Verlag, Heidelberg (1974).
13. W. R. Vogler and Y. K. Chan, Effect of Bacillus-Calmette Guérin (BCG) in prolongation of remissions in acute myeloblastic leukemia (AML). *Proc. Amer. Cancer Res.* **15,** 164 (1974).
14. J. E. Sokal and C. W. Aungst, Immunization with cultured cell BCG-mixtures. In *Investigation and Stimulation of Immunity in Cancer Patients* Recent Results in Cancer Research, No. 47 (Edited by G. Mathé and R. Weiner) Vol. 1, p. 496, Springer-Verlag, Heidelberg (1974).
15. J. U. Gutterman, C. McBride, E. J. Freireich, G. Mavligit, E. Frei III and E. M. Hersh, Active immunotherapy with BCG for recurrent malignant melanoma. *Lancet* **1,** 1208 (1973).
16. A. Z. Bluming, C. L. Vogel, J. L. Ziegler, N. Mody and G. Kamya, Immunological effect of BCG in patients with malignant melanoma. A comparison of two modes of administration. *Ann. intern. Med.* **76,** 405 (1972).
17. H. Takita and A. Brugarolas, Adjuvant immunotherapy for bronchogenic carcinoma: preliminary results. *Cancer Chemother. Rep.* Part 3 **4,** 293 (1973).

18. L. SCHWARZENBERG and G. MATHÉ, Results obtained with active immunotherapy using Corynebacteria (*C. parvum* or *C. granulosum*) in the treatment of acute lymphoid leukemia. Conference on Corynebacteria, Paris, May 1974, Springer-Verlag, Heidelberg, 1975.
19. G. MATHÉ, *Introduction to Cancer Immunotherapy*, Springer, Heidelberg, 1976.
20. G. MATHÉ, M. KAMEL, M. DEZFULIAN, O. HALLE-PANNENKO and C. BOURUT, An experimental screening for "systemic adjuvants of immunity" applicable in cancer immunotherapy. *Cancer Res.* **33,** 1987 (1973).
21. R. KELLER, B. M. OLGIVIE and E. SIMPSON, Tumour growth in nematode-infected animals. *Lancet* **1,** 678 (1971).
22. N. F. WEATHERLY, Increased survival of Swiss mice given sublethal infections of *Trichinella spiralis*. *J. Parasit.* **56,** 748 (1970).
23. J. B. HIBBS, L. H. LAMBERT and J. S. REMINGTON, Tumour resistance conferred by intracellular protozoa. *J. clin. Invest.* **50,** 45 (1971).
24. J. B. HIBBS, L. H. LAMBERT and J. S. REMINGTON, Macrophage mediated non specific cytotoxicity. Possible role in tumour resistance. *Nature New Biol.* **235,** 48 (1972).
25. I. J. HIU, Water soluble and lipid free fraction from BCG with adjuvant and antitumour activity. *Nature New Biol.* **238,** 241 (1972).
26. N. K. JERNE, A. A. NORDIN and C. C. HENRY, The agar plaque technique for recognizing antibody producing cells. In *Cell Bound Antibodies* **1,** p. 109, Wistar Institute, Philadelphia (1963).
27. M. BRULEY-ROSSET, I. FLORENTIN and A. M. KHALIL, *In vivo* and *in vitro* non-specific macrophage activation by systemic adjuvants of immunity. Submitted to International Archives of Allergy and Applied Immunology.
28. S. C. BANSAL, H. O. SJÖGREN, Effects of BCG on various facets of the immune response against polyoma tumors in rats. *Int. J. Cancer* **1,** 162 (1973).
29. D. W. DRESSER, R. N. TAUB and A. R. KRAWTZ, The effect of localized injection of adjuvant material on the draining lymph node. II. Circulating lymphocyte. *Immunology* **18,** 663 (1970).

Europ. J. Cancer Vol. 11, pp. 809–813. Pergamon Press 1975. Printed in Great Britain

The Histochemical Demonstration of Arylsulphatase in Human Tumours

JOHAN KOUDSTAAL,

Department of Pathology, "S.S.D.Z.", Stichting Samenwerking Delftse Ziekenhuizen, Reynier de Graefweg 7, Delft, Hollande

Abstract—*A membrane technique for the enzyme histochemical demonstration of the enzyme arylsulphatase is described. The activity of arylsulphatase in human tissues and in* 261 *malignant tumours in man has been investigated. Among the* 261, 59 *tumours showed slight to moderate enzyme activity. Arylsulphatase activity was found to be increased in a number of cancers of the colon and in some cases of gastric carcinoma.*

INTRODUCTION

ARYLSULPHATASES, according to enzymological nomenclature, arylsulphate sulphohydrolase (E.C. 3.1.6.1.), hydrolyse the esters of aromatic alcohols of sulphuric acid. Fromagot [1] categorized the sulphatases under four headings according to the nature of the organic moiety: phenylsulphatases, glucosulphatases, myrosulphatases and chondrosulphatases.

Seligman *et al.* [2] and Dodgson *et al.* [3] recommend the term *arylsulphatases* for the phenylsulphatases. Aryl is a chemical prefix indicating a radical belonging to the aromatic series.

Metal-salt methods and azo-dye methods [4, 5] have been used to demonstrate the localisation of the enzyme in tissues. As pointed out by Rutenberg *et al.* [6], the problems of demonstrating arylsulphatases suffers from some serious drawbacks. Much of the enzyme apparently remains soluble, even after fixation of the tissue. Enzyme activity is low, requiring long incubation periods, which provide the opportunity of diffusion of both the enzyme and the freed naphthol.

Meijer [7–9] described a method for the histochemical demonstration of some hydrolytic enzymes and oxidoreductases by using a semipermeable membrane interposed between the incubating solution and the tissue section. In this way diffusion of enzyme is prevented. On the other hand, substrate molecules and other components with a relatively low molecular weight necessary for the staining reaction may diffused through the semipermeable membranes. Application of this method to demonstrate activity of the enzyme arylsulphatase by the azo-dye method described by Rutenberg *et al.* [6] appeared to be a real improvement of the existing methods.

The present work was undertaken to investigate the activity of arylsulphatase in tissues and malignant tumours in man.

Accepted 7 April 1975.

MATERIAL AND METHODS

Human tissues, procured through surgical procedures were rapidly frozen. Excepting tissues of the central nervous system, of the bones and hemopoietic tissue, nearly all normal tissues could be investigated. In Table 2 (Results) the number and kinds of tumours are indicated. Sections 10 μ thick were cut on a cryostat microtome.

The following reagents were used: 6-bromo-2-naphthylsulphate obtained from Sigma (St. Louis, U.S.A.); Agar (special agar-noble), obtained from Difco (Detroit, U.S.A.). Semipermeable cellulose dialysis membranes with an average pore radius of 24 Å (supplier application) were obtained from Viking Co., (Chicago, U.S.A.)—Fast Blue B (Sigma).

Visking dialysis tubing was soaked in a solution of 0·100 g ethylene diamine tetra acetic acid in 250 ml of distilled water for 30 min and in three changes of distilled water for

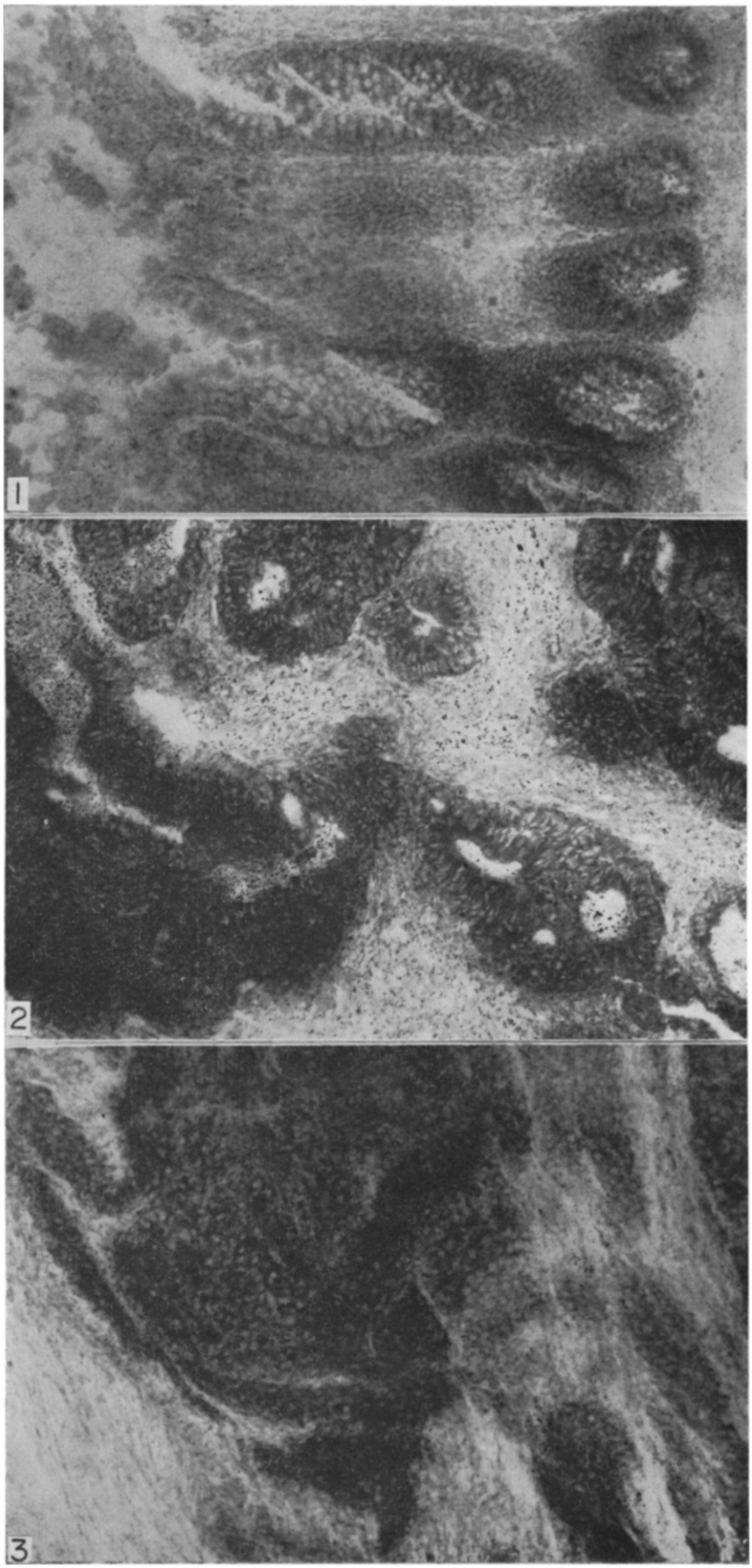


Fig. 1. *Enzyme activity of arylsulphatase in normal epithelium of the colon. The cells in the crypts showed the highest activity.*

Fig. 2. *Enzyme activity of arylsulphatase in carcinoma cells of the colon. A moderate, increased activity is seen only in carcinoma cells. Compare the activity with the activity in normal cells in Fig.* 1.

Fig. 3. *A moderate enzyme activity of arylsulphatase is shown in a carcinoma of the stomach.*

(to face p. 810)

Table 1. *Normal activity of the enzyme (aryl) sulphatase in human tissues*

Kidney	Proximal tubules	+ +/+
	Distal tubules	±
Liver	Periportal hepatocytes	+
	Pericentral hepatocytes	±
Pancreas	Acinar cells	+
Small intestine-epith cells		±
Large intestine-epith cells		+
Lung	Bronchial glands	±
Skin	Stratum granulosum	+
	Hair follicle cells	+/±
Ductus epididymis		+
Endometrium		±
Corpus luteum of ovary		±
Syncytio- and trophoblast placenta		+
Medulla of adrenal glands		±

Connective tissue, blood vessels, muscular tissue, lymfoid tissue, breast, prostate gland, a.o. no enzyme activity.
± = trace; + = slight; + + = moderate enzyme activity.

30 min. The membranes were then kept in distilled water until use.

Rings having a height of 2·5 cm were made out of a plastic tube with a diameter of 3 cm. One opening on each ring was closed with a piece of semipermeable membrane, 5 cm by 5 cm. After having been stretched tightly, the most membranes were, with the aid of elastics, fastened to the plastic rings. The incubating vessels were kept in distilled water until use.

The incubating solution consisted of:

(*a*) Agar-solution: 0·85 % sodium chloride 40 ml.
Agar 750 mg.
gently warming on a waterbath.

(*b*) Acetate buffer 0·5 M, pH 6·1, 10 ml.
6-bromo-2-naphthylsulphate 12·5 mg.
Other substrates, potassium 6-benzoyl-2-naphthylsulphate, 2-hyroxy-5-nitrophenyl-

Table 2. *Activity of (aryl) sulphatase in human malignant tumours*

	−	±	+	+ +	T
Carcinoma of the breast	69	10	3	1	83
Carcinoma of the colon/rectum	3	21	24	14	62
Carcinoma of the stomach	8	9	1	2	20
Carcinoma of the lung	16	—	1	—	17
Carcinoma of the kidney	1	7	2	—	10
Carcinoma of the renal pelvis	2	—	—	—	2
Carcinoma of the urinary bladder	1	—	—	—	1
Carcinoma of the bile ducts	1	1	—	—	2
Carcinoma of the pancreas	—	—	1	—	1
Hepatocellular carcinoma	—	—	1	—	1
Medullary thyroid carcinoma	1	—	—	—	1
Salivary gland carcinoma	1	—	—	—	1
Carcinoma of the skin (+ tongue)	2	—	—	—	2
Malignant melanoblastoma (+ metast.)	6	1	3	—	10
Carcinoma of the ovary	2	2	1	—	5
Granulosacelcarcinoma	1	—	—	—	1
Carcinoma of the endometrium	5	3	1	1	10
Carcinoma of the portio. ut.	2	—	—	—	2
Ca in situ portio. ut.	3	—	—	—	3
Carcinoma of the vulva	2	—	—	—	2
Teratocarc. of the testis	—	1	1	—	2
Embryonalcelcarcinoma	—	—	1	—	1
Seminoma	1	—	—	—	1
Carcinoid	—	1	—	—	1
Sarcoma	6	1	1	—	8
Hodgkin's disease	3	—	—	—	3
Malignant lymphoma	2	—	—	—	2
Reticul. celsarcoma	5	—	—	—	5
Histiocytose	1	—	—	—	1
M. Ewing	1	—	—	—	1

	−	±	+	+ +
Total: 261	145	57	41	18

− = no enzyme activity; ± = trace; + = slight;
+ + = moderate enzyme activity; T = Total.

sulphate = *p*-nitrocatecholsulphate, 1-naphthylsulphate, naphthol AS BI sulphate and 8 hydroxy chinoline hemisulphate dissolved in 4 drops of dimethylformamide has also been used.

(*c*) Just before use, Fast Blue B 17·5 mg is mixed with the solution obtained by mixing the solutions (*a*) and (*b*)

The incubating vessels containing substrate gel are placed in the cryostat, immediately after which the sections are mounted on the membranes. To prevent diffusion of the enzyme during the incubation period, the membrane must not only be dry, but also the sections must be free from moisture or ice.

The vessels with the mounted sections are incubated for 24 hr at room temperature.

After staining, the agar incubating-medium is removed from the vessels and the membranes with the sections are fixed with Macrodex-formal-calcium chloride. The sections are mounted with the membrane side on glass sides. Cover glass is mounted on the sections with glycerine-gelatine.

Controls consisted of sections treated with the same technique without substrates. In these cases, a coloured product never appeared. Pilot studies with a conventional method with the substrate K-6-benzoyl-2-naphthylsulphate [6] or with the substrate 6-bromo-2-naphthyl-sulphate [2], using incubations of 4 and 24 did not result in any coloured product. In another pilot study, post-coupling with Fast Blue B was applied. However, in these cases as well, no coloured product was obtained. Only the membrane technique resulted in a good, purple-coloured product. Empirically, it appeared that the unstable azo-dye Fast Blue B remained stable in the agar.

RESULTS

The enzyme histochemical method with 6-bromo-2-naphthyl sulphate gives the best results. The enzyme histochemical results with the substrate potassium 6-benzoyl-2-naphthyl sulphate were comparable to the forementioned substrate. However, the coloured product was too variable in intensity possibly caused by impurities of the substrate. With the other substrates, no activity could be shown under the described conditions.

In normal tissues only was enzyme activity observed in the epithelial cells of some organs (Table 1). In the liver the enzyme activity was moderate, especially in the periportal hepatocytes.

The activity of the enzyme is the highest in the less differentiated epithelial cells of the large intestine. The differentiated superficial cell keeps some activity of the enzyme (Fig. 1). The normal stomach showed hardly any enzyme activity. The pancreatic activity is only localized in the acinar cells. In the lungs we found only a trace of enzyme activity in some bronchial glands. Cells of the stratum granulosum and of the sheath of the hairfollicles of the skin showed a moderate enzyme activity. The strongest activity was seen in the proximal tubules of the kidney. Cells of the ductus epididymis possessed clear activity. Except for a trace of enzyme activity in the corpus luteum, the human ovaries showed no enzyme activity. The placenta had active cells in the form of syncytic- and cytotrophoblast cells. The adrenals showed only some activity in the cells of the medulla.

Activity of the enzyme arylsulphatases in human tumours is summarized in Table 2.

Generally, the enzyme activity is very low and decreased compared with the activity of normal corresponding cells. In one and the same tumour the activity is variable; generally we noted only the highest enzyme activity. A clear enzyme activity was noted in 38 of the 62 colonic-rectal carcinomas.

Thirty of the 62 carcinomas of the colon and of the rectum showed an increased activity compared with the activity of the neighbouring non-tumorous epithelium (Fig. 2). The activity was decreased in eleven tumours of this group. We also found some carcinomas of the stomach with increased activity (Fig. 3). Exceptionally, some other tumours showed a clear enzyme activity: 4 mammary carcinomas, 2 renal carcinomas, 1 pancreas carcinoma, 1 hepatocellular carcinoma, 2 carcinomas of the endometrium, 1 carcinoma of the ovary, 2 testis tumours and 3 malignant melanomas.

Non epithelial tumours generally showed no enzyme activity.

DISCUSSION

Although it is known that arylsulphatases are widespread in the organs of mammals [10,11] the biochemical significance and the localization in human tissues is not yet understood. Dodgson *et al.* [10] divided the arylsulphatase activity of ox, rat and human livers into three distinct enzymes: sulphatase A and B, readily solubilized and found in lysosomes [12]. Sulphatase C was found to be localized in the endoplasmatic reticulum of the rat liver cell [13]. All the human tissues examined contained both types of arylsulphatases. The pancreas, the kidney and the liver appeared to be the

richest sources of both types of enzyme [10]. Sulphatases A, B and C cannot be differentiated under the usual histochemical conditions [14].

In normal tissues only was there enzyme activity in epithelial cells of some organs and also some activity in the medulla of adrenal glands. Zaruba *et al.* [15] determined the arylsulphatase activity in crude skin homogenates of human embryos, 1 to 5 months old. Nitrocatecholsulphate served as a substrate of the enzyme activity. There appeared to be a correlation in time between the differentiation of the epidermis in fetal skin and the activity of arylsulphatase. The activity is correlated probably with the development of the stratum granulosum and cells of the sheath of hair follicles since we showed a considerable activity of arylsulphatase in these cells of the skin.

We did not have the opportunity to study enzyme activity in the human brain. Deficiency of the various sulphatases alone or in combination are known to be responsible for metachromatic leukodystrophy; Maroteaux-Lamy syndrome and the multiple sulphatase deficiency syndrome [16–17].

Among the 261 human malignant tumours, a number of 59 showed a slight to moderate enzyme activity. Thirty-eight colon–rectal carcinomas showed a slight to moderate enzyme activity. It should be considered remarkable, that in a number of cases, particularly in carcinomas of the colon and rectum, the enzyme activity was higher than in the normal epithelium in the neighbourhood.

Of the 95 human neoplasms examined by Rutenberg and Seligman [11] only two (a metastasis to the spleen from adenocarcinoma of colon and a carcinoma of stomach) showed a low order of sulphatase activity. Our enzyme histochemical method is comparable to the biochemical method of Rutenberg and Seligman. Comparing the results it seems that the enzyme histochemical method is more sensitive than the biochemical method.

Dzialoszynski *et al.* [18] determined activity of arylsulphatase in malignant tumours of the stomach, colon, breast and skin. The substrate for the biochemical determination was a 0·016 M solution of dipotassium 2-hydroxy-5-nitrophenylsulphate. The results obtained showed that the activity of arylsulphatase was increased considerably in malignant tissue. Arylsulphatase activity was found to be increased significantly in cancer of the colon. In all cases of gastric carcinoma the arylsulphatase activity increased markedly.

A strikingly high activity was found in malignant melanoma. Günther [19] investigated by enzyme histochemical methods, the activity of arylsulphatase in melanocytic nevi and malignant melanoblastoma of the skin. Malignant melanomas showed a high enzyme activity. Morgan *et al.* [20] reported a direct relationship between arylsulphatase, tyrosinase, DOPA oxidase and melanin production in the melanin cell cycle of human malignant melanomas. The more pigmented and differentiated, the higher the melanin and enzyme content and the more amelanotic and undifferentiated, the less enzymes. Of our 10 investigated malignant melanoms, 3 showed a slight enzyme activity. One of the negative malignant melanomas was heavily pigmented. This observation does not agree with the results of Morgan *et al.* [20].

Enzyme-histochemically we found no enzyme activity in carcinomas of the lung except one adenocarcinoma. Among the 11 cases of lung neoplasms studied biochemically by Morgan *et al.* [21] only one was comparable in enzyme activity to the normal tissue, the rest contained significantly less.

In two carcinomas of the renal pelvis and in a carcinoma of the urinary bladder, we found no enzyme activity. Boyland *et al.* [22] described that bladder cancer contained more sulphatase than the corresponding bladder mucosa. Our experience is too limited to discuss the activity in bladder tumours. Although a moderate enzyme activity is shown in normal tubular cells of the renal cortex, only a trace to slight enzyme activity is present in renal carcinomas. Morgan *et al.* [21] found a decrease in 4 renal neoplasms of arylsulphatase activity as compared to normal kidney cortex.

These authors studied also tumours of the liver, lung and kidney with a biochemical method. They found 6-benzoyl-2-naphthyl sulphate to be a poor substrate for human arylsulphatase whether used histochemically or quantitatively. When compared to 2-hydroxy-5-nitrophenyl sulphate in the quantitative essay, much lower values were obtained with the former. This means among the others that it is dangerous to compare results of different authors using different methods. During our study we used one method. In this orienting study it appeared that in some groups of tumours, (e.g. lung carcinomas) hardly any enzyme activity can be found. In the group of carcinomas of the breast, most tumours have no enzyme activity, in contrast to the tumours in the group of colonic-rectal carcinomas which have increased arylsulphatase activity. This agrees with the biochemical results of Dzialoszynski *et al.* [18]. In normal colon mucosa, the activity is the

highest in the epithelial cells of the deeper glands. The activity is decreased in the differentiated cells. In tumour cells of the colon, a high activity of arylsulphatase has been recorded. This finding is in accordance with the general rule that most tumour cells are less differentiated than the normal corresponding cells.

Further studies are necessary for more insight in the significance of the increase or decrease of arylsulphatase enzyme activity in tumour cells.

REFERENCES

1. C. Fromagot, Sulfatases. In *The Enzymes.* (Edited by J. B. Summer and K. Myrbäck), Vol. I, 517 Academic Press, New York (1952).
2. A. M. Seligman, M. M. Nachlass, L. H. Manheimer, O. M. Friedman and G. Wolf, Development of new methods for the histochemical demonstration of hydrolytic intracellular enzymes in a program of cancer research. *Ann. Surg.* **130,** 333 (1949).
3. K. S. Dodgson, B. Spencer and J. Thomas, The arylsulphatases of mammalian livers. *Biochem. J.* **59,** 29 (1955).
4. H. W. Deane, R. J. Barnett and A. M. Seligman, Enzymes. In *Handbuch der Histochemie.* (Edited by W. Graumann and K. Neumann) Vol. VII, part I. Gustav Fischer Verlag-Stuttgart (1960).
5. A. G. E. Pearse, *Histochemistry. Theoretical and Applied.* Third Edition, Vol. 2. Churchill Livingstone, Edinburgh and London. (1972).
6. A. M. Rutenberg, E. Cohen and A. M. Seligman, Histochemical demonstration of arylsulphatase. *Science* **116,** 539 (1952).
7. A. E. F. H. Meijer, Semipermeable membranes for improving the histochemical demonstration of enzyme activities in tissue sections—I. Acid phosphatase. *Histochemie* **30,** 31 (1972).
8. A. E. F. H. Meijer and A. H. T. Vloedman, Semipermeable membranes for improving the histochemical demonstration of enzyme activities in tissue sections. II. Nonspecific esterase and β-glucuronidase. *Histochemie* **34,** 127 (1973).
9. A. E. F. H. Meijer, Semipermeable membranes for improving the histochemical demonstration of enzyme activities in tissue sections. III. Lactate dehydrogenase. *Histochemie* **35,** 165 (1973).
10. K. S. Dodgson, B. Spencer and C. H. Wijnn, Studies on sulphatases. The arylsulphatases of human tissues. *Biochem. J.* **62,** 500 (1956).
11. A. M. Rutenberg and A. M. Seligman, Coloric estimation of arylsulphatase. Enzyme kinetics and distribution of the enzyme in seven mammals and in tumours. *Arch. Biochem.* **60,** 198 (1956).
12. R. Viala and R. Gianetto, The binding of sulphatase by rat-liver particles as compared to that of acid phosphatase. *Can J. Biochem.* **33,** 839 (1955).
13. D. M. Milsom and F. A. Rose, Distribution of arylsulphatase C in subcellular membranes of rat liver. *Biochem. J.* **118,** 22 P (1970).
14. A. B. Roy, The histochemical detection of arylsulphatases. *J. Histochem. Cytochem.* **10,** 106 (1962).
15. F. Zaruba, J. Uher, K. Lichá, V. Krs and H. Heringova, Arylsulfatase activity in human fetal skin. *Acta derm.-venereol. (Stockh).* **50,** 181 (1970).
16. J. Austin, Metachromatic leukodystrophy (sulfatide lipidosis). In *Lysosomes and Storage diseases.* (Edited by H. G. Hers and F. van Hoof) Academic Press, New York and London (1973).
17. D. A. Stumpf, J. H. Austin, A. C. Crocker and M. la France, Mucopolysaccharisosis Type VI. *Am J. Dis. Child* **126,** 747 (1973).
18. L. M. Dzialoszynski, J. L. Kroll and A. Fröhlich, Arylsulphatase activity of malignant tumours. *Clin. Chim. Acta.* **14,** 450 (1966).
19. W. Günther, Histopochemischer Nachweis der Arylsulfatasen A, B and C (Arylsulphate sulphydrolase EC 3.1.6.1.) in pigmentbildenden Geweben des Menschen. *Derm. Wschr.* **156,** 329 (1970).
20. L. R. Morgan, N. J. Reehlmann, B. Maddux, M. S. Samuels, E. T. Krementz, Arylsulphatase and the melanin pigment cell cycle. *Int. J. Biochem.* **1,** 257 (1970).
21. L. R. Morgan, N. J. Reehlmann, B. Maddux, L. Rodriquez, G. Gidman, M. S. Samuels and E. T. Krementz, Arylsulphatase activity of human lung, liver and kidney neoplasms. *Clin. Chim. Acta.* **32,** 316 (1971).
22. E. Boyland and D. M. Wallace, The activity of the enzyme sulphatases and β-glucuronidase in the urine, serum and bladder tissue. *Brit. J. Cancer.* **9,** 62 (1955).

Europ. J. Cancer Vol. 11, pp. 815–819. Pergamon Press 1975. Printed in Great Britain

Two Pseudodiploid Human Breast Carcinomas Studied with G-Band Technique*

JOACHIM MARK

The Departments of Pathology and Surgery, Central Hospital, Skövde, Sweden.

Abstract—*The cytogenetical observations in 2 human breast carcinomas studied with G-band technique are reported. Both tumours had a pseudodiploid stemline. One of these had the constitution* 46, *XX*, $2p-$, $+5$, $+8$, $9p-$, $16q-$, $17p+$, -20, -21; *the details of the structural rearrangements could be completely clarified. The other stemline had the constitution* 46, *XX*, $3q-$, -6, $+7$, -8, $11q+$, -15, $16q+$, $+2$ *different ring chromosomes; the details of the structural changes could be clarified to a considerable extent. A preferential pattern of chromosome variation in breast carcinoma is discussed on the basis of the present results and a review of the literature.*

INTRODUCTION

RECENTLY, Bodor *et al.* [1] discussed the chromosomal findings in human breast carcinomas studied with conventional staining methods. The authors presented a pseudodiploid case of their own, and pointed out the rarity of this chromosomal constitution in solid breast carcinomas. They also draw attention to the fact that, on the whole, comparatively few solid, particularly primary, mammary carcinomas have been studied cytogenetically. Technical reasons are undoubtedly responsible for the scarcity. The importance of these technical problems has increased with the introduction of the modern banding methods. Thus, in addition to a sufficient number of well-spread metaphases, the application of banding methods demands slightly contracted chromosomes with fairly straight arms. Hitherto, these demands have been met within two of our cases of breast carcinomas, and the cytogenetical observations are presented below. The results of this preliminary study will be discussed in connection with a brief survey of previous investigations of human mammary carcinomas.

Accepted 10 February 1975.

*The present study was supported by grants from the Swedish Cancer Society and from the Carlsson family.

MATERIAL AND METHODS

Both breast carcinomas were poorly differentiated, scirrhous neoplasms. One of the tumours (case 1) was located in the left breast of a 78-year-old woman, and the other carcinoma (case 2) was found in the right breast of a 74-year-old woman. Lymph node metastases (microscopical ones) were present only in the latter case. Both patients were alive 6 and 3 months, respectively, after operation.

The rounded tumours had a dia of 2–3 cm. The carcinomas were cut in thick slices. The surfaces of these were gently scraped with a sharp knife. The material on the blade of the knife was suspended directly in 10 ml of Parker's medium 199 with 0·5 μg colcemid (CIBA) per ml medium. After 1 hr of incubation at 37°C, the medium was removed, and the cells suspended in 8 ml 0·075 M KCL for 20 min. Then the hypotonic solution was removed, and 5 ml of Carnoy's fixative was added for 30 min. Later, the fixative was changed three or more times, and finally the cells were suspended in 2–3 ml of freshly prepared fixative. This was used for the chromosome preparations which were made with the air-drying method. Two to five days later, a few slides were stained with Giemsa for 5 min, and the remaining slides were used for banding. The banding was made by a slightly modified version of the trypsin–versene

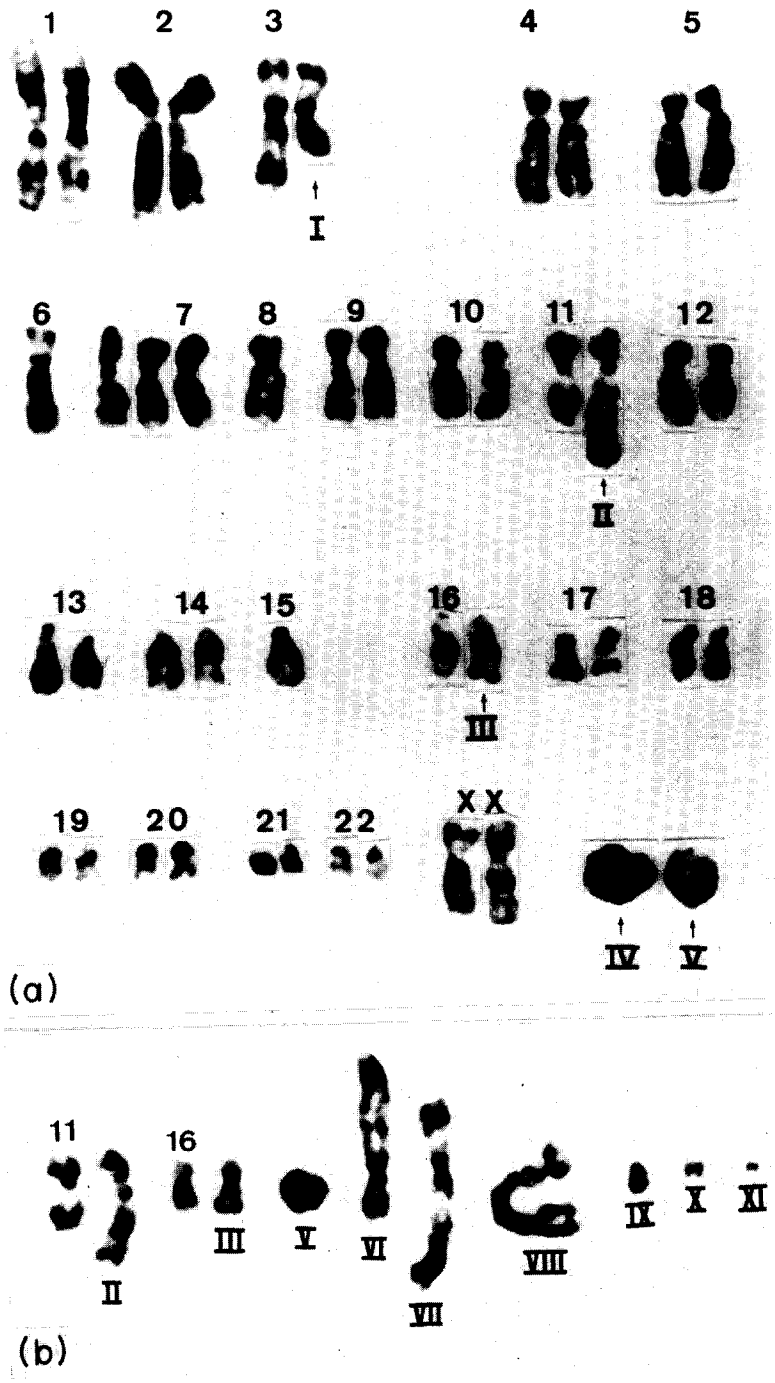

1
2
3
4
5
I
6
7
8
9
10
11
12
II
13
14
15
16
17
18
III
19
20
21
22
X X
IV
V
(a)
11
16
II
III
V
VI
VII
VIII
IX
X
XI
(b)

Fig 2.

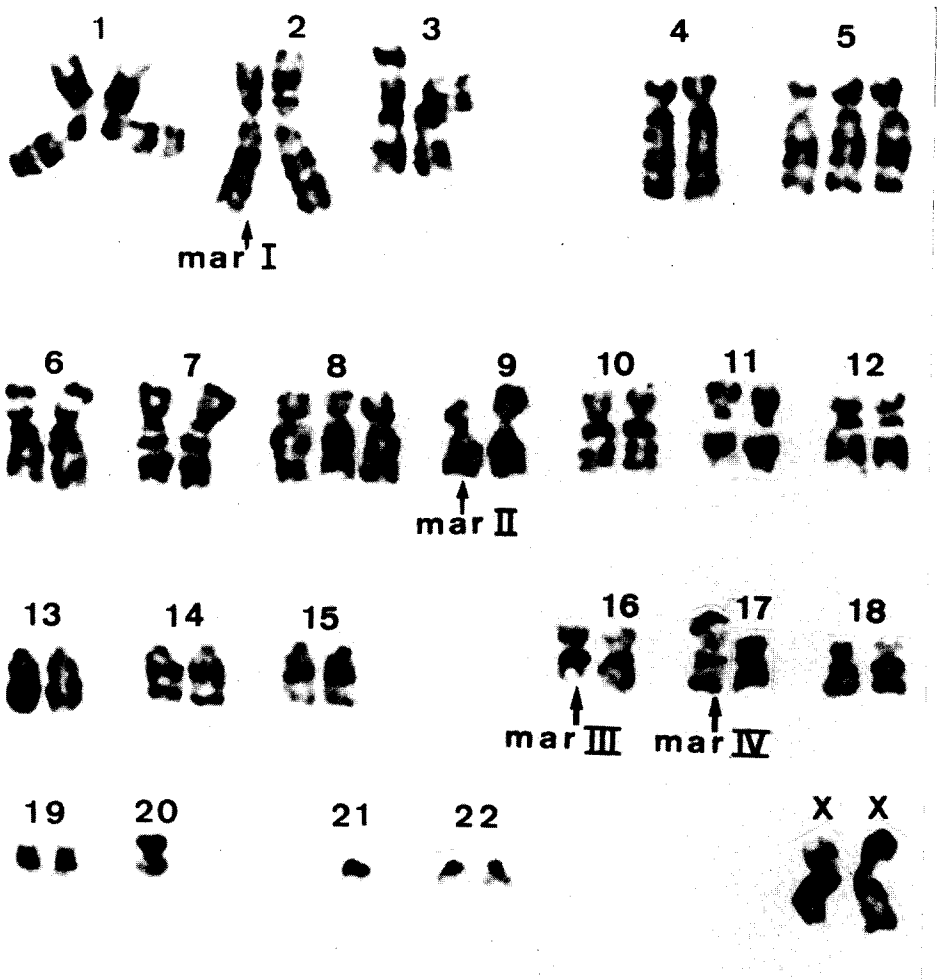


Fig 1.

(to face p. 816)

(EDTA)-Giemsa method described in detail earlier [2].

Fifty or more metaphases were counted in both tumours. Magnified photos were used for the karyotype analyses which comprised 27 or more cells. The nomenclature follows that of the Paris Conference [3] with two exceptions: (1) *S* is used as a symbol for the stemline; (2) all structurally changed chromosomes are termed markers (mar) regardless of whether or not their origin is wholly or partly clarified.

RESULTS

Table 1 shows the chromosome number distribution in the breast carcinomas. In both tumours there was a definite mode at $S = 46$, a moderate, predominating negative spread around the modal number, and no polyploid cells. The karyotypic findings in each tumour are treated separately below.

Table 1. *The chromosome number distribution in two breast carcinomas, case* 1 *and case* 2

Case no.	Chromosome numbers 41	42	43	44	45	46	47	48	Total cells
1	1	1	2	3	5	35	3	-	50
2	2	2	5	5	10	52	4	2	82

Case 1

A total of 27 cells were karyotyped. Nineteen of the analyzed cells had the *S*-number, i.e. 46. As many as 16 of these modal cells had the same karyotype, the stemline karyotype. This is shown in Fig. 1. The S-karyotype can be written as follows (deviations recorded in relation to a normal female complement): 2p−, +5, +8, 9p−, 16q−, 17p+, −20, −21. Thus, the S-karyotype included 4 structurally changed chromosomes, i.e. markers. The origin of all markers could be completely clarified, and each marker type is characterized in detail below.

mar I = 2p−: This marker was a deleted chromosome No. 2 which had lost approximately the distal quarter of its short arm. With regard to the mechanism for the rearrangement and the break points, the marker can be characterized by the formula del (2) (p24).

mar II = 9p−: This marker was a chromosome No. 9 with a shortened short arm. In size and banding it accorded with the short arm of a chromosome No. 17. The designation for the translocation should be t(9q17p).

mar III = 16q−: This small, metacentric marker was a chromosome No. 16 having lost approximately the distal third of its long arm. The designation for the anomaly should be del (16) (q21).

mar IV = 17p+: This metacentric marker was a chromosome No. 17 with a lengthened short arm. In size and banding pattern it accorded with the short arm of a chromosome No. 9 (cf. mar II). The designation for the translocation should be t(9p17q).

The deleted segments of chromosomes Nos. 2 and 16 were big enough to have been detected if they had been translocated onto any other chromosome. Thus, it can be concluded that these segments had been eliminated during the evolution of the S-karyotype.

In conventionally stained slides it was only possible to identify one marker, and this only in some very clear cases, namely mar I.

Eleven variant cells were analyzed in detail. All of them contained the markers I–IV; new marker types were not observed. Most variant cells showed also the same deviations among normal chromosome types as did the S-cells. The additional changes are summarized in Table 2. It is clear from this table, that the additional changes, gains and/or losses, concerned only a few chromosome pairs, and that these usually were involved in two or more variant cells. These findings spoke for a true variation and not one caused by broken cells.

Table 2. *Karyotypic findings in* 11 *variant cells in case* 1*; deviations recorded in relation to the stemline karyotype*

Chromosome number	chromosome pairs 5	7	8	10	11	14	19	22	Number of cells
47					+1				1
47	+1								2
46				+1			-1		2
46		+1					-1		1
45							-1		2
44						-1	-1		1
43			-1			-1		-1	1
42	-1	-1		-1				-1	1

Case 2

Altogether 44 cells were karyotyped. Twenty-five of these had the S-number, i.e. 46. No less than 23 had the same karyotype, the stemline karyotype (Fig. 2(*a*)). This S-karyotype can be written as follows: 3q−, −6, +7, −8, 11q+, −15, 16q+, +2 ring chromosomes (mar IV and mar V, cf. below). The five markers

included in the S-karyotype are characterized below.

mar I = 3q−: A chromosome No. 3 had lost almost its whole long arm, and the deleted segment was replaced by approximately the distal half of the long arm of a chromosome No. 11. The designation for the translocation should be t(3; 11) (q12; q14).

mar II = 11q+: The deleted segment of the above-mentioned chromosome No. 11 was replaced by almost the whole long arm of a chromosome No. 5. The designation for the translocation should be t(5; 11) (q12; q14).

mar III = 16q+: A chromosome No. 16 had been subjected to an almost terminal deletion in its long arm. A short segment of unknown origin was translocated onto the remaining part of its long arm. The formula for the translocation should be t(16q+; ?) (q23; ?).

mar IV and mar V = the ring chromosomes: The origin of both the monocentric ring chromosomes was uncertain. It should be mentioned, however, that the biggest ring chromosome, i.e. mar IV, had a size and banding pattern suggesting an origin from the missing chromosomes Nos. 6 and 15. Clear bands could not be discerned in the small ring chromosome, i.e. mar V.

The ring chromosomes were the only markers which could be regularly identified in conventionally stained preparations.

Out of the 21 variant cells analyzed, 9 were closely related to the S-cells. Table 3 shows the deviations in relation to the S-karyotype. The non-random involvement is obvious. The new, sometimes doubled, minute marker occurring in 4 cells, was similar to mar XI observed in the second group of variant cells.

Table 3. *Karyotypic findings in* 9 *variant cells (see text) in case* 2; *deviations recorded in relation to the stemline karyotype*

Chromosome numbers	Chromosome pairs 7	8	9	18	22	Minute marker	Number of cells
48						+2	1
47						+1	2
45		-1					2
44	-1				-1		2
44	-1		-1	-1		+1	1
41	-2	-1		-1	-1		1

The 12 karyotyped cells belonging to the second group of variant cells had chromosome numbers ranging from 41 to 46, and there was a minor mode at 45. These cells could be distinguished from those of the former group and the S-cells, because of their changed marker set. Thus, all cells had lost mar I and mar IV and gained 4–6 new marker types, i.e. mar VI–XI. The whole set of 9 markers (Fig. 2(*b*)) were seen in all the five 45-chromosome cells. They had the same karyotype, and it can be written as follows: mar Ip+, 3q+, −7, −8, −8, mar II, +mar IIq+, −13, −14, −15, mar III, +mar IV, +3 small markers (IX–XI). The new marker types, VI–XI, are characterized below.

mar VI = mar Ip+: This marker was derived partly from the missing mar I. Thus, a small, distal part of the preserved arm (short arm of No. 3) was lost and replaced by almost the whole long arm of a chromosome No. 8. With reference to previous description of mar I, this secondary rearrangement can be written as t(mar I; 8) (p25–26; q12).

mar VII = 3q+: The remaining chromosome No. 3 had lost a small distal part of its long arm, and this segment was replaced by almost the whole long arm of a chromosome No. 13. The designation for the translocation should be t(3; 13) (q26; q13–14).

mar VIII = mar IIq+: A very small, distal part of the long arm (cf. long arm of No. 5) of a mar II was lost and replaced by almost the whole long arm of a chromosome No. 14. As for mar VI, this secondary rearrangement can be written as t(mar II; 14) (q33–34; q12–13).

mar IX–XI: The origin of these 3 small markers could not be traced with certainty. It seemed plausible, however, that the main part of mar IX was formed by the short arm of the deleted chromosome No. 8 (cf. mar VI), and that mar X and mar XI were remnants of the deleted chromosomes Nos. 13 and 14 (cf. mar VII and mar VIII).

The other variant cells of this second group differed from those with $2n = 45$ by various combinations of gains affecting chromosomes Nos. 7 and 8 and losses involving chromosomes Nos. 6, 10, 13, 14, 22 and the markers X and XI. None of them contained marker types other than those seen in the 45-chromosome cells.

DISCUSSION

In earlier chromosomes studies, performed with conventional staining methods, malignant effusions have constituted a favourite material. The commonplace character of breast carcinomas and their strong tendency to spread to the serous cavities has led to a great number of these neoplasms being included in the previous studies of effusions. The compilations by Berger [4] and by Atkin [5] and the recent

report by Bodor *et al.* [1] furnish comprehensive lists of references to the earlier analyses. In summary, these investigations have shown that secondary breast carcinomas in effusion form often have their mode in the diploid region (about half of the cases) and that near-triploid stemlines outnumber near-tetraploid ones; in the diploid region pseudodiploid stemlines are as common as hypodiploid and hyperdiploid ones [6, 7]; the karyotype analyses have not shown a consistent involvement of any particular chromosome pair or group, nor are there any reports about a preferential pattern of deviations; one or more marker chromosomes with morphological features varying from case to case are found in about three-quarters of the effusions and several cases with ring chromosomes are on record [7–9]. Only a few secondary solid mammary carcinomas have been studied earlier [1, 4, 5, 9]. The results did not differ in any significant respect from those in the effusions.

Previous studies of primary breast carcinomas are much more scarce ([5], and references as above) and the reported cases are often cursorily treated. In summary, these studies have shown that lobular *in situ* carcinomas and a few invasive carcinomas have normal, diploid stemlines; the other invasive carcinomas have chromosomal characteristics similar to those observed in secondary cases, except for a predominance of hypodiploid stemlines in the diploid region [5].

Altogether these previous studies give a good idea regarding the numerical stemline pattern in mammary carcinomas, the incidence of some gross structural rearrangements (e.g. the formation of ring chromosomes), and a crude measure of the differences between primary invasive and non-invasive carcinomas and secondary cases. The inconsistent karyotypic observations, however, have a very restricted value in the light of the exactness of results obtained with banding methods. Thus, only a few cases, with unusually clear karyotype, will be referred to in the discussion of the present results.

The tumours (cases 1 and 2) of the present study were classified as poorly differentiated carcinomas. Cytogenetically both neoplasmas had a pseudodiploid stemline with a similar extent of deviations among normal chromosome types. The structural rearrangements, however, were much more complex in case 2, and, in addition, this carcinoma contained a secondary stemline, or sideline, with extensive superimposed changes resulting in a set of 9 markers. Cytogenetical differences, as those between the present tumours, are probably important reasons for the well-known variation in prognosis for patients with carcinomas with similar histopathological maturity.

The above-mentioned progressional differences between the two carcinomas shall not obscure the fact that several karyotypic similarities could be found: a gain of one No. 5 in case 1 and gain of almost the whole long arm of one No. 5 in case 2; involvement of No. 8 in both cases with gain and loss of one chromosome, respectively; a deleted long arm of one No. 16 in case 1 and a translocation onto the long arm of one No. 16 in case 2. Some results obtained with conventional staining methods are of special interest in this context. Thus. Bodor *et al.* [1] recently reported the chromosomal findings in a solid, perhaps secondary, mammary carcinoma; this tumour had a uniform pseudodiploid stemline with gain of one B chromosome and loss of one No. 16. Ishihara and Sandberg [7] described the chromosomal findings in 7 malignant effusions of breast carcinomas. In two of their cases there was a pseudodiploid stemline. One of these had the constitution 46XX, +A3, −B, +2C, −2D, −E16+1 big subtelocentric marker. The other stemline showed more extensive changes which can be summarized as 46XX, −2A1, −A2, −2B, −5C, −2D, +5E, +F, +6 big metacentric–subtelocentric markers. In a later series of effusions studied by Sandberg *et al.* [6] there were further pseudodiploid stemlines with a frequent involvement of B, C and E chromosomes. The observations in these cases selected from the literature add strength to the idea that at least a preferential pattern of chromosome variation might exist in human mammary carcinomas. This proposal would be in line with recent observations in meningiomas and in chronic myelogenous leukemia (CML). In the meningiomas there was a specific involvement of No. 22, and the additional changes were nonrandom with a variation preferentially affecting the chromosomes No. 8, 1, 14 and 9, in that order of frequency [2, 10]. In CML there was a specific involvement of No. 22 (the Ph^1 chromosome) and No. 9, and the chromosome variation preferentially affected No. 8, 17 and the Ph^1 chromosome [11]. The survey by Levan [12] gives further examples of a preferential chromosome pattern among additional human tumour types and also some animal neoplasms.

REFERENCES

1. F. Bodor, C. H. Håkansson and A. Norgren, Pseudodiploid karyotype in a breast carcinoma. *Acta path. microbiol. scand.* **82,** 386 (1974).
2. J. Mark, Karyotype patterns in human nemingiomas: A comparison between studies with G- and Q-banding technique. *Hereditas (Lund)* **75,** 213 (1973).
3. Paris Conference (1971), Standardization in Human Cytogenetics. In *Birth Defects: Original Article Series*, VIII: 7. New York: The National Foundation (1972).
4. R. Berger, Chromosomes et tumeurs humaines. *Path. et Biol.* **17,** 1133 (1969).
5. N. B. Atkin, Chromosomes in Human Malignant Tumors. In *Chromosomes and Cancer* (Edited by J. German, p. 375) John Wiley, New York (1974).
6. A. A. Sandberg, K. Yamada, Y. Kikuchi and N. Takagi, Chromosomes and causation of human cancer and leukemia. III. Karyotypes of cancerous effusions. *Cancer (Philad.)* **20,** 1099 (1967).
7. T. Ishihara and A. A. Sandberg, Chromosome constition of diploid and pseudodiploid cells in effusions of cancer patients. *Cancer (Philad.)* **16,** 883 (1963).
8. K. P. Katayama and T. Masukawa, Ring chromosomes in a breast cancer. *Acta Cytol. (Philad.)* **12,** 159 (1968).
9. J. Mark, Chromosomal characteristics of secondary human brain tumours. *Europ. J. Cancer* **8,** 399 (1972).
10. J. Mark, The human meningioma: a benign tumor with specific chromosome characteristics. In *Chromosomes and Cancer* (Edited by J. German, p. 497), John Wiley, New York (1974).
11. J. Rowley, Do human tumors show a chromosome pattern specific for each etiologic agent? *J. nat. Cancer Inst.* **52,** 315 (1974).
12. A. Levan, Chromosome patterns in tumors. In *Chromosome Identification*, Nobel Symposium 23, (Edited by T. Caspersson and L. Zech p. 144), Academic Press, London-New York (1973).

Europ. J. Cancer Vol. 11, pp. 821–827. Pergamon Press 1975. Printed in Great Britain.

Prognostic Correlations in Malignant Melanoma

A. L. CLAUDY, J. VIAC, N. PELLETIER, N. FOUAD-WASSEF, A. ALARIO and J. THIVOLET

Laboratoire d'Immunopathologie, Clinique Dermatologique—Pr. Thivolet, Hôpital E. Herriot, 69374 Lyon Cedex 2, France.

Abstract—*Fifty-two patients with malignant melanoma were studied histologically and by various assays to determine cell mediated immune responses. Delayed cutaneous reactivity to tuberculin, candidin and DNCB, PHA and PWM induced lymphocyte blastogenesis and total E rosette count are not statistically impaired compared to normal individuals. The active E rosette count drops significantly below 15% among patients whose tumour is growing rapidly.*

The results suggest that both the histopathological findings and active E rosette count may be valuable tests to help assess the prognosis in maligant melanoma patients.

INTRODUCTION

CELLULAR immune functions in human malignant melanoma have been studied by many authors [1–5]. Cell mediated immunity directed against soluble melanoma antigens have been demonstrated in animal [6] and man [7] by *in vivo* and *in vitro* techniques. Immunological reactions against tissue type specific melanoma antigens seem to take place during the course of the disease [8]. However, studies of this type are technically difficult as they involve the use of heterogeneous tissues and control tissue. Moreover, in the absence of syngeneic control subjects we cannot be certain that the reactivity detected is really tumour associated and not specific to the individual or a cross reaction between tumour associated antigens and normal antigens. Therefore, we tried to determine cell mediated reactivity against various antigens (tuberculin, candidin, DNCB, PHA, PWM, sheep erythrocytes) which are easier to deal with and more reliable than melanoma-derived tumour antigens.

Some attempts to compare the results obtained from different *in vivo* and *in vitro* assays have failed to show a significant impairment of cell mediated immunity during the evolution of malignant melanoma [9, 10]. Our main purpose was to establish a prognostic correlation between the clinical stage of the disease, the histopathological findings and the active E rosette count. Clark and Mihm [11] have already stressed that superficial spreading melanomas (S.S.M.) and lentigo maligna melanomas (L.M.M.) have a better prognosis than nodular melanomas (N.M.), but the prognostic value of the active E rosette test has not as yet been emphasized. Current evidence suggests that capacity to form rosette with sheep red blood cells (SRBC) under certain conditions (active E rosette) is a characteristic of T-type lymphocytes [12, 13]. A fall of the active E rosette count below 15% may correlate with dissemination of the disease a few weeks later. On the other hand, we failed to show any correlation between the presence of immune lymphocytes, as indicated by skin reactivity to tuberculin, candidin, DNCB, by PHA and PWM induced lymphocyte blastogenesis and by total E rosette count and the prognosis. These discrepancies suggest that a small quantitative defect in cellular immune function may be detectable only by the active E rosette assay.

Accepted 12 August 1975.

PATIENTS

Fifty-two patients with malignant melanoma were studied. Twenty-four were male and

twenty-eight female. The mean age of the patients was 52·3 years old. Tumour types according to the classification of Clark and Mihm [11] and the site of the primary lesion are shown in Table 1 and 2.

Table 1. Distribution of tumor types and depth of invasion in 52 melanoma patients

Tumour type	Depth of invasion* (level)	Number of patients
S.S.M.	2	2
	3	2
	4	2
	5	2
N.M.	3	7
	4	7
	5	14
L.M.M.		2
Undetermined		14
Total		52

Ref. * According to Clark's classification [11].
S.S.M. : superficial spreading melanoma
N.M. : modular melanoma.
L.M.N.: lentigo maligna melanoma.

Table 2. Site of the primary lesion in 52 melanoma patients

	Number of patients
Upper limb	6
Lower limb	21
Trunk	19
Face	3
Choroid	3
Total	52

Each patient was tested concurrently with at least two of the three assays. The number of patients tested with each assay is shown in Table 3.

The patients were treated in the following manner:

—a wide and deep excision (10 × 6 cm) of the tumour was performed, usually including the fascia of the underlying muscle,

—a lymph node dissection was only performed when the volume of the superficial nodes was too large for the patient's comfort,

—adjuvant immunotherapy with a low dose BCG ($6{\cdot}10^7$ viable organisms, Institut Pasteur) was administered by scarification to 22 patients with N.M. and 4 patients with histologically undetermined melanoma, once weekly for six weeks or more,

—eight patients with disseminated malignant melanoma were given chemotherapy alone: ThioTepa, 10 mg/day IV for 5 days, followed by procarbazine, 100 mg/day by mouth for 10 days monthly. All the patients were started on chemotherapy after the clinical discovery of dissemination.

The follow up rate was 90% over a period from 6 months to 5 years.

METHODS

Histopathological studies

The melanomas were classified according Clark [11] into three groups: S.S.M., N.M., L.M.M., and unclassifiable. Each of these groups has characteristic microscopic features and a typical developmental history. The survival data may be correlated with the depth of invasion, determined on a scale of invasion ranging from level 1 where all the tumour cells are above the basement membrane to level 5 where the subcutaneous tissues are invaded.

Delayed cutaneous reactivity

Intradermal skin tests to different agents were performed:

—tuberculin 10 and 50 units (IP 48 Institut Pasteur),

—candidin 1/100 (Institut Pasteur),

—DNCB sensitization: the patients were topically skin tested with 2000γ of dinitrochlorobenzene (DNCB) in equal volumes of oil and acetone applied to an area approximately 20 mm dia on the medial forearm of the arm not used for immunisation. The area was occluded with gauze and plastic tape for 48 hr. Reactions were read 24, 48 and 72 hr after the application of DNCB. Erythema and vesiculation at 48 hours were accepted as evidence of sensitization.

Immunological studies

Isolation of circulating lymphocytes. Forty millilitres of peripheral blood are drawn into a syringe containing 250 units of heparin (liquemine roche) lymphocytes are isolated on a layer of Ficoll Metrizoate by centrifugation at 500 ***g*** for 30 min. The cells are washed three times in phosphate buffered saline (pH 7·2) and the final concentration is adjusted appropriately for the different assays.

Mitogen induced lymphocyte blastogenesis. 10^6 lymphocytes were cultured with each of the following mitogens: 25 γ/ml of phytohemag-

Table 3. Results of 3 assays used to measure cell mediated immunity in 52 melanoma patients without metastasis and in controls

	Number of melanoma patients tested (n_B)	Normal number	Tests % (p_B)	Number of control patients tested (n_A)	Normal number	Tests % (p_A)	$\lvert\varepsilon\rvert$*	Statistical† significance
Delayed skin tests								
Tuberculin	51	32	63	43	31	72	0·12	N.S.
Candidin	48	33	69	31	22	71	0·28	N.S.
DNCB	40	30	75	13	10	77	0·14	N.S.
Mitogens								
PHA	38	27	71	16	12	75	0·30	N.S.
PWM	34	20	59	12	8	67	0·49	N.S.
Active E rosette	51	24	47	13	11	85	2·55	S.
Total E rosette	34	24	71	11	9	82	0·68	N.S.
EAC rosette	23	21	91	8	7	88	0·04	N.S.

*$\lvert\varepsilon\rvert = \dfrac{p_A - p_B}{\sqrt{\frac{pq}{n_A} + \frac{pq}{n_B}}}$

†N.S.: not significant
S. : significant

glutinin P (Difco), 500 γ/ml of pokeweed mitogen (Grand Island Biological Company). The blastogenic response was measured by tritiated thymidine incorporation and recorded as counts per mn/10^6 lymphocytes. Stimulation levels forty times higher than the controls were considered as positive.

Rosette formation by circulating lymphocytes. Active E rosette. 0·1 ml of a 2×10^7 cells/ml suspension of lymphocytes was incubated at 37°C for one hour with 0·1 ml of heat inactivated and absorbed fetal calf serum and 0·1 ml of SRBC at a concentration of 100 SRBC per lymphocyte. The mixture was centrifuged at 300 ***g*** for 3 min and gently resuspended. Four hundred lymphocytes were immediately counted under a light microscope at a magnification of × 100 and the percentage of lymphocytes that had formed rosettes with more than 3 SRBC calculated. The mean value of control patients was 27% (range 22–32). A test was considered abnormal when less than 15% of lymphocytes formed rosettes.

Total E rosette. The same technique was used except that after centrifugation the cells were incubated for 12 hr at +4°C. The mean value of control patients was 61% (range 47–75). A test was considered abnormal when less than 35% of lymphocytes formed rosettes.

EAC rosette. One volume of 5% concentration of SRBC was incubated with one volume of diluted 1: 100 IgG rabbit anti-SRBC at 37°C for 30 min. Our dilution of the antiserum gave no EA rosette-forming cells at 37°C. It is known that rosettes formed with IgG-EAC (or whole serum EAC) using properly diluted antiserum identify complement reactive lymphocytes and are not caused by synergism with Fc receptors [14]. After washing, one volume of mouse serum diluted 1:5 in veronal buffered saline was added in order not to give complete lysis of the SRBC, and incubated with the SRBC at 37°C for 30 min. The EAC complexes were washed and adjusted to a concentration of 1×10^8 SRBC per ml. The EAC complexes and the suspension of lymphocytes (2×10^6 cells/ml) were mixed in equal volumes and incubated for 20 min at 37°C. The cells were then resuspended on a whirlmixer and EAC rosettes were counted in the same way as for E rosettes. The mean value of control patients was 13% (range 8–18).

The average number of rosette tests performed in each of the fifty-two patients was 3·6 for the active E rosette count, 2·1 for the total E rosette count and 1·4 for the EAC rosette count. The first rosette counts were calculated during the week after the primary excision. Most of the tests were performed on patients not under chemotherapy. In terms of prognostic value, we did not take in account the rosette counts of treated patients.

Statistical analysis. Data comparing group tests (normal population and malignant melanoma patients) were analysed by the minimal spread for the probability $P = 0{\cdot}05$.

Healthy individuals were used as normal control for the *in vivo* and *in vitro* assays.

RESULTS

Results of individual assays. The histological types of melanoma were distributed as follows: eight S.S.M., twenty-eight N.M., two L.M.M. and fourteen histologically undetermined (Table 1).

Sixty-three per cent of melanoma patients tested with tuberculin, 69% tested with candidin and 75% tested with DNCB had a normal positive reactivity. These results are statistically identical to those of a control group (Table 3). The patients were tested at clinical stages I and II. Impaired reactions in the tests were associated, in most instances, with disseminated malignant melanomas treated with chemotherapy.

Seventy-one per cent of the melanoma patients had normal PHA responsiveness, and 59% normal PWM responsiveness (statistically identical to a control group, Table 3). We showed no change in lymphocyte reactivity to PHA and PWM during BCG treatment.

Seventy-one per cent of the melanoma patients had normal total E rosette counts (mean 52%, range 35–69). These results are statistically identical to a control group (mean 61%, range 47–75) (Table 3).

Fifty-three per cent had a low active E rosette count which is statistically non-identical to the control group.

EAC rosette counts were normal in most melanoma patients (91%) versus 88% for the control group. Mean value for melanoma patients 10%, range 8–12.

Results of the comparison between different assays. The relationships between active E rosette counts and histopathological findings are shown in Table 4. An abnormally low active E rosette count was encountered in 21 cases of N.M. and undetermined melanomas (50%), and in only two cases of S.S.M. (25%). The two cases of L.M.M. had a normal active E rosette count. None of these patients were on chemotherapy at the time of the tests.

The clinical course of the disease and the histopathological findings are inter-related (Table 5). Among 42 patients with N.M. or undetermined melanoma, twenty (47·6%) underwent rapid progression of the disease with metastasis or death within 6–18 months, while nine out of ten patients with S.S.M. and L.M.M have been stable for one to five years after the excision of the primary lesion. This correlates with the high percentage of normal active E rosette count in S.M.M. and L.M.M. (80%) and the low active E rosette count in many patients with N.M. and undetermined melanoma.

The statistical relationships between delayed skin tests, mitogen induced lymphocyte blastogenesis and total E rosette count in a control group and the group of malignant melanoma patients can be seen in Table 3. There were no significant differences among the various assays for melanoma patients and for control patients ($\varepsilon < 1{\cdot}96$, $P = 0{\cdot}05$), except for active E rosette counts which were subjected to larger variations showing a significant difference between normal and melanoma patients ($\varepsilon = 2{\cdot}55$, $P = 0{\cdot}01$). We would wish to emphasize that low active E rosette counts returned to normal quickly after starting chemotherapy and that, on the other hand, BCG therapy was uneffective to modify the counts.

The time span between excision of the primary lesion and metastasis was correlated to the

*Table 4. Number of patients with active E rosette counts below 15% correlated to histological findings**

Tumour type	Number of cases	Number of active E rosette count below 15% at the moment of excision	Number of active E rosette count subsequently below 15%	Percentage
S.S.M.	8	1	1	25
N.M.	28	11	3	50
L.M.M.	2	0	0	0
Undetermined	14	5	2	50
Total	52	17	6	44·2

Average time span in months between the drop of the count and clinical dissemination: 1·52.

*None of the patients were under treatment when E rosette count calculated.

Table 5. Number of melanoma patients who developed metastasis correlated with histological findings, treatment and drop of the active E rosette count

Tumour type	Number of cases	Untreated patients*	Treated patients* Chemotherapy	BCG	Metastasis*	Drop*
S.S.M.	8	7	1	0	1	2
N.M.	28	0	6	22	12	14
L.M.M.	2	2	0	0	0	0
Undetermined	14	8	2	4	8	7
Total	52	17	9	26	21	23

*Number of patients.

fall of active E rosette count (Table 4). In six cases, the fall of active E rosette count occurred on average 1·52 months before the clinical appearance of metastasis. In 17 patients, we did not register any such fall, the count being below 15% when first performed.

DISCUSSION

Cell mediated immunity seems to represent the main defense mechanism against tumour proliferation [15]. One approach to elucidate its role in malignant melanoma is to search for an *in vitro* marker accurate enough to detect impairment of the immunological functions of thymus dependent lymphocytes at an early phase. Since the recent introduction of E rosettes as a T cell marker of human lymphocytes [12, 13], few results have been reported concerning the value of counts of active E rosettes in cancer and especially in malignant melanoma [16, 17]. The E rosette technique is delicate and needs skill for correct performance. Many factors can explain the differences in the percentage of E rosette forming cells in normal human peripheral blood, which have been reported in the literature (temperature, incubation, dispersion of the pellet) [18]. However, in our hands, the active E rosette test is highly reproducible and yields a steady percentage in normal individuals [19]. The total E rosette test provides information about the percentage of T lymphocytes in the peripheral blood while the active E rosette test may indicate the activity of a subset of thymo-dependent lymphocytes. EAC rosettes are characteristic of B-type lymphocytes [20] and their percentage appears to be normal among melanoma patients. So far, only an active E rosette count below 15% is of significant value. No hypothesis can be advanced for the reason why a fall in the percentage of active E rosette forming cells in melanoma patients means a poor prognosis. Nor can we explain why the fall occurs just a few weeks before the clinical appearance of metastasis. More data will be needed to elucidate these findings.

The other immunological criteria studied in this series, such as delayed skin tests to tuberculin, candidin, DNCB, PHA and PWM lymphocyte stimulation, total E and EAC rosette count seem to have no prognostic value. They appear to be normal throughout the course of the disease and become abnormal only during chemotherapy or shortly before death. Cell mediated reactivity was reported to be detectable in a high percentage of melanoma patients, using the inhibition of melanoma target cell growth by lymphocytes from the patients, but the assay is still controversial [21].

The active E rosette test, providing care is given to its technical details, is highly reproducible. It may represent a useful tool to detect and may allow to treat the patients preventively before the appearance of disseminating melanomata.

We attempted further to subclassify the tumours according to the classification of Clark [11]. These data are particularly useful at an early stage of the disease. The survival may be correlated with the type of the tumour (S.S.M. and L.M.M. having a better prognosis than N.M.) and the depth of invasion as determined histologically. According to our data, 42·8% of N.M. develop metastases compared to 12·5% of S.S.M. during the same period.

A direct correlation between histological data and active E rosette count can be demonstrated; 75% of S.S.M. patients having both normal active E rosette counts and a long survival, while only 50% of N.M. patients have normal active E rosette counts and the N.M. group has a shorter survival.

According to this data, melanoma patients can be subdivided into four groups in terms of prognosis:

(1) N.M. with a deep level of invasion and a low active E rosette count: poor prognosis,

(2) N.M. with a high active E rosette count: careful follow up, uncertain prognosis,

(3) S.S.M. with a high active E rosette count: good prognosis,

(4) S.S.M. with a low active E rosette count: uncertain prognosis,

(5) L.M.M. with a constant high active E rosette count: excellent prognosis.

Histological data associated with the evaluation of the percentage of active E rosette forming cells may provide accurate guidance in the management of melanoma patients allowing an increase in the remission rate and an improved prognosis.

Acknowledgements—The authors are grateful to Miss M. Chaix for her skilful assistance and are indebted to Dr. J. Wybran for his advice and criticism to whom they express their appreciation.

This project is supported by grant INSERM, ATP 19-75-42.

REFERENCES

1. I. Hellstrom and K. E. Hellstrom, Some recent studies on cellular immunity to human melanomas. *Fed. Proc.* **32,** 156 (1973).
2. A. W. Kopf, Host defense against malignant melanoma *Hosp. Prac.* **6,** 116 (1971).
3. D. A. Clark and L. Nathanson, Cellular immunity (C.M.I.) in malignant melanoma. *Proc. Amer. Ass. Cancer Res.* **13,** 79 (1972).
4. A. J. Cochran, U. W. Jehn and B. Gothoskar, Cell mediated immunity in malignant melanoma. *Lancet* **i,** 1340 (1972).
5. B. Mukherji, L. Nathanson and D. A. Clark, Studies of humoral and cell mediated immunity in human melanoma. *Yale Biol. Med.* **46,** 681 (1973).
6. W. Henderson, K. Fukuyama, W. L. Epstein and L. E. Spitler, Demonstration of cellular immunity to tumor-specific antigens of malignant melanoma in hamsters by inhibition of macrophage migration. *J. invest. Derm.* **58,** 229 (1972).
7. D. H. Char, A. Hollinshead, D. H. Cogan, E. J. Ballentine, M. J. Hogan and R. B. Herberman, Cutaneous delayed hypersensitivity reactions to soluble melanoma antigen in patients with ocular malignant melanoma. *New Engl. J. Med.* **291,** 274 (1974).
8. R. B. Herberman, *In vivo* and *in vitro* assays of cellular immunity to human tumor antigens. *Fed. Proc.* **32,** 160 (1973).
9. J. L. Ziegler, M. A. Lewis, J. M. S. Luyombya and J. W. M. Kiryabwire, Immunologic studies in patients with malignant melanoma in Uganda. *Brit. J. Cancer* **23,** 729 (1969).
10. C. M. Pinsky, H. F. Oettgen, A. Eldomeiri, L. J. Old, E. J. Beattie and J. H. Burchenal, Delayed hypersensitivity reactions in patients with cancer. *Proc. Amer. Ass. Cancer Res.* **12,** 100 (1971).
11. W. H. Clark Jr., F. Lynn, E. A. Bernardino and M. C. Mihm, The histogenesis and biologic behaviour of primary human malignant melanomas of the skin. *Cancer Res.* **29,** 705 (1969).
12. J. Wybran, M. C. Carr and H. H. Fudenberg, The human rosette forming cell as a marker of a population of thymus derived cells. *J. clin. Invest.* **51,** 2537 (1972).
13. J. Wybran and H. H. Fudenberg, Thymus derived rosette forming cells. *New Engl. J. Med.* **288,** 1072 (1973).
14. G. Holm, D. Pettersson, H. Mellstedt, E. Hedfors and B. Bloth, Lymphocyte subpopulations in peripheral blood of healthy persons. Characterization by surface marker and lack of selection during purification. *Clin. exp. Immunol.* **20,** 443 (1975).
15. J. Stjernswärd and A. Levin, Delayed hypersensitivity induced regression of human neoplasms. *Cancer (Philad.)* **28,** 628 (1971).
16. J. Wybran and H. H. Fudenberg, Thymus derived rosette forming cells in various human disease states: cancer, lymphoma, bacterial and viral infections and other diseases. *J. clin. Invest.* **52,** 1026 (1973).
17. J. Wybran, L. E. Spitler and H. H. Fudenberg, Rosette-forming cells and melanoma. *Clin. Res.* **21,** 655 (1973).

18. H. E. Heier, Influence of mechanical force on the rosette test for human T lymphocytes. *Scand. J. Immunol.* **3,** 677 (1973).
19. A. Claudy, N. Fouad-Wassef, N. Pelletier, Valeur du test des rosettes thymodépendantes dans la surveillance des mélanomes malins. Etude préliminaire a propos de 25 cas. *Ann. Derm. Syph. (Paris)* **102,** 69 (1975).
20. C. Bianco, R. Patrick and V. Nussenzweig, A population of lymphocyte bearing a membrane receptor for antigen–antibody–complement complexes. I Separation and characterization. *J. exp. Med.* **132,** 702 (1970).
21. G. H. Heppner, L. Stolbach, M. Byrne, F. J. Cummings, E. McDonough and P. Calabresi, Cell mediated and serum blocking reactivity to tumour antigens in patients with malignant melanoma. *Int. J. Cancer* **11,** 245 (1973).

Europ. J. Cancer Vol. 11, pp. 829–830. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer — *November, 1975*

J. H. Dean, J. L. McCoy, L. W. Law, B. A. Maurer and E. Appella: Cellular immune reactivity *in vitro* and tumour rejection provided by tumor-associated antigens of Friend virus induced leukemia.

S. Canevari, G. Fossati, G. Della Porta and G. P. Balzarini: Humoral cytotoxicity in melanoma patients and its correlation with the extent and course of the disease.

G. de Thé, J. H. C. Ho, D. V. Ablashi, N. E. Day, A. J. L. Macario, M. Cl. Martin-Berthelon, G. Pearson and R. Sohier: Nasopharyngeal carcinoma. IX. Antibodies to EBNA and correlation with response to other EBV antigens in Chinese patients.

A. A. Stavrovskaya and B. P. Kopnin: Colcemic-induced polyploidy and aneuploidy in normal and tumour cells *in vitro*.

L. Milas, H. D. Kogelnik, I. Basic, K. Mason, N. Hunter and H. R. Withers: Combination of *C. parvum* and specific immunization against artificial pulmonary metastases in mice.

G. Parmiani and G. Invernizzi: Alien histocompatibility determinants on the cell surface of sarcomas induced by methylcholanthrene. I. *In vivo* studies.

L. M. Shabad, T. S. Kolesnichenko and N. I. Golub: The effect produced by some carcinogenic nitrocompounds on organ cultures from human embryonic lung and kidney tissues.

M. Ahmed, W. Korol, D. L. Larson, K. R. Harewood and S. A. Mayyasi: Interactions between endogenous baboon type-C virus and oncogenic viruses. I. Syncytium-induction and development of infectivity assay.

B. H. Dorsett, H. L. Ioachim, L. Stolbach, J. Walker and H. R. K. Barber: Isolation of tumor-specific antibodies from effusions of ovarian carcinomas.

H. R. Glatt, F. Oesch, A. Frigerio and S. Garattini: Epoxides metabolically produced from some known carcinogens and from some clinically used drugs: I. Differences in mutagenicity.

R. J. Elkort, A. H. Handler and P. J. Mozden: Preneoplastic changes in rabbit pancreatic duct cells produced by dimethylhydrazine.

J. S. Haskill, Y. Yamamura and L. Radov: Host responses within solid tumors: Non-thymus-derived specific cytotoxic cells within a murine mammary adenocarcinoma.

D. P. Evenson, D. M. Pla, D. Beju and E. de Harven: A quantitative comparison between *in vivo* and *in vitro* derived Friend leukemia virus.

J. S. Rhim, H. Y. Cho, M. L. Vernon, P. Arnstein, R. J. Huebner, R. V. Gilden and W. A. Nelson-Rees: Characterization of nonproducer human cells induced by Kirsten sarcoma virus.

I. Hellström, K. E. Hellström and J. T. Nepom: Increased lymphocyte mediated destruction of tumor cells in microcytotoxicity assays seen after addition of inhibitors of protein synthesis.

F. Vánky, E. Klein, J. Stjernswärd and G. Trempe: Lymphocyte stimulation by autologous tumor cells in the presence of serum from the same patient or from healthy donors.

J. F. Evermann and T. Burnstein: Immune enhancement of the tumorigenicity of hamster brain tumor cells persistently infected with measles virus.

D. E. Tracey, H. F. Pross, M. Jondal and I. P. Witz: Antibody-dependent cell-mediated cytotoxic activity in syngeneic mouse ascites tumors.

British Journal of Cancer *November, 1975*

R. F. Mould and J. W. Boag: A test of several parametric statistical models for estimating success rate in the treatment of carcinoma cervix uteri.

R. Bomford: Active specific immunotherapy of mouse methylcholanthrene-induced tumours with *Corynebacterium parvum* and irradiated tumour cells.

W. H. McBride, S. Tuach and B. P. Marmion: The effect of gold salts on tumour immunity and its stimulation by *Corynebacterium parvum*.

P. R. Armitstead and G. Gowland: The leucocyte adherence inhibition test in cancer of the large bowel.

P. M. Wilkinson, C. Sumner, I. W. Delamore, C. G. Geary and G. R. Milner: Granulocyte function in myeloblastic leukaemia.

J. Hilfrich, H. Haas, N. Knoch, R. Montesano, U. Mohr and P. N. Magee: The modification of the renal carcinogenicity of dimethylnitrosamine by Actinomycin D and A protein deficient diet.

J. Hilfrich: Comparative morphological studies on the carcinogenic effect of 7, 12, dimethylbenz(a).

A. W. Pound and T. A. Lawson: Partial hepatectomy and toxicity of dimethylnitrosamine and carbon tetrachloride, in relation to the carcinogenic action of dimethylnitrosamine.

A. Flaks and P. Sims: The activities of some polycyclic hydrocarbons and their "K-region" epoxides in an *in vitro–in vivo* carcinogenicity test system.

N. J. McNally: The effect of an electron-affinic radiosensitizer on tumour growth delay and cell survival.

W. R. Miller, T. Hamilton, H. R. Champion, K. W. J. Wallace, A. P. M. Forrest, R. J. Prescott, E. D. Cameron and K. Griffiths: Urinary aetiocholanolone in patients with early breast cancer from South East Scotland and South Wales.

Co-Ordinating Committee Report: New Edinburgh primary breast cancer trials.

Book Reviews:

R. C. S. Pointon: The biology and clinical management of bladder cancer.

C. G. Beardwell: New Techniques in tumour localisation and radioimmunoassay.

J. M. A. Whitehouse: La chimiotherapie des cancer leucemies hematosarcomes et tumeurs solides.

M. Moore: Cancer and the immune response.

Europ. J. Cancer Vol. 11, pp. 831–840. Pergamon Press 1975. Printed in Great Britain

Friend Leukemia: A Model for the Physiopathology of Human Chronic Leukemia*

F. SMADJA-JOFFE, C. JASMIN, C. KERDILES and B. KLEIN
Institut de Cancérologie et d'Immunogénétique (INSERM et Association Claude-Bernard), Hôpital Paul-Brousse, 14 avenue Paul-Vaillant-Couturier, 94800 Villejuif, France

Abstract—*We have reviewed from a critical point of view, available experimental data concerning the physiopathology of the erythroblastosis induced by the Friend virus in mice. From these data it appears that the presence of neoplasic cells, characterized by an autonomous capacity for growth, is not necessary to explain the disease. On the contrary, it seems that the main role of Friend virus is to induce a permanent differentiation of erythropoietin responsive cells (ERC) into short-lived proerythroblastic cells: most of these cells die rapidly and the others differentiate into mature red blood cells. These mechanisms of cell elimination cannot compensate for the overproduction of proerythroblastic cells, which is maintained by* (1) *the virus which is replicating continuously in the infected tissues of the host,* (2) *the continuous supply of target cells (ERC) stimulated by almost normal feed-back mechanisms of control.*

INTRODUCTION

NORMAL hemopoiesis is a well controlled process which supplies mature blood cells with finite lifespans from a pool of stem cells. The proliferative capacity of precursor blood cells and their progressive maturation into more specialized cells are two distinct mechanisms which characterize normal differentiation. If these mechanisms fail to respond to the control exerted by the organism, via humoral and cellular regulators, a leukemia may develop. A leukemia was defined by Dameshek [1] as a "generalized abnormal, neoplastic, self perpetuating proliferation (slow or rapid) of one of the leucocyte tissues, often associated with abnormal white blood cell counts, and eventually leading to anemia, thrombocytopenia and death". This definition, which is currently accepted, makes it obvious that the concept of leukemia is often associated with the existence of clones of pathological cells characterized by an autonomous and unlimited capacity to proliferate.

Clinical criteria have led to a distinction between acute and chronic leukemias. In acute leukemia, the normal hematopoietic cells are gradually replaced by a population of immature precursors, and different forms of acute leukemia have been described according to their morphological similarities with normal hematopoietic blast cells: lymphoid, myeloid, monocytoid and myelomonocytoid. These acute disorders seem to be characterized by a defective maturation of pathological cells and their spontaneous evolution results rapidly in death.

On the contrary, chronic leukemias are mainly characterized by an overproduction of differentiated cells which are not very different morphologically from normal cells, although some functional or chromosomal abnormalities have been described. Chronic leukemias have a clinical evolution slower than acute leukemias; some of them, namely the granulocytic leukemia (CGL) and sometimes polycythemia vera (PV) end in a blastic crisis followed by a

Accepted 10 September 1975.
*This work was supported by a grant from INSERM (ATP no. 36).

rapid death. (The classification of polycythemia as a chronic leukemia is not accepted by all authors. However, as will be shown later, this disease has many physiopathological features in common with other chronic leukemias.)

The data concerning physiopathology of human leukemias remain partial, and often concern only the advanced stages of the disease. However, a limited knowledge of the *in vivo* kinetics of the cellular proliferation and maturation has been obtained with the use of radioactive components such as tritiated thymidine, ^{51}Chromium and ^{59}Iron; soft-gel cultures have allowed a better understanding of the physiology of proliferation and differentiation of leukemic cells, and a partial evaluation of the committed stem cell compartment [2, 3].

It appears, therefore, that a comprehensive study of the physiopathology of human leukemias requires good experimental models. However, until now, a correlation between human and animal hemopathies appeared difficult to establish, namely because of immunological, anatomopathological and etiological differences: for instance, Gross leukemia of mice resembles a lymphosarcoma more than an acute lymphoid leukemia. Many virus-induced leukemias have a short preleukemic phase and usually no blastic crisis is observed. But the lack of experimental models is also due to our poor understanding of their pathology. Friend leukemia, a murine erythroleukemia induced by an oncornavirus, appears to be the most studied. A great amount of valuable data has been accumulated in the last few years, which enables us to understand most of its physiopathological processes.

The purpose of this paper is to summarize these data and to propose a scheme which can explain the evolution of the disease. We will also compare this model with data concerning human leukemia.

Actual model of normal erythropoiesis

A model of erythropoiesis has been proposed recently by Lajtha [4]. In this model a definite number of pluripotent stem cells, whose turn-over rate is principally under their own control, undergoes a first step by differentiating into an erythroid committed cell population (ECP). The factors regulating this first step in differentiation are as yet unknown, but experimental data suggest that local influences might be involved (HIM: hemopoietic inductive microenvironment). The ECP cells are not a self-maintaining population; they are an amplifying transitional population characterized by an age structure which undergoes a maturation process. This means that daughter cells have a reduced capacity for proliferation compared to progenitor cells. The most mature cells of this population are the ERC (erythropoietin responsive cells). The ECP cells may divide 4 to 5 times in normal mice, this number being increased to 8–10 times on demand [5]. This maturation process is influenced by erythropoietin (EPO) which directly controls the differentiation of ERC into proerythroblasts and also affects their proliferation rate. EPO seems also to act on some further stages of erythroid differentiation, but during the more differentiated stages the capacity of the cells to undergo mitosis may be mainly controlled by the level of hemoglobin in the cytoplasm.

Furthermore, feed-back mechanisms probably control the first step in differentiation as well, since removal of a large number of ECP

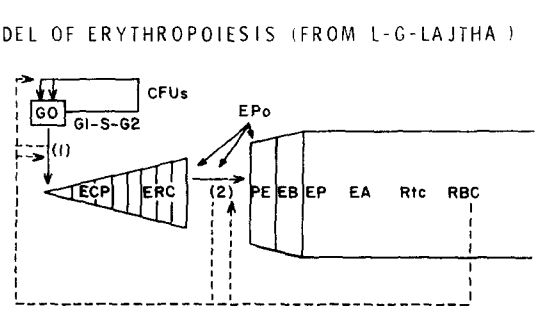


Fig. 1. Model of erythropoiesis (taken from Lajtha, L. G. [5]).

CFUs: pluripotent stem cells.
In Go: non-cycling Go state, and in G_1-S-G_2: cell-cycle.
Their proliferation is probably regulated by their own control, but their rate of turnover seems to be influenced by feed-back mechanisms.

ECP: erythroid committed population:
This population has a large amplification capacity and an age structure: ECP cells endow a maturation process, the last stage of which is the ERC (erythropoietin responsive) stage.

PE: proerythroblasts.

EB: basophilic erythroblasts (EB_1: early; EB_2: late).

EP: polychromatophilic erythroblasts.

Rtc: reticulocytes.

RBC: mature red blood cells.

$\xrightarrow{1}$: "first step" differentiation of pluripotent stem cells to erythroid committed cells. The factors controlling this step are unknown. Feed-back mechanisms and microenvironmental factors may influence it.

$\xrightarrow{2}$: "second step" differentiation: ERC cells differentiate into proerythroblasts. This step is induced by erythropoietin, which probably stimulates ERC proliferation also, and has a promoting effect on proerythroblastic maturation.

$\dashrightarrow$: Stages probably under the control of feed-back mechanisms.

EPO: erythropoietin.

into proerythroblasts apparently increases the rate of differentiation of the pluripotent stem cells, which respond to this increased stem cell removal for differentiation by an increased turnover. This model of erythropoiesis is summarized in Fig. 1, taken from L. G. Lajtha [4].

Some quantitative studies of erythropoiesis using labeling techniques with ^{59}Fe, ^{55}Fe, ^{51}Cr, ^{3}H-TdR, and cytological observations have been performed in rats [6], mice [7] and man [8]. They mainly concern the maturation process from proerythroblasts into mature red blood cells, the kinetics of proliferation and differentiation of the earlier precursor cells being less understood.

Models for the physiopathology of Friend leukemia

Friend disease is characterized by a splenomegaly (the weight of the spleen is increased by a factor of about 20), followed by an hepatomegaly and a late increase in the number of nucleated cells in the peripheral blood (from about 3000 nucleated cells per mm^3 in normal mice to more than 200,000 during the advanced stage of the disease). Anemia or polycythemia is observed according to the strain of Friend virus (FVA or FVP respectively) which has been injected, and there is an important increase in the plasma volume, which is multiplied respectively by 5 and 3 at the end of the diseases induced by FVP and FVA [9–12]. These clinical disorders are mainly due to an erythroblastosis and are detectable very early after the injection of the virus, the first pathological cells (hyperbasophilic cells, which are immature proerythroblasts) being observed in the red pulp of the spleen (under the capsule and along the trabeculae) 36 hours after injection of high doses of FVP [13]. This lag-phase is an inverse function of the dose of virus injected. The megakaryocytic and granulocytic cell lines do not appear to be perturbed by the virus, although some maturation deficiency has been demonstrated for the former [14]. Lymphocytosis has been described in spleen and peripheral blood [10], but quantitative data are still lacking. The absolute number of stem cells (CFU-s) is enhanced, especially in the peripheral blood (their number is multiplied by 800 in the polycythemic variety of leukemia) and in the spleen, where their increase parallels the splenomegaly [15, 16]. These data are summarized in Table 2.

A specific action of FV on the erythropoietin responsive cells (ERC) was suggested a few years ago [17], and recently demonstrated [18]. FVP is able to provoke the appearance of hyperbasophilic cells and splenomegaly in mice where most of the pluripotent stem cells (CFU-s) but only a few ERC have been killed with appropriate doses of myleran. Since the capacity of FV (even after multiple injections of virus) to induce leukemia is strongly reduced when the ERC population has been depleted by polycythemia in myleran-treated mice, it has been concluded that FV acts mainly by committing ERC cells to differentiate into more mature erythroid cells (hyperbasophilic cells), but it does not appear to have the capacity to restore the ERC compartment. This erythropoietin independent erythropoiesis shows strong similarities with the normal process: hyperbasophilic cells are able to mature into erythrocytes. In the spleen, erythroblast precursors are observed at different stages of their maturation [19], and the number of erythrocytes in the peripheral blood increases regularly, their total production being multiplied by a factor of about 13 at the terminal phase of the disease induced by the FVP [12]. This pathological erythropoiesis also remains sensitive, at least partially, to normal controls. Despite the fact that the virus and target cells are present in the bone marrow and in the spleen, the first hyperbasophilic cells are observed in the spleen [20], which is the main site of the pathological proliferation; furthermore, addition of erythropoietin accelerates the development of splenomegaly, and artificial polycythemia (or bleeding) delays it [21, 22].

However, several morphological and functional anomalies have been described: proerythroblasts are hyperbasophilic [20], some asynchrony between maturation of the nucleus and rate of cytoplasmic hemoglobinization is observed (personal observation), the erythropoietic maturation is only partially sensitive to normal homeostatic controls [21, 22]; but the main defect has been shown by autoradiographic studies using ^{3}H-TdR labeling which has demonstrated that hyperbasophilic cells die rapidly, in great numbers, probably after one or two mitoses [23]. This death involves at least 2/3 of the hyperbasophilic cells produced in the spleen [24].

The increase of the pluripotential stem cell compartment seems to follow the continuous depletion of the ERC compartment, and is provoked by an almost normal feed-back mechanism. Several experimental data support this hypothesis: infection of the CFU-s by FV does not confer on them a greater capacity for proliferation: infected CFU-s have an impaired ability to seed in the spleens of irradiated

normal recipients [25], the depression of the *f* factor being greater for CFU-s obtained from Friend leukemic spleen [15]. Furthermore, they remain multipotential, since splenic CFU-s transplanted into irradiated recipients are able to provide granulocytic, megakaryocytic and erythroid colonies, in the same proportion as normal [14–16]. The functional

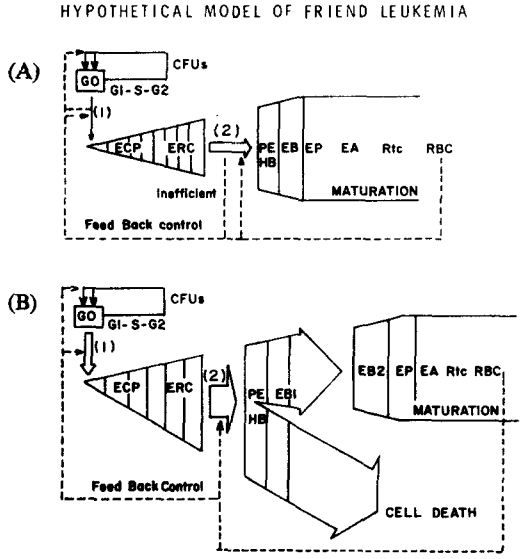


Fig. 2. Hypothetical model of Friend leukemia. (A) *Early action of the virus.*

The Friend virus acts mainly on the "second step" differentiation. It provokes a pathological differentiation of ERC cells into proerythroblastic cells. This differentiation escapes from the normal controls: feed-back mechanisms and EPO action. But feed-back mechanisms continue to control the "first step" differentiation[1].

HB: hyperbasophilic cells which are pathological proerythroblasts or closely related precursors.

For legends, see Fig. 1.

(B) *Pathological process of the disease (exponential growth phase).*

The Friend disease is characterized by (1) *an overproduction of proerythroblastic cells (PE or hyperbasophilic cells HB) which is mostly inefficient since at least* 2/3 *of the PE die rapidly and since RBC are short lived. This overproduction results in* (2) *a continuous depletion of the ERC compartment and therefore the production of precursor cells: CFUs, ECP, ERC, is stimulated, apparently, by feed-back mechanisms of control. The balance between the production of PE and their death (with or without maturation) is positive.*

For legends: see Fig. 1.

deficiencies which are observed, namely in their capacity to produce thrombocytes, may be due to the fact that these CFU-s, as all the cells of the organism [9], are infected by the FV. However, this infection does not result in a neoplasic process at the level of the CFU-s.

From all these experimental data it appears that the abnormal overproduction of erythroid cells provoking Friend leukemia may only be due to a permanent differentiation of ERC into hyperbasophilic cells. This differentiation is induced and maintained (1) by the virus which is replicating continuously in the infected tissues of the host; (2) by the continuous supply of target cells (ERC) resulting from the action of "normal" feed-back mechanisms of control. It seems, therefore, unnecessary to postulate the existence of "neoplasic" clones having an unlimited capacity of proliferation. On the contrary, Friend leukemia cells are characterized by a massive cell death, but this mechanism of cell elimination is unable to compensate for the great overproduction of erythroid cells (proerythroblastic, erythroblastic and mature red blood cells). This model of the disease is summarized in Fig. 2.

If neoplasic cells are not necessary to explain the disease, do they exist in Friend leukemia? The main characteristics of malignant cells are correlated with their unlimited potential of multiplication: *in vitro*, malignant cells are generally capable of producing colonies when they are seeded in semi-solid agar culture medium and/or multiplying permanently in a liquid nutrient medium [26, 27]. *In vivo*, they grow and provoke death when injected into non-irradiated isogenic recipients. Numerous chromosome anomalies (aneuploidy) are also currently observed [28]. None of these characteristics have been observed in Friend leukemic cells: they cannot be cultivated *in vitro*, they are usually not transplantable into isogenic non-irradiated recipients [9, 10], and no karyotype anomalies have been observed [29].

The presence of tumor cells, transplantable into non-irradiated recipients, has been described by Thomson and Axelrad [30] as appearing early after virus injection. But only morphological criteria have been invoked, and no enhanced capacity of transplantability has been demonstrated. However, several laboratories [31–33] have obtained transplantable tumors by injecting subcutaneously pieces of leukemic spleens: in these cases, the success of the graft is exceptional (frequency 0·1%) and depends on the use of special conditions, i.e., the injection of large pieces of "old" leukemic spleen. These tumor cells have often a capacity of erythropoietic differentiation when cultivated *in vitro* [34], but they differ from the Friend leukemic cells: they have chromosomal markers, they are frequently aneuploid [33] and they are indefinitely transplantable. Therefore, they appear to be true malignant clones, arising at a late stage of the disease.

Table 1. *Clinical and physiological characteristics of human acute and chronic leukemia (excluding lymphoid leukemias)*

(A) *Clinical characteristics*

		Pathological cell lines	Infiltration by leukemic cells		
			Spleen	Bone marrow	Peripheral blood
CHRONIC LEUKEMIA	Granulocytic CGL	Granulocytic	+ + +	Increase of the granulocytic line	+ + +
	Polycythemia vera PV	Erythrocytic	+ +	Increase of the erythroid line	+ + + (plasma volume increased)
	Monocytic	Monocytic	Often enlarged	+ + +	+ +
ACUTE LEUKEMIA	Erythroleukemia	Erythroblastic	+ + +	+ +	+ + +
	Myeloblastic	Myeloblasts	Often enlarged	+ + +	+ +
	Monoblastic	Monoblasts	Often enlarged	+ + +	+ +
	Myelomonoblastic	Myloblasts and monoblasts	Often enlarged	+ + +	+ +

(B) *Physiological characteristics*

		Karyotype	Labeling indices	CFUc+		
				Number	Proliferation	Maturation
CHRONIC LEUKEMIA	Granulocytic CGL	Diploid Philadelphia chromosome	Increased in the spleen	Increased in bone marrow & peripheral blood	CSA dependent	CSA dependent
	Polycythemia vera PV	Diploid	ND++	Normal or decreased; very increased in peripheral blood	CSA dependent	CSA dependent
	Monocytic	Usually diploid	ND	ND	ND	ND
ACUTE LEUKEMIA	Erythroleukemia	Aneuploid (one case)	ND	ND	ND	ND
	Myeloblastic	Aneuploid	Normal or decreased in bone marrow	Decreased	Abortive CSA dependent	Abortive
	Monoblastic	Usually diploid	ND	ND	ND	ND
	Myelomonoblastic	Diploid or aneuploid	ND	ND	ND	ND

+In man, only CFUc can be quantified by *in vitro* techniques
++ND = not determined.

Comparison with the physiopathology of human leukemias

As it has been already outlined, the physiopathology of human leukemia is poorly understood. Clinical evolution and the degree of cellular differentiation have enabled chronic leukemias to be distinguished from acute leukemias. The clinical and physiopathological characteristics of the main categories of leukemias are summarized in Table 1. We have excluded lymphoid leukemias, which are less understood than the others, mainly because the physiology of the lymphocyte remains obscure: in contrast to other hemopoietic cells the

Table 2. Comparison between the physiopathological data concerning murine Friend leukemia, human chronic granulocytic leukemia and polycythemia vera

			Friend leukemia (Polycythemic variety)		Chronic granulocytic leukemia		Polycythemia Vera	
Clinical features	Main localization of pathological proliferation		Spleen		Spleen and bone marrow		Spleen	
	Plasma volume		Increased		ND+		Increased	
Physiological characteristics of the leukemic cells	Autonomous growth (*in vitro* and *in vivo*)		—		—		ND	
	Differentiation capacity		+		+		+	
	Death		+ + +		ND		ND	
	Caryotype		Diploid		Diploid (Philadelphia chromosome)		Diploid	
Physio-pathological studies	Stem cells in		No. of CFUs	Dif.+++ pattern	No.++	Dif.+++ pattern	No.++	Dif. +++ pattern
		Spleen	Increased	Almost normal	ND	ND	ND	
		Bone marrow	Normal	Normal	Normal or moderately increased	Normal	Decreased	Normal
		Peripheral blood	Very increased	Almost normal	Very increased	Normal	Very increased	Normal
	Labeling index++++	Spleen	High		High		ND	
		Peripheral blood	Low		Low		ND	

+ND = Not determined
++Number of CFUc
+++Diferentiation pattern
++++Percentage of labeled cells one hour after ^{3}H-TdR injection.

lymphocyte is a morphologically differentiated cell which can transform into a lymphoblast, and very few data regarding the physiology of this phenomenon are available. A distinction between leukemias of the hematopoietic and the immunopoietic systems is also justified by the probable existence of different stem cells for the two systems in the adult organism [4].

From this table, it appears that many physiopathological parameters have not been studied. However, available data on chronic leukemias, mainly granulocytic leukemia and polycythemia vera, and to a certain extent erythroleukemia (its classification is still a subject of controversy), have concordant features. All stages of the differentiation of the pathological cell line are found in the bone marrow, the spleen and the peripheral blood. These cells usually have a normal morphology and physiology: in the case of CGL, observations on cyclic granulocyte production [35] provide indirect evidence for the existence of positive and negative regulatory influences. The sensitivity of leukemic cells to the inhibitory effect of the granulocytic chalone has been reported [36]. In polycythemia vera it has been shown that erythropoietic differentiation was stimulated, namely in the spleen, and was accompanied by an increased plasma volume [37].

However, anomalies of the maturation process have been described: asynchrony of maturation of the nucleus and the cytoplasm in CGL and polycythemia vera [38, 39], and decreased levels of leucocyte alkaline phosphatase in CGL [40]. Cellular death has not yet been studied, but in CGL, an increase of the proportion of early precursor cells (myeloblasts, promyelo-

cytes, myelocytes) has been observed in the bone marrow of patients in relapse [41], and perhaps hyperuricemia, which is frequently observed [40], may be correlated with cell death. Defects of other cell lines usually accompanied the proliferation of the pathological cells.

There are no definite signs of malignancy: a diploid caryotype has always been found. An anomaly of the 22nd pair of chromosomes (Philadelphia chromosome) is associated with 95% of CGL [42], but whether this anomaly represents a malignant stigma is not known. However, it should be pointed out that this anomaly persists during the clinical and hematological remission of the disease, and is also found in erythroblastic and megaracaryocytic cells which are not believed to be malignant. With the exception of one report in the literature [43], and in spite of repeated attempts, the Philadelphia chromosome is lost from bone marrow and blood cells from CGL when cultured in liquid medium [44]. Peripheral blood of patients with CGL not in remission was transfused into patients with various states of leucopenia: the presence of Ph^+ cells in the bone marrow of these patients has been reported, but this "graft" has always been transient, and no induction of CGL has been reported using this procedure [45].

In the two chronic leukemias which have been the best studied (CGL and PV), the total number of CFU-c is increased as compared to normal values, namely in the spleen (CGL) and peripheral blood (CGL and PV) [39, 46]. These CFU-c keep normal characteristics of maturation and proliferation in soft-agar culture medium, and the presence of colony stimulating factor (CSF) is necessary [47].

The labeling indices (LI) observed one hour after *in vivo* injection of ^{3}H-TdR have been measured in the spleen of CGL: in this instance, high values were found, which correlated well with the stage of the disease. On the contrary, LI of the bone marrow has no indicative value [48].

Therefore, all these data concerning CGL, although incomplete, are compatible with the Friend model as shown in Table II. The most striking similarities are: absence of autonomous proliferation of the leukemic cells, the existence of a quasi-normal pattern of differentiation, an important increase in the number of committed stem cells whose capacity for proliferation and differentiation *in vitro* remain normal and CSF dependent, and privileged localization of the pathological proliferation in the spleen. A study of the cell death in CGL remains necessary to demonstrate the value of this model. In addition, it should be mentioned that Rauscher leukemia, which is an erythroleukemia almost indistinguishable from Friend leukemia, may end like CGL in blastic crisis, a myeloblastic or lymphoblastic leukemia occurring in the bone marrow [49, 50].

The clinical picture is very different in acute leukemias. There is a failure of differentiation in the pathological cell line; aneuploidy has usually been found in AML [28], the number of CFU-c is normal or decreased and there is an *in vitro* defective capacity for proliferation and maturation when seeded in soft-agar medium, although the CFU-c remain apparently dependent on CSF [51]. It is not known whether the leukemic cells can grow in liquid medium because in culture they lose distinctive features [52]. Low labeling indices have been regularly found, and abnormal cells generally have a prolonged cell cycle time [53, 54]; but most of these kinetic data are difficult to interpret since they show the existence of dividing and non-dividing cells (in AML) whose proportions vary as a function of the patient and the stage of the disease.

It is obvious that a model for studying the physiopathology of acute leukemia is needed because of the limitations of investigations in human patients. It is striking that models of myeloblastic leukemias have never been studied *in vivo*. This could perhaps be explained by the experimental difficulties due to a long latency period, and mainly because this type of leukemia was difficult to reproduce regularly in mice. The recent availability of a purified strain of myelogenous leukemia-inducing virus [55] will perhaps provide an appropriate model for the study of the physiopathology of human acute myeloblastic leukemia.

Acknowledgements—The authors are greatly indebted to P. Tambourin and F. Wendling for valuable discussions and wish to thank A. Beaudiment for secretarial assistance.

REFERENCES

1. W. Dameshek, *Leukemia*, p. 14. Grune & Stratton, New York (1964).
2. D. H. Pluznik and L. Sachs, The cloning of normal mast cells in tissue culture. *J. cell. comp. Physiol.* **66,** 319 (1965).

3. T. R. Bradley and D. Metcalf, The growth of mouse bone marrow cells *in vitro*. *Aust. J. exp. Biol. med. Sci.* **44,** 287 (1966).
4. L. G. Lajtha and R. Schofield, On the problem of differentiation in haemopoiesis. *Differentiation* **2,** 313 (1974).
5. L. G. Lajtha and R. Schofield, Regulation on stem cell renewal and differentiation: possible significance in aging. *Advanc. in gerontol. Res.* **5,** 131 (1971).
6. R. G. Tarbutt and N. M. Blackett, Cell population kinetics of the recognizable erythroid cells in the rat. *Cell Tiss. Kinet.* **1,** 65 (1968).
7. V. Covelli, G. Briganti and G. Silini, An analysis of bone marrow erythropoiesis in the mouse. *Cell Tiss. Kinet.* **5,** 41 (1972).
8. Y. Najean, J. Dionio and C. Dresch, Modèle de l'érythropoiese obtenu à partir de l'utilisation de ^{3}H-Thymidine par la moèlle *in vitro* et sa vérification par l'étude de la cinétique du radio-fer *in vivo*. I—Resultats obtenus chez des sujets normaux. *Rev. franç. Et. clin. biol.* **6,** 575 (1969).
9. C. Friend, Cell free transmission in Swiss mice of a disease having the character of a leukemia. *J. exp. Med.* **105,** 307 (1957).
10. D. Metcalf, J. Furth and R. F. Buffett, Pathogenesis of mouse leukemia caused by Friend virus. *Cancer Res.* **19,** 52 (1953).
11. E. A. Mirand, T. C. Prentice, J. G. Hoffman and J. T. Grace, Effect of Friend virus in Swiss and DBA_2 mice on ^{59}Fe uptake. *Proc. Soc. exp. Biol. (N.Y.)* **106,** 423 (1961).
12. P. E. Tambourin, D. Gallien-Lartigue, F. Wendling and D. Hualme, Erythrocyte production in mice infected by the polycythaemia-inducing Friend virus or by the anaemia-inducing Friend virus. *Brit. J. Haematol.* **24,** 511 (1973).
13. P. Tambourin and F. Wendling, Malignant transformation and erythroid differentiation by polycythemia-inducing Friend virus. *Nature New Biol.* **234,** 230 (1971).
14. F. Wendling, P. E. Tambourin and P. Jullien, Hematopoietic colony forming units in mice infected by the polycythemia-inducing Friend virus. III—Pattern of blood recovery in irradiated mice grafted with normal or infected spleen cells. *J. nat. Cancer Inst.* **51,** 179 (1973).
15. F. Wendling, P. E. Tambourin and P. Jullien, Hematopoietic CFU in mice infected by the polycythemia-inducing Friend virus. Number of CFU and differentiation pattern in the spleen colonies. *Int. J. Cancer* **9,** 554 (1972).
16. J. P. Okunewick and E. L. Phillips, Change in marrow and spleen CFU compartments following leukemia virus infection: comparison of Friend and Rauscher virus. *Blood* **42,** 885 (1973).
17. E. A. Mirand, R. A. Steeves, R. D. Lange and J. T. Grace, Virus-induced polycythemia in mice: erythropoiesis without erythropoietin. *Proc. Soc. exp. Biol. (N.Y.)* **128,** 844 (1968).
18. T. Fredrikson, P. E. Tambourin, F. Wendling, C. Jasmin and F. Smadja-Joffe, The target cell of the polycythemia-inducing Friend virus: studies with myleran. *J. nat. Cancer Inst.* **55,** 443–446 (1975).
19. J. Lobue, P. Alexander, T. N. Fredrickson, E. F. Schultz, A. S. Gordon and L. J. Johnson. Erythrokinetics in normal and disease states. Virally-induced murine erythroblastosis, a model system in *Regulation of Erythropoiesis*; 1st international conference on hematopoiesis. Capri 13, 14 oct., 1971. Edited by A. S. Gordon, M. Condoralli, C. Peschle. p. 89. The Publishing House "Il Ponte", Milano (1971).
20. F. Zajdela, Contribution à l'étude de la cellule de Friend. *Bull. Cancer* **49,** 351 (1962).
21. E. A. Mirand, R. A. Steeves, R. D. Lange and J. T. Grace. Virus-induced polycythemia in mice: erythropoiesis without erythropoietin. *Proc. Soc. exp. Bio. Med. (N.Y.)* **128,** 844 (1968).
22. S. Sassa, F. Takaku and K. Nakao Regulation of erythropoiesis in the Friend leukemia mouse. *Blood* **31,** 758 (1968).
23. F. Smadja-Joffe B. Klein. M. C. Kerdiles, L. Feinendegen and C. Jasmin, Study of cell death in Friend leukemia. *Cell Tiss. Kinet.* in press (1975).
24. F. Smadja-Joffe, C. Jasmin, E. P. Malaise and C. Bournoutian, Study of the cellular proliferation kinetics of Friend leukemia. *Int. J. Cancer* **11,** 300 (1973).
25. F. Wendling, P. Tambourin and P. Bucau-Varlet, Capacité de prolifération des cellules souches (CFU-s) de la rate de souris leucémiques de Friend. *C.R. Acad. Sc. Paris* **278,** 2385 (1974).

26. L. MONTAGNIER and I. MACPHERSON, Croissance sélective en gélose de cellules de hamster transformées par le virus du polyome. *C.R. Acad. Sc. Paris* **258,** 4171 (1964).
27. G. J. TODARO and S. A. AARONSON, Properties of clonal lines of murine sarcoma virus transformed Balb/3T3 cells. *Virology* **38,** 174 (1969).
28. M. C. CORTINAS DE NAVA, *Les anomalies chromosomiques au cours des hemopathies malignes et non malignes. Une étude de* 171 *cas.* Thèse, Edited by l'Expansion Paris (1968).
29. A. TAKADA, Y. TAKADA and J. L. AMBRUS, Proliferation of spleen cells from mice infected with Friend virus in the spleens of unirradiated and irradiated mice. *Experientia (Basel)* **27,** 315 (1971).
30. S. THOMSON and A. A. AXELRAD, A quantitative spleen colony assay method for tumor cells induced by Friend leukemia virus infection in mice. *Cancer Res.* **28,** 2105 (1968).
31. R. F. BUFFET and J. FURTH, A transplantable reticulum cell sarcoma variant of Friend's viral leukemia. *Cancer Res.* **19,** 1063 (1959).
32. C. FRIEND and J. R. HADDAD, Tumor formation with transplants of spleen or liver from mice with virus-induced leukemia. *J. nat. Cancer Inst.* **25,** 1279 (1960).
33. W. OSTERTAG, H. MELDERIS, G. STEINHEIDER, N. KLUGE and S. DUBE, Synthesis of mouse haemoglobin and globin mRNA in leukaemic cell cultures. *Nature New Biol.* **239,** 231 (1972).
34. G. B. ROSSI and C. FRIEND, Erythrocytic maturation *in vitro* of murine (Friend) virus-induced leukemic cells. *Nat. Cancer Inst. Monogr.* **22,** 505 (1966).
35. H. VODOPICK, E. M. RUPP and C. L. EDWARDS, Spontaneous cyclic leucocytosis and thrombocytosis in chronic granulocytic leukemia. *New Engl. J. Med.* **286,** 284 (1972).
36. T. RYTOMAA, Role of chalone in granulopoiesis. *Brit. J. Haematol.* **24,** 141 (1973).
37. C. PARMENTIER, Personal communication.
38. J. V. ULLYOT and D. F. BAINTON, Azurophil and specific granules of blood neutrophils in chronic myelogenous leukemia: an ultrastructural and cytochemical analysis. *Blood* **44,** 469 (1974).
39. J. W. ADAMSON and C. A. FINCH, Erythropoietin and the regulation of erythropoiesis in Di Guglielmo's syndrome. *Blood* **36,** 590 (1970).
40. M. M. WINTROBE, *Clinical Haematology*, Lea & Febiger, Philadelphia (1968).
41. L. BRANDT and C. R. SCHNELL, Granulopoiesis in bone marrow and spleen in chronic myeloid leukaemia. *Scand. J. Haematol.* **6,** 65 (1967).
42. J. WHANG, E. FREI and J. H. TJIO, The distribution of the Philadelphia chromosome in patients with chronic myelogenous leukemia. *Blood* **22,** 664 (1963).
43. C. B. LOZZIO and B. B. LOZZIO, Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood* **45,** 321 (1975).
44. A. M. VENUAT, C. ROSENFELD and M. J. TESTI, Etude cytogénétique comparative de lignées lymphoblastoïdes humaines normales et leucémiques au cours de leur établissement, in *La Différentiation des Cellules Eucaryotes en Culture.* Edited by "Colloque de l'INSERM" Vol. **19,** 165 (1973).
45. E. E. MORSE, E. J. FREIREICH, P. P. CARBONE, W. BRONSON and E. FREI, The transfusion of leukocytes from donors with chronic myelocytic leukemia to patients with leukopenia. *Transfusion* **6,** 183 (1966).
46. J. M. GOLDMAN, K. H. TH'NG and R. M. LOWENTHAL. *In vitro* colony forming cells and colony stimulating factors in chronic granulocytic leukemia. *Brit. J. Cancer* **30,** 1 (1974).
47. M. A. S. MOORE, N. WILLIAMS and D. METCALF, *In vitro* colony formation by normal and leukemic human hematopoietic cells: interaction between colony forming and colony-stimulating cells. *J. nat. Cancer Inst.* **50,** 591 (1973).
48. L. BRANDT, Differences in the proliferative activity of myelocytes from bone marrow, spleen and peripheral blood in chronic myeloid leukemia. *Scand. J. Haematol.* **6,** 65 (1969).
49. M. BOIRON, J. P. LEVY, J. LASNERET, S. OPPENHEIM and J. BERNARD, Pathogenesis of Rauscher leukemia. *J. nat. Cancer Inst.* **35,** 865 (1965).
50. F. J. RAUSCHER, A virus-induced disease characterized by erythrocytopoiesis and lymphoid leukemia. *J. nat. Cancer Inst.* **29,** 515 (1962).
51. M. A. S. MOORE, G. SPITZER, W. WILLIAMS, D. METCALF and J. BUCKLEY, Agar culture studies in 127 cases of untreated acute leukemia according to *in vitro* growth characteristics. *Blood* **44,** 1 (1974).

52. K. K. SANFORD, Biologic manifestations of oncogenesis *in vitro*: a critique. *J. nat. Cancer Inst.* **53,** 1481 (1974).
53. A. M. MAUER and V. FISCHER, Characteristics of cell proliferation in four patients with untreated acute leukemia. *Blood* **28,** 428 (1966).
54. S. A. KILLMAN, E. P. CRONKITE, J. S. ROBERTSON, T. M. FLIEDNER and V. P. BOND, Estimation of phases of the life cycle of leukemic cells from labeling in human beings *in vivo* with tritiated thymidine. *Lab. Invest.* **12,** 671 (1963).
55. M. P. MCGARRY, R. A. STEEVES, R. J. ECKNER, E. A. MIRAND and P. J. TRUDEL, Isolation of a myelogenous leukemia-inducing virus from mice infected with the Friend virus complex. *Int. J. Cancer* **13,** 867 (1974).

Europ. J. Cancer Vol. 11, pp. 841–844. Pergamon Press 1975. Printed in Great Britain

Preliminary Clinical and Absorption Studies with Prednimustine* in Patients with Mammary Carcinoma

I. KÖNYVES,‡ B. NORDENSKJÖLD,† G. PLYM FORSHELL,‡ A. DE SCHRYVER† and H. WESTERBERG-LARSSON‡

†*Radiumhemmet, Karolinska Hospital*, 104 01 *Stockholm*
‡*Research Laboratories, AB Leo*, 252 42 *Helsinborg*

Abstract—*Prednimustine is a prednisolone ester of chlorambucil with known activity against lymphocytic leukemia and lymphoma. Its therapeutic effect against mammary carcinoma was investigated after daily oral doses of* 20–100 *mg to* 15 *patients with measurable soft tissue metastases. Seven of these had previously received hormone therapy or chemotherapy. Two complete and five partial remissions were observed. The side effects were dose and time dependent. The most frequent side effect was moderate leukopenia and thrombocytopenia.*

Preliminary absorption studies were performed. When tritiumlabelled prednimustine was administered to one patient no unchanged drug was found in the urine, whereas in faeces the total amount of radioactivity was found to be prednimustine. When single oral doses of prednimustine were given to six patients, the amounts of unchanged drug recovered in faeces indicate that 45–100% *was absorbed.*

INTRODUCTION

PREDNIMUSTINE (INN) (pregna-1, 4-diene-3, 20-dione, 11,17,21-trihydroxy, 21-4-*p*(bis-(2-chloroethyl)-amino) phenylbutyrate (Fig. 1) is a

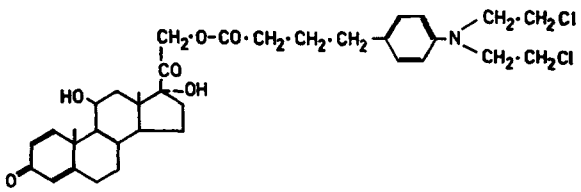


Fig. 1.

member of a class of steroid esters with alkylating properties synthesized at Leo Research Laboratories. The synthesis and the relation between chemical structure and cytostatic activity of this class of cytostatic compounds were previously reported [1].

Accepted 10 September 1975.

*Prednimustine (INN) = Leo 1031, NSC 134087, EORTC 1502.

The drug is active against several animal tumors with a generally increased therapeutic index as compared to that of chlorambucil. Furthermore, the ratio LD50:ED50 obtained with prednimustine in rats bearing Walker carcinoma 256 was increased as compared to that of an equimolar mixture of prednisolone and chlorambucil. Prednimustine was also found to be a potent inhibitor of DMBA induced mammary adenocarcinoma and spontaneous mammary tumors. The toxicity, hormonal activities and cytotoxic activities of the drug were defined in several species [1,2].

Evenaar *et al.* [3] measured the activity of several chemotherapeutic agents on mouse leukemia L1210 and normal bone marrow stem cells. Prednimustine was 23 times more active against leukemia cells than against bone marrow stem cells.

Clinical studies have demonstrated activity of prednimustine in chronic lymphocytic leukemia and lymphocytic lymphoma [4–6].

Combination of prednisolone and chlorambucil was successfully used against human

Table 1. Results in individual patients

Pat. no.	Age	Previous[a] treatment	Time in months and mg/day	Clinical[b] response	Toxicity
1	55	h	2 (100)	II	Gastritis, leukopenia, cutaneous infiltration.
2	55	r	5 (32-80)	II	Leukopenia, trombocytopenia, diarrhoea.
3	55	c+h+r	3 (32-56)	IV	Anemia.
4	56	r	4 (20-80)	III	Leukopenia.
5	59	r	5 (32)	II	Leukopenia.
6	59	c+h	3 (24-48)	IV	Moderate hypertension.
7	65	h	2 (40-100)	III	Leukopenia, fever, exanthema.
8	69	r	3 (100)	III	Anemia, leukopenia.
9	70	r	7 (24)	II	Leukopenia.
10	73	r	2 (100)	II	Leukopenia, fever, cutaneous infiltration.
11	79	h	3 (20-48)	IV	Decreased sense of well-being.
12	79	c+h+r	4 (16-24)	III	None.
13	80	h+r	2 (32)	I	Psychosis, glucosuria.
14	81	-	2 (100)	I	Glucosuria, dizziness.
15	82	h+r	1 (100)	III	Leukopenia, trombocytopenia.

a) c = chemotherapy
h = hormone therapy
r = radiotherapy

b) See page 2 "Evaluation" for definitions.

mammary carcinoma [7]. This article describes the clinical effect and toxicity of prednimustine in patients with advanced mammary carcinoma. In a few cases the oral absorption of prednimustine was studied.

MATERIAL AND METHODS

Fifteen patients all postmenopausal were seen as outpatients or hospitalized at Radiumhemmet. Fourteen had locally recurrent or inoperable mammary carcinoma while one had measurable lung metastasis. Fourteen of the patients had been treated with post operative radiotherapy or chemotherapy or endocrine therapy as indicated in Table 1.

Evaluation

Tumor diameter, clinical status and peripheral blood cell counts were recorded at one to two weeks interval. Chest X-ray, serum GOT, GPT, Bilirubin and alkaline phosphatase were obtained at one to two months intervals. Results were defined according to EORTC-protocol [8] and grouped into: I complete remission; II partial remission; III stationary disease and IV progression.

Administration of drug

The drug was available as 8 and 20 mg tablets and was given orally in doses of between 24 and 100 mg/day divided into three portions (Table 1).

ABSORPTION STUDIES

Single oral doses of 20 or 100 mg were administered to six patients. Faeces was quantitatively collected during four days and kept frozen until analyzed. The samples were weighed and extracted with 3×100 ml of methanol per 100 g of faeces. Aliquots of the methanol extracts were applied to columns of Al_2O_3 and prednimustine was eluated with ethanol–diethyl ether (1:5 by volume). Quantitative determination was then achieved by gas-liquid chromatography.

RESULTS

Therapeutic effect

The therapeutic effects on the tumors are illustrated in Tables 1 and 2. Seven patients responded to therapy (Response categories I and II). Two patients had complete remissions; one of these was treated for a local recurrence (patient no. 13); the other (no. 14) for lung metastases.

Table 2. Results of therapy

Response category	Previous, hormonal or cytotoxic treatment	No previous hormonal or cytotoxic treatment	Total
Complete remission (I)	1	1	2
Incomplete remission (II)	1	4	5
Stationary (III)	3	2	5
Progression (IV)	3	-	3

Therapy was discontinued when side effects (see below) were recorded. After therapy, the median duration of complete or partial remission was approximately two months.

Patients previously treated with chemotherapy or hormone therapy showed poor or no response. None of the two responders in this category had received corticosteroids. Five out of seven patients without previous hormonal therapy responded.

Side effects

The side effects were time and dose dependent. Leukopenia ($\leqq$ 2000 wbc/mm^3) or thrombocytopenia ($\leqq 10^5$ thrombocytes per mm^2) were dose limiting in nine of the patients. One patient (no. 3) who had osseous metastases died with moderate leukopenia and anemia. In all other patients the leukopenia and thrombocytopenia were reversible and the cell counts were normalized within 3–6 weeks. In several patients the white cell counts continued to decrease during the first 2–3 weeks after removal of the drug. Hair loss was not frequent nor complete. Mucositis was not recorded.

One patient had hypertension as dose limiting side effect at 48 mg/day while another with 100 mg/day developed symtoms reminiscent of gastritis or gastric ulcer but X-ray examination failed to reveal any ulcer. One patient had diarrhoea. Several of the patients experienced a marked decrease of their general sense of well-being. One patient developed diabetes and psychosis at 32 mg/day. These last findings, which were reversible, are interpreted to indicate prednisolone effects. Two patients had cutaneous infiltrations which disappeared after discontinuation of therapy.

Absorption

The absorption of prednimustine was investigated in animals and in humans. Initial experiments with ^{3}H-labeled prednimustine in rats, dogs and one patient demonstrated that the unabsorbed fraction of orally administered drug was excreted unchanged. Moreover, drug that was absorbed was metabolized and not excreted as unchanged ester (not illustrated). To estimate the absorption six patients (not participating in the clinical study) received single oral doses of prednimustine and the faecal excretion of unchanged drug was measured. The data show considerable variation in excretion. Four patients that were in good general condition excreted 1–35% (Table 3, subjects 1, 3, 5 and 6) as unchanged drug while two patients (Table 3, subjects 2 and 4) in severe clinical condition with hepatic metastasis excreted 47–55% as unchanged drug.

Table 3. Recovery of unchanged prednimustine in faeces after a single oral dose; cumulative excretion for 72 hr

Subject	Dose mg	Found in faeces mg	%
1	20	1.2	6
1	100	10.2	10
2	100	46.7	47
3	20	4.7	24
4	100	55.2	55
5	100	0.3	0.3
6	20	7.0	35

DISCUSSION

Our data show that prednimustine has activity in man typical for alkylating agents. This conclusion is based on the tumor regression observed, thrombocytopenia and leukopenia. The drug also has some prednisolone-like activity which is concluded from the corticosteroid side effects and the development of cushingoid appearance of patients under prolonged treatment. This steroid effect is important to consider since it means that the drug should be gradually removed or alternatively a suitable dose of a corticosteroid should be given after discontinuation of prednimustine therapy. In all patients but one (no. 3) who had also osseous metastases the side effects were reversed within a few weeks after discontinuation of therapy.

In animal systems prednimustine has properties different from chlorambucil and prednisolone and different from the two compounds in equimolar mixture. This difference might possibly be due to the difference in lipid solubility or alternatively to different biological specificity. In animals, prednimustine has a higher therapeutic index than chlorambucil, prednisolone and their mixture. Our data do not allow the conclusion that this difference in therapeutic index is true also for human mammary carcinoma. The data do, however, suggest that further work are motivated to estimate the value of prednimustine in the treatment of mammary carcinoma.

Our data on drug absorption indicate considerable interindividual differences in absorption of prednimustine. The data must, however, be interpreted with care since our method gives only indirect information on the absorption of prednimustine. Pharmacokinetic studies with labeled prednimustine are in progress.

REFERENCES

1. I. Könyves, H. Fex and B. Högberg. Novel corticosteroid esters with alkylating properties. G. K. Daikos, Ed. Vol III: Antineoplastic chemotherapy, 791, Proc. 8th Int. Congr. Chemotherapy, Athens (1974).
2. Leo 1031, Brochure, 4th Edn., Dec., 1974, AB Leo, Helsinborg, Sweden. (Obtainable from AB Leo.)
3. A. H. Evenaar, E. H. R. Wins and L. M. van Putten. Cell killing effectiveness of an alkylating steroid (Leo 1031). *Europ. J. Cancer* **9,** 773 (1973).
4. L. Brandt, I. Könyves and T. R. Möller. Therapeutic effect of Leo 1031, an alkylating corticosteroid ester in lymphoproliferative disorders—I. Chronic lymphocytic leukaemia. *Acta med. Scand.* **197,** 317 (1975).
5. T. R. Möller, L. Brandt, I. Könyves and L. G. Lindberg. Therapeutic effect of Leo 1031, an alkylating corticosteroid ester in lymphoproliferative disorders—II. Lymphocytic lymphoma. *Acta med. Scand.* **197,** 323 (1975).
6. C. W. Aungst, A. Mittelman and G. P. Murphy. Treatment of chronic lymphocytic leukemia and lymphosarcoma with a new chlorambucil ester of prednisolone (Leo 1031) (NSC 134087). Submitted for publication.
7. H. A. Freckman, H. L. Fry, F. L. Mendez and E. R. Maurer. Chlorambucil-prednisolone therapy for disseminated breast carcinoma. *J. Amer. med. Ass.* **189,** 111 (1964).
8. E.O.R.T.C., Early clinical trial cooperative group: General protocol for cooperative chemotherapy screening study in human cancer. (1973).

Europ. J. Cancer Vol. 11, pp. 845–854. Pergamon Press 1975. Printed in Great Britain

Changes of Molecular Properties Associated with the Development of Resistance Against Methotrexate in Human Lymphoblastoid Cells*

D. NIETHAMMER and R. C. JACKSON†
Department of Paediatrics, University of Ulm, Germany
and
Department of Applied Biochemistry, Institute of Cancer Research, Sutton, Surrey, United Kingdom

Abstract—*W1-L2 human lymphoblastoid cells were exposed, in culture, to progressively increasing concentrations of methotrexate. By this procedure a series of sublines was obtained with varying degrees of resistance to this drug. Membrane transport of folates and antifolates and the activities of folate dependent enzymes were studied in these sublines. Three mechanisms of drug resistance were observed. As in many previous studies, elevated cellular activities of dihydrofolate reductase were found; in some sublines the activity increased over 200-fold. A detailed study was made of the electrophoretic pattern of dihydrofolate reductase in the resistant cells, and the increase in activity was found to be of two kinds; initially electrophoretic form I increased, whereas in cells with the highest enzyme activities predominantly form II was found. The other enzymes tested, thymidine kinase, thymidylate synthetase, serine hydroxymethyltransferase, 10-formyltetrahydrofolate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase, did not change greatly during the course of development of resistance. Serine hydroxymethyltransferase and thymidylate synthetase were both inhibited by methotrexate but these inhibitions were weak. The contributions made by these secondary sites of action to the cytotoxicity of the drug are discussed. A relatively high frequency of mutants was found with impaired membrane transport of methotrexate; these mutants appeared rather late in the course of development of resistance, and in all these cases enzyme and transport mutations occurred together. As in L1210 mouse leukaemia cells, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and methotrexate shared a common pathway for uptake into the cell; in contrast to L1210 cells, we found the predominant change in the V_{max}, rather than the K_m, of the transport system of resistant mutants. It seems that resistance involving impaired methotrexate transport during treatment of human malignant disease may be a comparatively frequent phenomenon, and may occur together with elevation of dihydrofolate reductase activity.*

Accepted 9 September 1975.

*Experimental work in this paper was supported by grants from Deutsche Forschungsgemeinschaft (SFB 112) and from the Ludwig Institute for Cancer Research (London Branch).

†Present address: Laboratory for Experimental Oncology Indiana University School of Medicine, Indianapolis 46202, U.S.A.

‡Abbreviations used: MTX, methotrexate; FH_2, dihydrofolate; FH_4, tetrahydrofolate.

INTRODUCTION

THE DEVELOPMENT of resistance to methotrexate (MTX)‡ has been studied in numerous experimental tumours. The known mechanisms of acquired resistance involve either an elevated level of the target enzyme, dihydrofolate (FH_2) reductase [1–8], increased activity of thymidylate synthetase [9, 10], impaired

membrane transport of the drug [7, 8, 11–15], or utilisation of the "salvage pathway" for biosynthesis of thymidylate [16]. Our own studies with L1210 mouse leukaemia cells [17] and experiments by others [14] have shown that defects of cellular uptake of MTX tend to appear early in the course of development of resistance; these mutants are then outgrown by cells containing high dihydrofolate reductase levels [17, 18]. The relationship of the multiple electrophoretic forms of dihydrofolate reductase has been studied in detail in *Lactobacillus casei* [19]. Preliminary studies with L1210 cells have explored the relation of these different forms to the increased total reductase activity found in MTX-resistant cells [17, 20, 21]. As resistance developed, electrophoretic form I increased first, but the highly resistant cells which emerged later contained predominantly form II of the enzyme. Corresponding studies have not been reported with human cells.

A complete understanding of resistance of human cells to MTX will also require a fuller knowledge of the molecular properties of folate transport systems in the membrane of human cells. Kessel, Hall and Roberts [22] demonstrated that permeability to MTX of human leukaemic leucocytes was correlated with clinical response to the drug. At low drug concentrations MTX entered the cells by a saturable, temperature-sensitive process of facilitated diffusion [22], but at extracellular concentrations greater than 2×10^{-5} M a significant amount of the drug could enter the cells by passive diffusion [23]. Much greater concentrations than this may be achieved during high-dose MTX infusions. Experiments with transport mutant L1210 cells [17] indicated that ability to transport 5-methyltetrahydrofolate was lost in parallel with MTX transport activity, but that folic acid transport was unimpaired; the V_{max} for uptake of MTX was similar to that of the drug-sensitive line, whereas K_m increased by a factor of 3·5. The present report describes analogous studies with human cells, and considers the relevance of the results to the treatment of human malignant disease.

MATERIAL AND METHODS

^{3}H-MTX, 5-methyl-(^{14}C)-tetrahydrofolate and ^{3}H-folic acid were obtained from the Radiochemical Centre, Amersham. Their purity, as monitored by thin layer chromatography, was about 98%. Unlabelled MTX was a product of Lederle, enzyme substrates and cofactors were from Boehringer, and reagent chemicals were obtained from the usual commercial sources, analytical grades being used where available.

Cell culture

The W1-L2 human lymphoblastoid cell line was originated in 1968 from the spleen of a patient with spherocytic anaemia [24]. The cells were maintained in continuous suspension culture using RPMI 1640 medium, supplemented with 10% foetal calf serum and antibiotics. Cells were subcultured every second day into Falcon plastic tissue culture flasks, using an inoculum of $6–8 \times 10^4$ cells/ml. The generation time under these conditions was about 14 hr and the cultures reached a final density of about $1·5 \times 10^6$ cells/ml. The dose–response curves were obtained by adding the required concentrations of MTX to triplicate cultures at zero time, and counting the cultures in a haemocytometer after 48 hr. What is measured under these conditions is thus inhibition of cell growth, not cell lysis or decrease in viability. Resistant sublines were obtained by growing sensitive W1-L2 cells in the continuous presence of MTX. The initial concentration used was 2×10^{-8} M. Under these conditions more than 70% of the cells died. After 48 hr the surviving cells were transferred to fresh drug-containing media. Over the course of 5–10 culture generations the cell growth rate increased and eventually no dead cells were seen. When the cell growth rate was almost back to normal the drug concentrations was doubled and the procedure was repeated. Five parallel lines were maintained in this way, and the total duration from step 1 to step 9 was about four months. The studies here described are based on the final two stages in the development of resistance of each line (steps 8 and 9). Terminology is as follows: WS indicates the original drug-sensitive line, and WR the resistant lines; the first number indicates the stage of resistance, and the second refers to the cell line (e.g. WR9-3 is line 3 at the 9th stage of resistance). The WR8-lines were maintained in the presence of $1·6 \times 10^{-6}$ M MTX, and the WR9-lines in the presence of $3·2 \times 10^{-6}$ M MTX.

Transport experiments

Cells were collected during logarithmic growth and resuspended in phosphate-buffered saline [25] pH 7·2 at 37° at a concentration of about 5×10^6 cells per ml. Radioactive substrate was added at zero time and 2 ml aliquots were withdrawn at the indicated times and were blown into 9 ml of ice-cold physiological saline which stopped transport immediately

[23]. During the course of the experiments the pH of the medium did not change, no cell lysis was seen, and the integrity of the membrane, as monitored by trypan blue, remained unimpaired. The pellet was washed twice in ice-cold saline and was digested overnight in 1 ml Protosol (New England Nuclear). After adding 10 ml of scintillation counting fluid (5 g PPO and 0·3 g dimethylPOPOP/l of toluene) the samples were poured into plastic vials and counted in a refrigerated scintillation counter. Counting efficiences under these conditions were 40·5% for tritium and 70% for ^{14}C. Extracellular volume was determined by the use of ^{14}C-inulin, which is not taken up by the cells. Radioactivity already present at zero time was probably due to a rapid non-specific adsorption to the outside of the cell membrane [23]. Data are calculated as nmoles of folate compound taken up per 10^9 cells (approx. 0·92 g wet weight).

Enzyme assays

All enzyme assays were performed at 30° on cell free preparations. For this purpose cells were disrupted with a Branson sonifier (position 6·20 sec) in ice-cold 0·05 M Tris–chloride buffer pH 7·5, and the lysate was centrifuged at 25000 × ***g*** for 20 min to remove particulate material. Dihydrofolate reductase was measured according to the method of Matthews and Huennekens [26], thymidylate synthetase by the method of Wahba and Friedkin [27], thymidine kinase by the method of Voytek and co-workers [28], serine hydroxymethyltransferase by the method of Scrimgeour and Huennekens [29], 5·10-methylene tetrahydrofolate dehydrogenase according to Ramasastri and Blakley [30] and 10-formyltetrahydrofolate synthetase as described by Rabinowitz and Pricer [31]. Polyacrylamide gel electrophoresis was carried out by the procedure of Davis [32], and gels were stained for dihydrofolate reductase activity as described by Dunlap and co-workers [19], and scanned in a gel scanner attached to a Gilford model 2400-S spectrophotometer. Affinity chromatography was performed by the method of Whiteley and co-workers [33].

RESULTS

Dose–response curves for the various resistant lines and for the sensitive line are shown in Fig. 1 and Fig. 2 respectively. Though the five lines of each stage of resistance were obtained in the same way, and kept at the same maintenance concentration of drug, their actual degree of resistance showed wide variation. The most resistant line obtained, WR9-4, was capable of growth at 70% of the normal rate at the highest MTX concentration tested, $2·5 \times 10^{-5}$ M.

Dihydrofolate reductase was measured in cell-free preparations of each cell line. All ten resistant sublines showed elevations of this

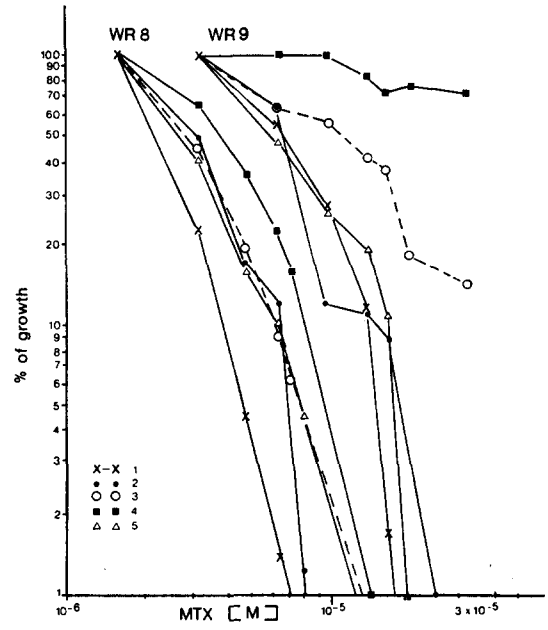


Fig. 1. Dose–response curves of the ten resistant sublines, measured in culture. Ordinate: cell count after 48 hr growth in presence of drug as percent of uninhibited control; Abscissa: MTX concentration (log scale).

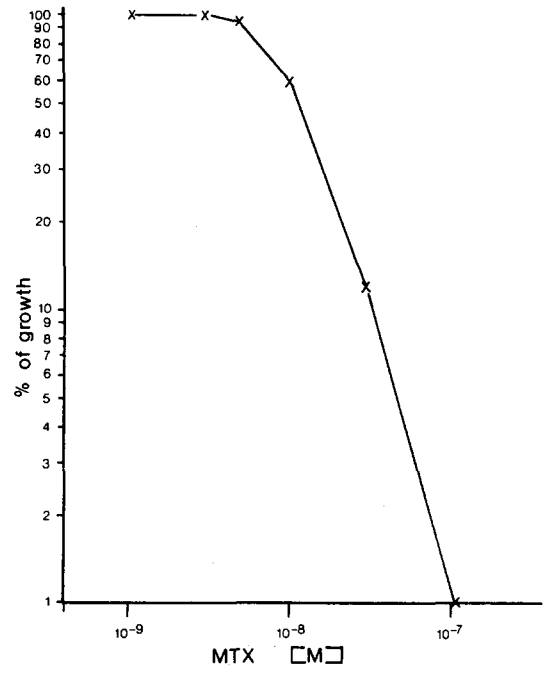


Fig. 2. Dose–response curve for the drug-sensitive W1-L2 cells. Details as for Fig. 1.

enzyme activity ranging from 20-fold to over 200-fold relative to the sensitive line (Table 1). Figure 3 shows scans of polyacrylamide gels stained for dihydrofolate reductase activity; enzyme from the sensitive line and three of the resistant lines (showing varying degrees of elevation of the activity) was examined in this way. The major peak at R_f = 0·24 is electrophoretic form I, and the double peak at R_f = 0·39 and 0·44 corresponds to electrophoretic form II; (although this peak is a doublet, both components are termed "Forms II" in order to conform with the terminology used by previous workers). This electrophoretic pattern corresponds closely to that previously described for mouse L1210 cells and human leukaemic leucocytes [17, 18, 34, 35]. It should be noted that the band widths shown are much wider than on the original gels, because of diffusion during the activity staining process. The two minor peaks at R_f = 0·17 and 0·61 developed also in the presence of 10^{-4} M MTX, suggesting that these bands represent NADPH oxidase activity. In the sensitive line form II predominated with over 60% of the total activity. In line WR9-3, with a 26-fold elevation of enzyme activity the increase has been predominantly in form I, now 63% of the total activity. In line WR9-4 (total elevation 126-fold) the further increase was apparently in Form II, and the proportions of the two forms are now close to those of the parent line. In WR9-1, with a total dihydrofolate reductase elevation of 234-fold, the increase in form II again accounted for most of the extra enzyme. It is noteworthy that in this highly resistant line Form II is no longer a doublet, but consists entirely of material with R_f = 0·39. Following affinity chromatography of the dihydrofolate reductase from crude extracts of WR9-1 cells all the activity appeared as Form I (Fig. 3). Since recovery from the column was 70%, and 66% of the original activity before chromatography was Form II, it is clear

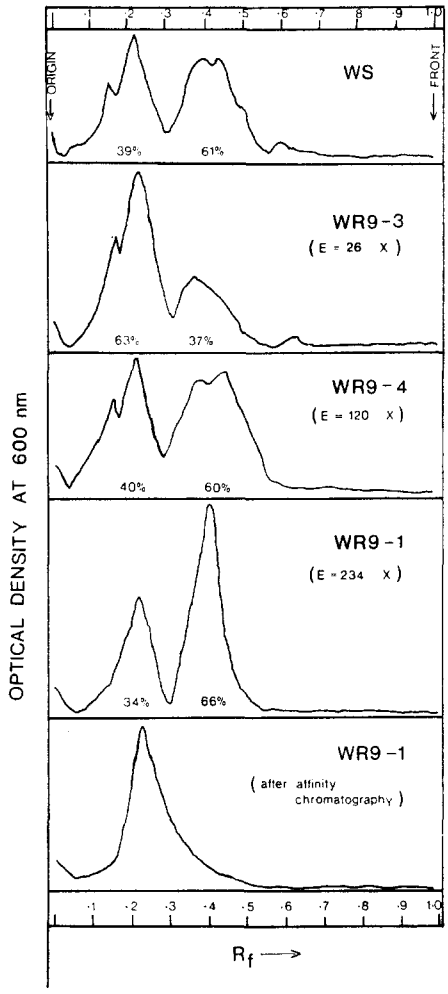


Fig. 3. Optical density scans of polyacrylamide gel electrophorograms stained for dihydrofolate reductase activity. The numbers under the peaks indicate the percentage of each form. E = elevation of total dihydrofolate reductase activity (relative to WS) for each line.

Table 1. Properties of some MTX-resistant W1-L2 sublines

Line	ID_{50} [μM]	Resistance – fold	Dihydrofolate reductase IU/10^9 cells*	Dihydrofolate reductase elevation – fold	Transport % of control*
WS	0·013	(1)	0·22	(1)	(100)
WR 8–1	2·2	170	48·1	222	108·3
WR 8–2	3·2	240	17·9	83	93·1
WR 8–3	3·0	230	4·5	21	19·7
WR 8–4	3·8	290	21·7	100	78·5
WR 8–5	2·8	220	30·6	141	93·8
WR 9–1	6·8	520	50·7	234	107·4
WR 9–2	6·9	530	4·9	23	27·3
WR 9–3	11·0	840	5·6	26	11·2
WR 9–4	>16·0	>1230	27·3	126	10·2
WR 9–5	6·1	440	14·9	69	76·5

*Values quoted are means for duplicate or triplicate determinations.

that at least 50% of the original Form II enzyme was converted to Form I during purification. The molecular relationship and biological significance of the electrophoretic forms of dihydrofolate reductase are discussed later. Table 2 records the activities of five other folate enzymes in the sensitive line and three of the resistant sublines. No large changes were detected; activity of thymidylate synthetase was slightly decreased in all three lines, and thymidine kinase showed increases of 21–60%. The biological significance of these small changes in cellular thymidine kinase is difficult to assess; no thymidine was present in the culture medium, but it is possible that traces of thymidine were present in the serum used to supplement the medium. The activities of serine hydroxymethyltransferase and 5·10-methylenetetrahydrofolate dehydrogenase were high, indicating a considerable potential for utilizing serine as a source of one-carbon groups for purine biosynthesis; the activity of 10-formyltetrahydrofolate synthetase was rather lower, suggesting that formate may be a less important one-carbon group source in these cells. In the W1-L2 cell dihydrofolate reductase is the only component known to interact strongly with MTX; the K_i value for the W1-L2 reductase with MTX has been estimated to be $7{\cdot}3 \times 10^{-12}$ M (Jackson, Hart and Harrap, unpublished results). Table 3 summarizes the inhibition of some other enzymes of folate metabolism by MTX in the sensitive cell line. Thymidylate synthetase and serine hydroxymethyltransferase from W1-L2 cells were also inhibited, though much more weakly, by MTX.

Transport of MTX was measured in the sensitive line and in all resistant sublines. Figure 4 shows the progress curves for cellular uptake of ^{3}H-MTX with time. Only one of the sublines of stage 8 (WR8-3) showed significant impairment of transport capacity, the other sublines at this stage having approximately normal rates. At the final stage 9 all but one of the cell lines (WR9-1) showed significant impairment of transport. Whereas drug uptake in the parental line ceased after 20 min, at which time the rates of uptake and efflux had become equal, such a steady state was not attained with the mutant lines, during the time of the experiments. The amount of bound MTX present in the cell at the steady state is

Table 2. Enzyme activities in W1-L2 cell sublines

	WS	WR9–I	WR9–3	WR9–4
Thymidylate synthetase	0·031	0·023	0·029	0·021
Thymidine kinase	0·056	0·068	0·070	0·090
10-Formyltetrahydrofolate synthetase	0·14	0·22	0·15	0·12
Serine hydroxymethyl transferase	1·14	1·64	1·50	1·10
5,10-Methylenetetrahydrofolate dehydrogenase	0·49	0·39	0·55	0·55

Activities are given as I.U./10^9 cells, when 1 I.U. = μmole of substrate converted per minute at 30°.

Table 3. Inhibition of some enzymes of folate metabolism in W1-L2 cells

Enzyme	Activity I.U./10^9 cells	Concentration of MTX required to give 50% inhibition
Dihydrofolate reductase	0·22	$3{\cdot}1 \times 10^{-9}$M*
Thymidylate synthetase	0·02	$2{\cdot}2 \times 10^{-5}$M
10-formyltetrahydrofolate synthetase	0·14	–†
Serine hydroxymethyl-transferase	1·14	$3{\cdot}2 \times 10^{-3}$M
5,10-methylenetetrahydrofolate dehydrogenase	0·49	–†

*Enzyme concn. used in this experiment was $1{\cdot}5 \times 10^{-9}$M.
†No detectable inhibition by MTX at 10^{-3}M.

determined by the number of binding sites, and clearly the increased amount of dihydrofolate reductase in the resistant lines may account for the increased amount of MTX taken up by the cells. The transport mutants are very stable, since their properties have not changed over six months continuous growth in the presence of MTX.

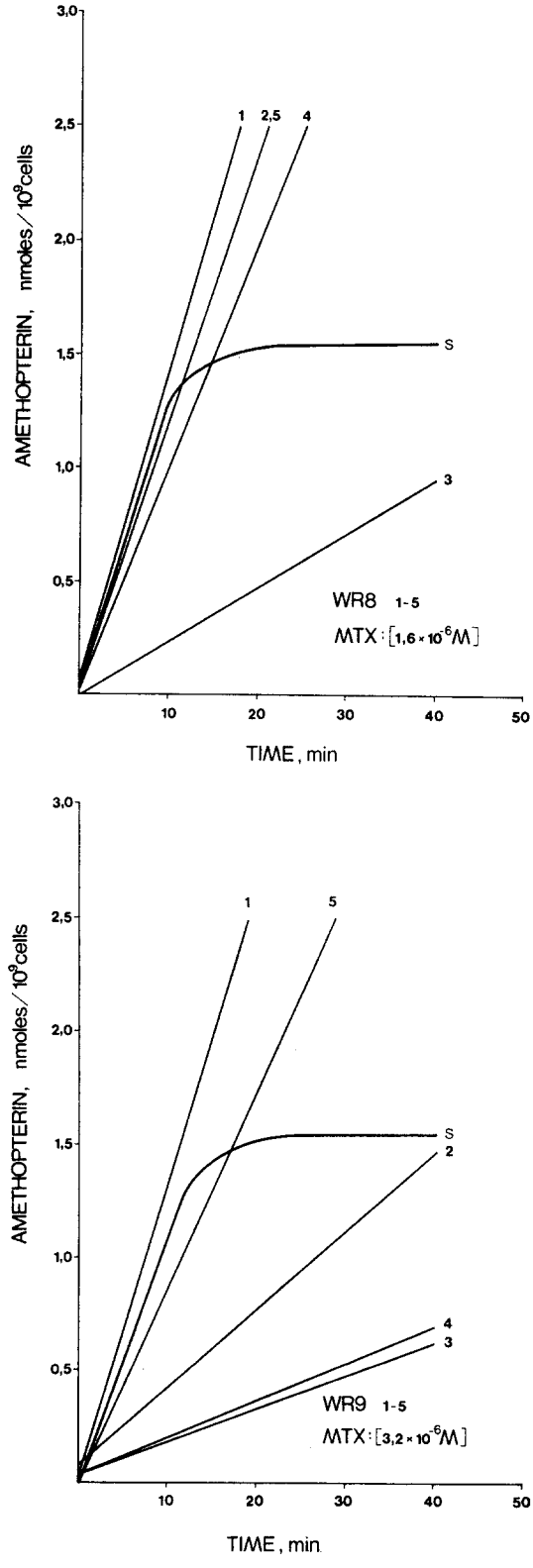


Fig. 4. Uptake of MTX by W1-L2 sublines. Extracellular concentration of MTX was 1 μM. (a) *Sublines WR8-1 to WR8-5,* (b) *Sublines WR9-1 to WR9-5.*

Figure 5 shows the uptake curves for MTX, folic acid and 5-methyltetrahydrofolate in the transport mutant WR9-4 compared with the sensitive line. The uptake of 5-methylFH_4 is impaired to the same extent as that of MTX whereas the influx of folic acid is identical in both lines. *p*-Choromercurisulphonate (20 μM) blocked the influx of MTX and 5-methylFH_4 completely in both lines, but did not change the rate of uptake of folic acid. Table 4 summarizes some of the kinetic data for the MTX transport system obtained with the parental W1-L2 line, and with WR9-4. 5-FormylFH_4 (folinic acid) competitively inhibited uptake of MTX, suggesting that it may enter the cell by the same route. In the mutant line WR9-4, the K_m for MTX of the transport system was not significantly altered from the normal value, but the V_{max} was decreased by a factor of more than seventeen.

Finally, Table 1 summarizes relative rates of MTX transport, when the extracellular MTX concentration was 1 μM, for all the sublines, together with the dihydrofolate reductase activities. It is clear that the greatest drug resistance is associated with an effective transport mutation in combination with large amounts of dihydrofolate reductase Form II (WR9-4). Rather lesser degrees of drug resistance are associated with *either* the possession of very large amounts of dihydrofolate reductase, Form II, but normal transport properties (WR8-1, WR9-1) *or* with an effective transport mutation, but lower activities of dihydrofolate reductase, and that preponderantly Form I (WR9-3). It is interesting to note that in two populations (2 and 5) the increase in drug resistance from stage 8 to stage 9 which was accompanied by a reduction in transport activity was also associated with reduction in the dihydrofolate reductase activity. This is probably the consequence of a change in cell population as cells containing the transport mutation but modest activities of the reductase outgrow cells with very high reductase levels but normal drug transport. By contrast, the development of the most resistant cells of all is probably the result of the simultaneous aquisition of the two mutations by the same cell.

DISCUSSION

The biochemistry and pharmacology of MTX have been studied in great detail in many

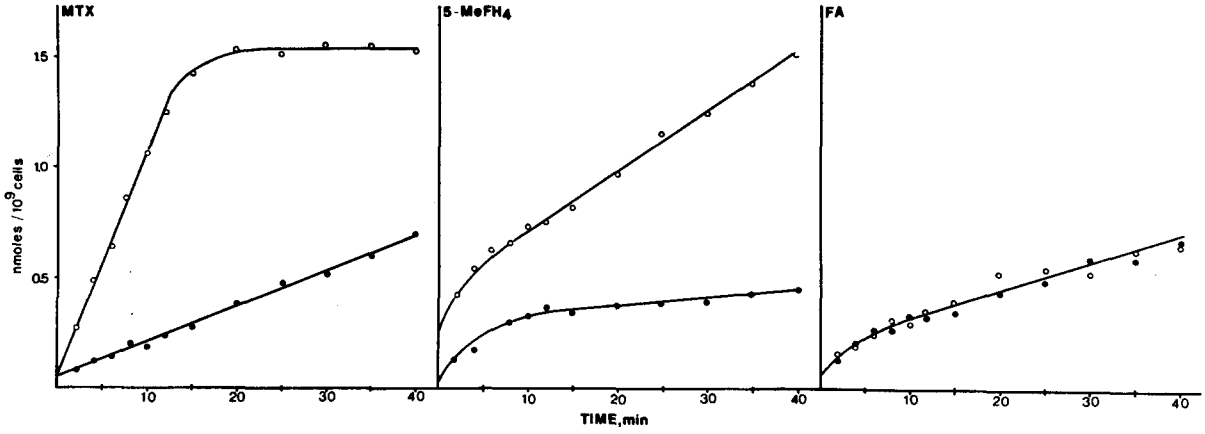


Fig. 5. Uptake of MTX (1 μM), 5-methyltetrahydrofolate (0·8 μM) and folic acid (5 μM) in the sensitive cell line (○) and the resistant subline WR9-4 (●).

experimental systems and a huge literature exists; however, a number of questions remain as topics of current debate. Some such questions are (i) Are the conclusions drawn from studies with mouse L1210 cells, which indicate that two membrane transport systems exist for folate compounds, also applicable to human cells? (ii) What is the significance of the multiple electrophoretic forms of dihydrofolate reductase? (iii) How important is the direct inhibition of thymidylate synthetase by MTX? The experiments described in this paper are attempts to answer these questions within the context of our experimental model of human lymphoblastoid cells grown in culture.

The first of these questions alone admits of a conclusive answer. From the results here outlined it is clear that 5-methyltetrahydrofolate and MTX share a common carrier, but that folic acid enters the cell by a separate mechanism. This is suggested by the simultaneous and quantitatively similar loss of MTX- and 5-methylFH$_4$-transporting activity in the drug resistant sublines, in which, however, folic acid uptake remains unimpaired. The effect of *p*-chloromercurisulphonate mimics the transport mutation: MTX and 5-methylFH$_4$ transport activity are again lost together, and folic acid transport is again unaffected. The kinetic results suggest that the MTX transport pathway may also be shared by 5-formylFH$_4$. With regard to the development of MTX-resistance by virtue of changes in membrane permeability, three aspects of the present study should be emphasized. Firstly although previous kinetic studies of transport mutants (with mouse L1210 cells) have shown increases in the K_m of the transport protein for MTX, with very little change in the transport V_{max}, the present results with subline WR9-4 indicate the reverse: an appreciable decrease was found in V_{max}. This is of clinical significance, since the effect of a K_m-type transport mutation may be largely overcome by increasing the extracellular drug concentration, but the effect of a V_{max}-type transport mutation cannot be reversed in this way. Secondly, the frequency and stability of the transport mutations in these human lines must be stressed. Studies with mouse L1210 cells [14, 17] have suggested that transport mutations occur rather early in the selection process, that they are less frequent than the high dihydrofolate reductase mutants, and that they tend to be overgrown or replaced by the latter type of MTX-resistant cell. By contrast, our results with the W1-L2 sublines indicate that transport mutations are in this case rather frequent, and very stable. This may be partly explained by the continued effectiveness of the V_{max}-type of transport mutation at high drug concentrations, in contrast to the K_m-type transport mutations found in L1210 cells. Thirdly, the fact that human lymphoblasts have a pathway for folic acid uptake separate from that for MTX suggests the desirability of having antifolate drugs which enter the cell by the folic acid pathway, and which would thus circumvent MTX-resistance resulting from transport mutations.

The molecular relationship of the different electrophoretic forms of dihydrofolate reductase in higher organisms is not yet entirely clear. In *Lactobacillus casei*, Form II consists simply of the apoenzyme, Form I, to which is bound an equimolar amount of NADPH [19]. The situation in mammalian cells is more complex: Form II appears on the polyacrylamide gels as a doublet: attempts to interconvert Form I into Form II, or vice versa, (using enzyme from mouse L1210 cells) by incubation with NADPH or FH_2 respectively did result in some interconversion, but this was slow and incom-

plete, unlike the situation in *L. casei*, (R. C. Jackson, unpublished work) and furthermore, experiments with the cell line L1210/R6, a mutant line containing an 80-fold elevation of dihydrofolate reductase, largely Form II, seemed to indicate that the cells did not contain enough NADPH for all the dihydrofolate reductase to exist as holoenzyme [17]. Moreover, the enzyme from L1210/R6 appeared to have slightly different kinetic properties from the normal L1210 dihydrofolate reductase [17]. The present study has indicated that only Form I is observed after purification of the enzyme by affinity chromatography, and the total recovery of activity is sufficiently great that at least half of the Form II must be converted to Form I on the affinity column. The only explanation consistent with all the observations is that "Form II" consists of two entities, one of which is Form I plus an equimolal amount of NADPH, and the other of which is a structural modification, and that this latter modification is not recovered under the conditions of affinity chromatography.

The change in electrophoretic pattern of dihydrofolate reductase as MTX resistance develops appears to be a consistent one. Earlier work with L1210 cells, both *in vivo* [20] and *in vitro* [17] has shown a rapid increase in Form I, followed, as higher degrees of drug resistance develop, by a rise in Form II. The present study, although not following changes in the electrophoretic pattern with time, has shown that in those sublines with moderate increases in total dihydrofolate reductase activity this increase is mainly in Form I, but that the highly resistant sublines showing the greatest total reductase activity have predominantly Form II. Two suggestions may be advanced for the biological significance of Form II in highly MTX-resistant cells. One suggestion is that the drug is preferentially bound by Form II, thus leaving Form I to function normally; evidence to support this suggestion has been obtained with L1210 cells, where the Form II enzyme from the highly resistant clone L1210/R7A has a K_i for MTX of $2{\cdot}0 \times 10^{-12}$ M, compared to $5{\cdot}3 \times 10^{-12}$ M for the normal Form I L1210 enzyme [36]. An alternative suggestion is that Form II has a longer cellular half-life, which confers a selective advantage on the cell in the presence of MTX; this possibility is supported by direct measurements of enzyme half-lives in both *L. casei* and L1210 cells [21].

Finally, the present study has attempted briefly to consider the importance to human cells of sites of action of MTX other than dihydrofolate reductase. The great activity of serine hydroxymethyltransferase, and the feebleness of its inhibition by MTX make it most unlikely that this enzyme ever becomes rate-limiting in the presence of MTX. For thymidylate synthetase, the situation is much harder to assess: the inhibition by MTX, although two orders of magnitude stronger than that for serine hydroxymethyltransferase, is many orders of magnitude weaker than for dihydrofolate reductase; on the other hand the V_{max} of thymidylate synthetase is much lower than that of dihydrofolate reductase. McBurney and Whitmore [37] have recently reported that hamster cells in the presence of growth-inhibitory concentrations of MTX showed no accumulation of FH_2, and thus suggested that thymidylate synthetase may be rate-limiting in these conditions. As yet, no measurements have been made of FH_2 in MTX-inhibited W1-L2 cells, but the known kinetic parameters of the folate enzymes in these cells make possible a tentative prediction of the behaviour of the system. The intracellular concentration of dihydrofolate reductase is about 0·19 μM (given that mol. wt. = 21,000 and specific activity = 89 I.U./mg). To reduce the potential steady-state flux through the pathway by 50% (to half the V_{max} of the rate-limiting enzyme) will require inhibiting dihydrofolate reductase by 95·45%. From the Straus–Goldstein equation [38] we may calculate that this will require a total cellular MTX concentration of 0·226 μM (given K_i = 7·3 pM, K_m for FH_2 = 0·13 μM, and cellular FH_2 = 37 μM; this makes the pessimistic assumption that 80% of total cellular folate accumulates as FH_2: if this is *not* true, slightly less MTX will be required). At this level of total MTX, the *free* MTX concentration will be 0·044 μM [38]. At the normal cellular level of 5,10-methyleneFH_4 (about 6 μM) this low concentration of free MTX will cause negligible inhibition of thymidylate synthetase. However, in the highly resistant subline WR9-1 the situation is rather different; the reductase activity is 234-fold higher, and to make it rate-limiting it must be inhibited 99·96%. Consider a total intracellular MTX level of 60 μM: this would give 99·68% inhibition of dihydrofolate reductase (not enough to make it rate-limiting). Under these conditions the *free* MTX concentration would be 15·5 μM. This would give appreciable inhibition of thymidylate synthetase.

We conclude that although a direct interaction of MTX with thymidylate synthetase is probably not a major determinant of cytotoxicity in the parental W1-L2 cell line, it may become important in those resistant sublines

with greatly elevated dihydrofolate reductase activity. The validity of these conclusions leans on the assumption that the kinetic parameters for the polyglutamyl forms which constitute a major proportion of cellular folate coenzymes are similar to those measured for the monoglutamyl folates. Some authors [39, 40] have stressed the importance of free cellular MTX as a determinant of toxicity of this drug. The above calculations indicate that even in the sensitive W1-L2 line, where inhibition of dihydrofolate reductase is probably the major mode of action, the extensive degree of inhibition required inevitably entails measurable concentrations of free drug.

In summary, the response of human lymphoblastoid cells to MTX is reassuringly similar, in general, to that of the useful murine model systems, although a few interesting differences exist. Years of research have made MTX one of the most completely documented of all antitumour drugs, but the last word has yet to be written about this interesting compound.

Acknowledgements—The authors are grateful for the skilled technical assistance of Miss C. Bullmann and Mr. G. A. Taylor.

REFERENCES

1. J. R. Bertino, D. R. Donohue, B. W. Gabrio, R. Silber, A. A. Lenty, M. Mayer and F. M. Huennekens, Increased level of dihydrofolate reductase in leucocytes of patients treated with amethopterin *Nature (Lond.)* **193,** 140 (1962).
2. S. Futterman and M. Silverman, The inactivation of folic acid by liver. *J. biol. Chem.* **224,** 31 (1957).
3. M. J. Osborn, M. Freeman and F. M. Huennekens, Inhibition of dihydrofolic reductase by aminopterin and amethopterin. *Proc. Soc. exp. Biol. (N.Y.)* **97,** 429 (1958).
4. D. Roberts, I. Wodinsky and T. C. Hall, Studies on folic reductase III. The level of enzyme activity and response to methotrexate of transplantable mouse tumours. *Cancer Res.* **25,** 1899 (1965).
5. J. P. Perkins, B. L. Hillcoat and J. R. Bertino, Dihydrofolate reductase from a resistant subline of the L1210 lymphoma. *J. biol. Chem.* **242,** 4771 (1967).
6. J. L. Biedler, A. W. Schrecker and D. J. Hutchison, Selection of chromosomal variant in amethopterin-resistant sublines of leukaemia L1210 with increased levels of dihydrofolate reductase. *J. nat. Cancer Inst.* **31,** 575 (1963).
7. G. A. Fischer, Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells. *Biochem. Pharmacol.* **7,** 75 (1961).
8. U. D. K. Misra, S. R. Humphreys, M. Friedkin, A. Goldin and E. J. Crawford, Increased dihydrofolate reductase activity as a possible basis of drug resistance in leukaemia. *Nature (Lond.)* **189,** 39 (1961).
9. D. Roberts, T. C. Hall and D. Rosenthal, Coordinated changes in biochemical patterns: the effect of cytosine arabinoside and methotrexate on leucocytes from patients with acute granulocytic leukaemia. *Cancer Res.* **29,** 571 (1969).
10. W. Wilmanns, Effects of amethopterin treatment on thymidylate synthesis in human leucocytes and bone marrow cells. *Ann. N.Y. Acad. Sci.* **186,** 365 (1971).
11. J. R. Bertino, The mechanism of action of the folate antagonists in man. *Cancer Res.* **23,** 1286 (1963).
12. G. A. Fischer, Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolite in L5178Y cells. *Biochem. Pharmacol.* **11,** 1233 (1962).
13. D. Kessel, T. C. Hall, D. Roberts and I. Wodinsky, Uptake as a determinant of methotrexate response in mouse leukaemias. *Science* **150,** 752 (1965).
14. F. M. Sirotnak, S. Kurita and D. J. Hutchison, On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukaemia. *Cancer Res.* **28,** 75 (1968).
15. W. C. Werkheiser, L. W. Law, R. A. Roosa and C. A. Nichol, Further evidence that selective uptake modifies cellular response to 4-amino-folate antagonists. *Proc. Am. Assoc. Cancer Res.* **4,** 71 (1963).
16. M. T. Hakala, S. F. Zakrezewski and C. A. Nichol, Relation of folic reductase to amethopterin resistance in cultured mammalian cells. *J. biol. Chem.* **236,** 962 (1961).

17. R. C. Jackson, D. Niethammer and F. M. Huennekens, Enzymic and transport mechanisms of amethopterin resistance in L1210 mouse leukaemia cells. *Cancer Biochem. Biophys.*, in press.
18. F. M. Huennekens, J. I. Rader, V. Neef, F. Otting, R. C. Jackson and D. Niethammer, Folate antagonists: transport and target site in leukemic cells. In: *Erythrocytes, Thrombocytes, Leukocytes.* (Edited by E. Gerlach, K. Moser, E. Deutsch and W. Wilmanns) p. 496, Thieme, Stuttgart (1973).
19. R. B. Dunlap, L. E. Gundersen and F. M. Huennekens, Interconversion of the multiple forms of dihydrofolate reductase from amethopterin-resistant *Lactobacillus casei. Biochem. Biophys. Res. Commun.* **42,** 772 (1971).
20. N. G. L. Harding, M. F. Martelli and F. M. Huennekens, Amethopterin-induced changes in the multiple forms of dihydrofolate reductase from L1210 cells. *Arch. Biochem. Biophys.* **137,** 295 (1970).
21. R. C. Jackson and F. M. Huennekens, Turnover of dihydrofolate reductase in rapidly-dividing cells. *Arch. Biochem. Biophys.* **154,** 192 (1973).
22. D. Kessel, T. C. Hall and D. Roberts, Modes of uptake of methotrexate by normal and leukaemic human leucocytes *in vitro* and their relation to drug response. *Cancer Res.* **28,** 564 (1968).
23. I. D. Goldman, The characteristics of the membrane transport of amethopterin and the naturally-occurring folates. *Ann. N.Y. Acad. Sci.* **186,** 400 (1971).
24. J. A. Levy, M. Virolainen and V. Defendi, Human lymphoblastoid lines from lymph node and spleen. *Cancer* **22,** 517 (1968).
25. R. Dulbecco and M. Vogr, Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99,** 167 (1954).
26. C. K. Mathews and F. M. Huennekens, Further studies on dihydrofolic reductase. *J. biol. Chem.* **238,** 3436 (1963).
27. A. J. Wahba and M. Friedkin, Direct spectrophotometric evidence for the oxidation of tetrahydrofolate during the enzymatic synthesis of thymidylate. *J. biol. Chem.* **236,** PC. 11 (1961).
28. P. Voytek, P. K. Chang and W. M. Prusoff, Purification of deoxythymidine kinase by preparative disc gel electrophoresis and the effects of various halogenated nucleoside triphosphates on its enzymatic activity. *J. biol. Chem.* **246,** 1432 (1971).
29. K. G. Scrimgeour and F. M. Huennekens, Dihydrofolic reductase. In: *Methods in Enzymology.* (Edited by S. P. Colowick and N. O. Kaplan) Vol. VI, p. 364. Academic Press, London (1963).
30. R. V. Ramasastri and R. L. Blakley, 5,10-Methylenetetrahydrofolate dehydrogenase from baker's yeast. *J. biol. Chem.* **237,** 1982 (1962).
31. J. C. Rabinowitz and W. E. Pricer, Formyltetrahydrofolate synthetase. In: *Methods in Enzymology.* (Edited by S. P. Colowick and N. O. Kaplan) Vol. VI, p. 375. Academic Press, London (1963).
32. B. J. Davis, Disc electrophoresis II. Method and application to human serum protein. *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).
33. J. M. Whiteley, R. C. Jackson, G. P. Mell, J. H. Drais and F. M. Huennekens, Folate antagonists covalently linked to carbohydrates: synthesis, properties and use in the purification of dihydrofolate reductases. *Arch. Biochem. Biophys.* **150,** 15 (1972).
34. G. P. Mell, M. F. Martelli, J. Kirchner and F. M. Huennekens, Multiple forms of dihydrofolate reductase. *Biochem. Biophys. Res. Commun.* **33,** 74 (1968).
35. F. M. Huennekens, G. P. Mell, N. G. L. Harding, L. E. Gundersen and J. H. Freisheim, Structure and catalytic mechanism of dihydrofolate reductase. In: *Chemistry and Biology of Pteridines* (Edited by K. Iwai, M. Akino, M. Goto and Y. Iwanami) p. 329 International Academic, Tokyo (1970).
36. L. I. Hart, R. C. Jackson and K. R. Harrap (unpublished).
37. M. W. McBurney and G. F. Whitmore, Mechanism of growth inhibition by methotrexate. *Cancer Research* **35,** 586 (1975).
38. O. H. Straus and A. Goldstein, Zone behaviour of enzymes, *J. gen. Physiol.* **26,** 559 (1943).
39. J. C. White and I. D. Goldman, Free intracellular methotrexate is a critical factor in the inhibition of dihydrofolate reduction in Ehrlich ascites tumour cells *in vitro. Proc. Am. Assoc. Cancer Res.* **16,** 45 (1975).
40. F. M. Sirotnak and R. C. Donsbach, The intracellular concentration dependence of antifolate inhibition of DNA synthesis in L1210 leukaemia cells. *Cancer Res.* **34,** 3332 (1974).

Europ. J. Cancer Vol. 11, pp. 855–860. Pergamon Press 1975. Printed in Great Britain

Serum Blocking Factor as an Index of Metastatic Spread Following Primary Tumour Excision*

A. E. BRAY†§ and P. G. HOLT‡
University Departments of Pathology† and Microbiology,‡
Perth Medical Centre, Shenton Park, 6008, Western Australia

Abstract—*Individual mice were monitored for serum blocking factor (SBF) activity prior to and after excision of their primary spontaneously metastasing melanomas. SBF activity was measured by the leukocyte adherence inhibition (LAI) microtest. Mice which maintained high levels of SBF activity on the third day after excision subsequently died with metastases in the lung. In contrast, mice which exhibited markedly decreased SBF activity on the third day post-excision did not develop metastases. The data are discussed in terms of the efficacy of SBF monitoring as a prognostic index in cancer.*

INTRODUCTION

FOLLOWING the demonstration that tumour antigen was one of the major components of serum blocking factor (SBF) [1–4] in tumour-bearing hosts, it suggested that the quantitative analysis of SBF may be valuable in the diagnosis of cancer. Despite this possibility there are no definitive reports regarding the potential role of SBF as a prognostic index after tumour excision. In fact some reports cast doubt on its application, because patients with a poor prognosis or animals which developed recurrent disease have been shown to lose SBF following immunotherapy or chemotherapy [5–7]. As both the latter procedures may reduce the size of the host's tumour, SBF activity may also be expected to decrease commensurately. Consequently the conventional, relatively insensitive assays may not have discriminated between reduced SBF activity which accompanies a decrease in tumour mass, and the complete disappearance of SBF which may accompany the successful excision of a primary tumour from a host free of metastatic spread.

A recent assay of cell-mediated immunity (CMI), originally described by Halliday and Miller [8] and modified by Holt *et al.* [9] has enabled blocking factor to be measured with greater sensitivity than conventional cytotoxicity assays [9, 10]. In this study, individual mice were monitored after excision of a metastasizing melanoma to determine if the measurement of SBF would differentiate between those animals who would die with metastatic disease from those surviving.

MATERIAL AND METHODS

Mice

All mice used were females of the C57 Black/6J strain, aged 6–8 weeks.

Tumour cell lines

B16 Melanoma. This melanoma originally arose spontaneously in a C57 BL/6J mouse in 1954 [11]. It spontaneously metastisized to the

Accepted 3 September 1975.

*Part of the work was sponsored by the Australian Tobacco Research Foundation of Australia.
§Recipient of a National Health and Medical Research Council of Australia Post-graduate scholarship.

lungs. These pigmented metastases were readily detected macroscopically.

Lewis lung tumour (LLT)

This arose spontaneously in C57 BL/6J mouse in 1951 [12]. *In vivo* experiments in this laboratory have shown little cross-reactivity between this tumour and the B16 melanoma. Both tumours were maintained by serial subcutaneous passage every three weeks.

Tumour cell inocculation

Single cell suspensions of melanoma and LLT cells were prepared by mincing fleshy pieces of tumour tissue in phosphate buffered saline (PBS). 5×10^4 viable melanoma cells were inocculated into the left foot-pad of twenty mice. Mice used to obtain immune spleen cells were inocculated with 1×10^5 melanoma or LLT cells subcutaneously.

Tumour antigen preparation

Melanoma antigen was extracted by 3 M KCl according to the method of Mavligit *et al.* [13] and fractionated on a Biogel P150 column. Each fraction was assayed in the LAI-microtest (over a wide concentration range) employing spleen cells from "immune" mice, i.e. those carrying a small subcutaneous melanoma. The fractions in the range of 150,000–200,000 MW specifically inhibited the adherence of spleen cells from melanoma bearing mice, but not from LLT bearing mice. The active (i.e., specifically antigenic) fractions were pooled, and used in the assay to detect SBF activity.

LLT antigen was prepared by homogenizing a suspension of 1×10^7 viable LLT cells/ml in PBS at 4°C. The homogenate was then centrifuged at 30,000 ***g*** for 60 min and the supernatant collected. This preparation was tested in the LAI system over a wide range of concentrations, employing spleen cells from animals bearing a small LLT. Maximal adherence inhibition was obtained at a final dilution of 1 in 50. Adherence inhibition was not observed when spleen cells from melanoma-bearing or normal mice were used.

Leukocyte adherence inhibition (LAI) microtest

This assay was used to measure SBF in the various sera. The method was described previously [9, 14]. Immune leukocytes were obtained from the spleens of mice which had a tumour of 1 cm dia growing subcutaneously on the abdomen. Leukocyte suspensions (from minced spleens) were separated from erythrocytes by isopyknic sedimentation, on a ficoll-hypaque gradient [15]; the separated leukocytes were washed three times in PBS. The cells were then suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 buffered according to the formula of Croce *et al.* [16]. 5 μl of the cell suspension was dispensed into each well of a Falcon 3034 microtest tray (Grand Island Biological N.Y.); each well had previously received 1 μl of PBS or 1 μl of appropriate antigen preparation. Following these additions, each well received 5 μl of RPMI 1640 supplemented with 20% foetal calf serum (FCS). The final concentration of serum in each well was thus 10%.

The trays were incubated for 2 hr at 37°C in $5°CO_2$ and inverted for 15 min. to allow non-adherent cells to float off the bottom of the wells. Non-adherent cells were then washed away by flooding the trays with PBS. The remaining adherent cells were fixed with methanol and stained with Giemsa stain. The number of cells in 10 fixed fields/well were counted, and the mean number of adherent cells in each well of triplicate cultures calculated.

Leukocyte adherence inhibition was calculated according to the formula:

$$\text{LAI} = 1 - \frac{\text{mean number of cells adhering with antigen}}{\text{mean number of cells adhering with PBS}}$$

This was expressed as a percentage.

To assess SBF activity similar preparations of "immune" spleen cells were used. Triplicate wells containing the sera under test (final concentration 10%) were set-up with and without antigen) in parallel to wells containing FCS (see above). The trays were incubated and processed as above. SBF activity was then determined by comparing the degree of antigen-specific adherence inhibition obtained with both types of sera:

$$\%\ \text{SBF activity} = \left\{1 - \frac{\%\ \text{LAI with test sera}}{\%\ \text{LAI with control sera}}\right\} \times 100.$$

Preliminary experiments indicated that identical results were obtained when 10% FCS or 10% normal mouse serum was used, and hence either of these sera would serve as an adequate control.

Experimental design

Fifiteen mice were individually monitored for SBF activity prior to and at 3 and 26 days after the left footpad was amputated. Thirteen

of the mice had melanomas of diameters 0·4 to 0·6 cm growing in the left foot, and the other two were normal mice (Nos 14 and 15). Sera was obtained by bleeding from the retro-orbital venous plexus on the days indicated; all samples were heat-inactivated (56°C for 30 min) and then stored at −20°C, The left foot-pad was excised under nembutal anaesthesia leaving the draining lymph node intact. Mice were observed for a further 30 days after collection of the last blood sample. Those mice dying were autopsied to confirm the presence of metastases to the lung. Surviving mice were sacrificed 55 days after tumour excision and examined for metastatic spread to the lungs.

RESULTS

When spleen cells from mice bearing a melanoma were tested against the melanoma and LLT antigen preparations, decreased adherence was observed only in the presence of the melanoma preparation. Similarly spleen cells from mice bearing an LLT reacted only against the LLT antigen preparation. Spleen cells from normal mice did not react against either antigen preparation (Tables 1 and 2).

In the presence of FCS there was a 25% reduction in the number of adherent "melanoma-immune" spleen cells in wells containing the melanoma antigen, compared to the number of adherent cells with PBS. When sera from normal or LLT bearing mice were substituted for FCS (Table 1), there was no significant change in the degree of adherence inhibition. However, when sera from a melanoma bearing mouse was added, this antigen-specific adherence inhibition was abolished; in fact, more adherent cells were observed in wells containing the melanoma antigen than in wells

Table 1. *Specificity of SBF in the melanoma system*

Spleen Cell Donor	Sera	Mean Number of Adherent Leukocytes/Well		% LAI	Significance*	% SBF Activity
		with PBS	with Melanoma Antigen			
Normal	FCS	148 ± 16·1	152 ± 16·5	— 3	NS°	
LLT bearer	FCS	140 ± 15·6	132 ± 10·8	6	NS	
Melanoma bearer	FCS	126 ± 10·1	94 ± 9·1	25	<0·01	
Melanoma bearer	Normal	121 ± 9·3	95 ± 8·1	22	<0·01	12
Melanoma bearer	LLT	115 ± 11·4	87 ± 8·9	24	<0·01	4
Melanoma bearer	Melanoma	129 ± 10·8	141 ± 12·2	— 9	NS	>100

Spleen cells from C57 BL mice bearing a melanoma were tested for melanoma-antigen specific LAI in the presence of FCS, serum from normal mice and serum from mice bearing a melanoma or LLT. Spleen cells from normal mice and LLT bearers were also tested for reactivity to melanoma antigen in the presence of FCS.

*Students *t* test.
°N.S. Not significant.

Table 2. *Specificity of SBF in LLT system*

Spleen Cell Donor	Sera	Mean Number of Adherent Leukocytes/Well		% LAI	Significance*	% SBF activity
		with PBS	with LLT antigen			
Normal	FCS	175 ± 8·5	181 ± 11·6	— 3	NS°	
Melanoma bearer	FCS	149 ± 8·2	159 ± 11·0	— 7	NS	
LLT bearer	FCS	161 ± 12·2	119 ± 8·3	26	<0·01	
LLT bearer	Normal	152 ± 7·6	106 ± 3·8	30	<0·001	—15
LLT bearer	Melanoma	159 ± 11·3	110 ± 12·3	31	<0·01	—19
LLT bearer	LLT	164 ± 3·6	166 ± 13·9	— 1	NS	>100

Spleen cells from C57 BL mice bearing an LLT were tested for LLT antigen specific LAI in the presence of FCS, serum from normal mice and serum from mice bearing a melanoma or LLT. Spleen cells from normal mice and melanoma bearers were also tested for reactivity to melanoma antigen in the presence of FCS.

*Students *t* test.
°N.S. not significant.

containing PBS (Table 1). When serum from melanoma bearing mice was added to spleen cells from normal mice not carrying this tumour (not shown here), cell adherence remained unaffected by antigen [17], and the results obtained were analogous to those shown for FCS (first line, Table 1).

Similarly spleen cells from LLT bearing mice exhibited a 26% LAI in the presence of the LLT antigen preparation, and only sera from LLT bearing mice abolished this decreased adherence, (Table 2).

When sera from the experimental mice were tested for SBF activity using the same system, there was a change in SBF activity after the tumour was excised in those mice that remained free of metastatic disease. The results are shown in Table 3. All tumour bearing mice (Nos 1–13) had blocking activity greater than 70% before tumour excision, whereas sera from normal mice had no blocking effect.

Three days after the left foot-pad was excised there was a decrease in SBF activity in the sera of mice (Nos. 2, 3, 11, 12, 13) which subsequently survived. All mice with SBF activity greater than 100% at this stage subsequently died with metastases. Sera from mouse number 9 had only 27% SBF activity, but this mouse subsequently died with metastases 35 days later. There was no change in SBF activity in the two normal mice (Nos 14 and 15).

Twenty-six days after tumour excision a similar pattern was observed, except that sera from mice numbers 12 and 13 had SBF activity 100% and 82% respectively, yet these mice had no evidence of metastatic spread at autopsy. Sera from mouse No. 9 (which developed metastases) increased in SBF activity from 27% on day 3 to 50% on day 26 post-excision.

DISCUSSION

The possibility that SBF may be a useful diagnostic-index of malignancy was suggested by Hellstrom *et al.* [18] when they found that patients with cancer exhibited CMI and SBF specific to the site of tumour origin. This was supported by the observation that melanoma patients who lost SBF activity after tumour excision had a favourable prognosis [19]. Further reports on the reliability of this approach for the detection of residual malignancy are not available. Currie *et al.* [6], doubted whether SBF would be useful for this purpose because patients treated by surgery or immunotherapy lost SBF, yet their prognosis was still poor.

In experimental systems it was demonstrated that SBF was lost over a period of 1–15 days following tumour excision [20–22]. It appeared that the rate of decrease of SBF activity was partly related to the size of the tumour at

Table 3. SBF activity and metastases following tumour excision

Mouse Number	Tumour Diam. Cm.	Days Survival after tumour excision	Pre-op Sera % SBF Activity	Day 3 Post-Excision Sera % SBF Activity	Day 26 Post-Excision Sera % SBF Activity
1	0·55	41	82	>100	58
2	0·46	N.M	>100	50	0
3	0·48	N.M	>100	9	0
4	0·53	50	>100	>100	>100
5	0·50	45	>100	>100	>100
6	0·60	20	91	>100	mouse died
7	0·62	45	>100	>100	>100
8	0·50	55*	>100	>100	>100
9	0·60	38	>100	27	50
10	0·58	46	72	>100	>100
11	0·58	N.M	>100	27	9
12	0·42	N.M	>100	0	>100
13	0·40	N.M	91	0	82
14	No tumour	—	0	0	0
15	No tumour	—	0	0	0

C57 BL mice bearing a spontaneously metastasing melanoma in the footpad were monitored for SBF activity before and after tumour excision.

N.M.—No metastases at autopsy 55 days after primary tumour excision.
*Autopsy revealed lung metastases when killed on day 55.

excision since more rapid loss of SBF was observed in systems where the tumour was small at the time of excision, i.e. the degree of SBF activity may be directly proportional to total tumour load. In addition it was shown employing both cytotoxicity and LAI microtest assays, that SBF was not lost when the primary tumour was removed after gross spontaneous metastases had developed [23].

The reliability of SBF as a measure of metastatic spread at the time of primary tumour excision may therefore depend to a large degree on the sensitivity of the SBF assay, as the procedure employed must be capable of detecting the low levels of SBF activity associated with small metastases. In earlier experiments, it was shown that the LAI microtest correlated well with the conventional cytotoxicity assay in terms of SBF assessment in a number of syngeneic tumour models [9, 17]. In addition, the LAI-microtest appeared considerably more sensitive than the cytotoxicity assay (10) and was capable of detecting SBF activity employing blocking sera at concentrations as low as 0·2% [17]. However, whether SBF activity as detected in these two tests is due to the same serum factor(s), must await further investigation.

The results obtained in these experiments were preliminary. However, if one assumed that SBF activity above 50% in the sera collected from animals on the third day after tumour excision indicated the presence of residual tumour, it was possible to make fourteen correct predictions as to the survival of the animals and one false negative. At 26 days post-excision it was possible to make twelve correct predictions with two false positives and one false negative by this criterion. The SBF activity detected on day 26 in those metastasis free mice could have been due to antibody, as the latter has been shown to develop over a similar time-scale following immunization with tumours in their allogeneic hosts [24]. However, antibody was also shown to block CMI *in vitro* in a syngeneic tumour model [21].

These results suggest that SBF determination may be a useful index to differentiate between hosts which had a tumour completely excised from those with residual metastases. Since the LAI assay has been successfully employed to measure SBF in some human cancers [25, 26], it is possible that similar findings to those reported here may be obtained in human tumour systems. In view of the apparent success of early adjuvant therapy [27] this approach requires further investigation, since it may accurately detect those patients with metastatic disease who require treatment after surgery.

Acknowledgements—The technical assistance of Mrs. Cheryl Caligero and Miss Lyn Roberts is gratefully acknowledged.

REFERENCES

1. H. O. Sjogren, I. Hellstrom, S. C. Bansal and K. E. Hellstrom. Suggestive evidence that the "blocking antibodies" of tumor bearing individuals may be antigen-antibody complexes. *Proc. nat. Acad. Sci., U.S.A.* **68,** 1372 (1971).
2. G. A. Currie and C. Basham, Serum mediated inhibition of the immunological reactions of the patient to his own tumour: a possible role for circulating antigen. *Brit. J. Cancer* **26,** 439 (1972).
3. R. W. Baldwin, M. R. Price and R. A. Robins. Inhibition of hepatoma-immune lymph-node cell cytotoxicity by tumour-bearer serum and solubilized hepatoma antigen. *Int. J. Cancer* **11,** 527 (1973).
4. G. Currie. The role of circulating antigen as an inhibitor of tumour immunity in man. *Brit. J. Cancer* **28,** suppl. I, 153 (1973).
5. J. G. Sinkovics, J. R. Cabiness and C. C. Schullenberger. Disappearance after chemotherapy of blocking serum factors as measured *in vitro* with lymphocytes cytotoxic to tumour cells. *Cancer (Philad.)* **30,** 1428 (1972).
6. G. A. Currie. Effect of active immunization with irradiated tumour cells on specific serum inhibitors of cell-mediated immunity in patients with disseminated cancer. *Brit. J. Cancer* **28,** 25 (1973).
7. G. Steele, H. O. Sjögren and J. Ankerst. The effect of cyclophospamide on *in vitro* correlates of tumour immunity. *Int. J. Cancer* **14,** 743 (1974).
8. W. J. Halliday and S. Miller. Leukocyte adherence inhibition: a simple test for cell-mediated tumour immunity and serum blocking factors. *Int. J. Cancer* **9,** 477 (1972).
9. P. G. Holt, A. E. Bray, L. M. Roberts and D. Keast. Detection of cell-mediated immunity and associated blocking factors employing inhibition of leucocyte adherence. *I.R.C.S.* **2,** 1484 (1974).

10. A. E. Bray, P. G. Holt, L. M. Roberts and D. Keast. Early onset of serum blocking in a murine melanoma. *Int. J. Cancer* **16,** 777 (1975).
11. E. L. Green. *Handbook on Genetically Standardized JAX Mice.* Edited by Bar Harbor Times Publishing Company, 1968, page 53.
12. K. Sugiura and C. C. Stock. Studies in a tumour spectrum III. The effect of phosphoramides on the growth of a variety of mouse and rat tumours. *Cancer Res.* **15,** 38 (1955).
13. G. M. Mavligit, V. Ambus, V. V. Gutterman, E. M. Hersh and C. M. McBride. Antigen solubilized from human solid tumours: Lymphocyte stimulation and cutaneous delayed hypersensitivity. *Nature New Biol.* **243,** 188 (1973).
14. P. G. Holt, L. M. Roberts, P. J. Fimmel and D. Keast. The LAI microtest: a rapid and sensitive procedure for the demonstration of cell-mediated immunity *in vitro. J. immunol. Methods,* in press (1975).
15. R. J. Penper, T. W. Zee and M. M. Mickelson. Purification of lymphocytes and platelets by gradient centrifugation. *J. lab. clin. Med.* **72,** 842 (1968).
16. C. M. Croce, M. Koprowski and H. Eagle. Effect of environmental pH on the efficiency of cellular hybridization. *Proc. nat. Acad. Sci., U.S.A.,* **69,** 1953 (1972).
17. P. G. Holt, J. Chalmer and D. Keast. Development of two manifestations of T-lymphocyte reactivity during tumour growth, altered kinetics associated with elevated growth rates. In press (1975).
18. I. Hellstrom, H. O. Sjögren, G. Warner and K. E. Hellstrom. Blocking of cell-mediated tumour immunity by sera from patients with growing neoplasia. *Int. J. Cancer* **7,** 226 (1971).
19. I. Hellstrom, G. A. Warner, K. E. Hellstrom and H. O. Sjogren. Sequential studies on cell-mediated tumor immunity and blocking serum activity in ten patients with malignant melanoma. *Int. J. Cancer* **11,** 280 (1973).
20. G. H. Heppner. *In vitro* studies on cell-mediated immunity following surgery in mice sensitized to syngeneic mammary tumours. *Int. J. Cancer* **9,** 119 (1972).
21. R. W. Baldwin, M. J. Embleton and R. A. Robins. Cellular and humoral immunity to rat hepatoma-specific antigens correlated with tumour status. *Int. J. Cancer* **11,** 1 (1973).
22. A. E. Bray and D. Keast. Changes in host immunity following excision of a murine melanoma. *Brit. J. Cancer* **31,** 170 (1975).
23. A. E. Bray. The use of serum blocking factor as an indicator of residual tumor following primary excision. *J. Surg. Res.* in press (1975).
24. N. Kaliss. Immunological enhancement: conditions for its expression and its relevance for grafts of normal tissues. *Ann. N.Y. Acad. Sci.* **129,** 155 (1966).
25. A. Maluish and W. J. Halliday. Cell-mediated immunity and specific serum factors in human cancers: the leukocyte adherence inhibition test. *J. nat. Cancer Inst.* **52,** 1415 (1974).
26. W. J. Halliday, A. Maluish and W. H. Isbister. Detection of anti-tumour cell-mediated immunity and the serum blocking factors in cancer patients by the leukocyte adherence inhibition test. *Brit. J. Cancer* **29,** 31 (1974).
27. B. Fisher, P. Carbone, S. G. Economose, R. Frelick, A. Glass, H. Lorner, C. Redmond, M. Zelen, P. Band, D. L. Kafrych, N. Wolmark and E. R. Fisher. L-Phenylalanine mustard in the management of primary breast cancer. *New Engl. J. Med.* **292,** 117 (1975).

Europ. J. Cancer Vol. 11, pp. 861–865. Pergamon Press 1975. Printed in Great Britain

Alterations in the Deoxyadenylate Regions of the DNA from four Human Breast Tumors*

RONALD W. PERO,† TOMAS BRYNGELSSON,† ALLAN NORGREN‡ and ADAM DEUTSCH†

†*Department of Biochemistry, Nucleic Acids Research Group, University of Lund, Box* 740, 220 07 *Lund, Sweden*

‡*Department of Radiation Therapy, University Hospital, Lund, Sweden*

Abstract— *The levels of (dA) regions in 4 breast tumor DNAs were compared with the level in normal DNA. Although the (dA) regions in normal DNA remained constant in breast tissue, lymphocytes and placenta, (dA) alterations from the normal pattern were observed in the DNA from all four breast tumors. Tumor histology varied and was parallelled by a corresponding variation in the (dA) alterations following a quantitative analysis of the (dA) rich and (dA) "pure" sequences. The (dA) alterations associated with 2 of the tumor DNAs were distributed into both A+T rich and G+C rich DNA fractions.*

INTRODUCTION

THE ETIOLOGIC factors involved in breast cancer are probably quite heterogenous and, as yet, cannot be clearly identified or classified into major groups of tumor inducing agents [1, 2]. Recently homology between human breast tumor RNA and mammary tumor viruses from the Mason–Pfizer monkey [3] and the mouse [4] have been shown. This work is suggestive of the involvement of viruses in the etiology of breast cancer, and is to this date the best biochemical evidence for establishing a cause and effect relationship between an etiologic agent and human mammary carcinogenesis.

It has now been established in the Chinese hamster [5], mouse [6], rat [7], duck [8], and human [9] that eukaryotic DNA contains small deoxyadenylate (dA) rich stretches which hybridize to polyuridylic acid (poly U). Current speculation is that RNA transcripts from these (dA) regions are involved in the processing of genetic information [10]. Our laboratory has shown that the (dA) regions in DNA from both 7,12-dimethylbenz(a)anthracene (DMBA)-induced and Rous sarcoma virus-induced rat tumors varied considerably from the (dA) regions in normal rat DNA [7]. Likewise, similar results were found when the (dA) regions in normal human and chronic lymphocytic leukemic (CLL) DNAs were compared (to be published). These results imply that changes occur in the (dA) regions of DNA from neoplastic tissues as an inherent part of the carcinogenic process, irrespective of the oncogenic agent involved. We now report the application of poly(U)-DNA hybridization techniques to the study of (dA) regions in the DNA of four tumors found in the female human breast. A correlation between the disturbances in the (dA) regions of these tumor DNAs and their corresponding tumor histology was observed, suggesting that specific qualitative and quantitative (dA) alterations could distinguish tumors of different tissue origin.

Accepted 18 August 1975.

*This work was supported by grants from the Swedish Natural Sciences Research Council (to A.D.) and the Swedish Cancer Society (to A.N.).

MATERIAL AND METHODS

Normal tissue material

Human female breast tissue was obtained following plastic surgical operations on

apparently healthy individuals. Placental tissue from healthy mothers was collected within 4 hr of fetal birth. Lymphocytes were isolated from normal heparinized venous blood using density centrifugation through a sodium metrizoate-ficoll solution [11].

Tumor material

The tumor material was obtained from four women admitted to the hospital because of a tumor of the breast. All tumors were solid, and sampling was made from different regions of the tumors. Occasional areas of necrosis or areas with haematoma were excluded. The wet weight of the material was recorded to the nearest 0·1 g. Representative pieces were fixed in Bouin's solution. Paraffin sections were cut at 5–10 μm thickness and stained with haematoxylin and eosin for histological diagnosis.

Table 1. *Description of tumor material used in this study.*

Case	Age	Histology	Wet weight (g) of tumor analyzed
E.H.	50	Cystosarcoma Phyllodes, malignant	10·5
M.N.	52	Infiltrating mammary duct carcinoma with productive fibrosis	24·0
O.O.	90	Low differentiated mammary carcinoma	3·7
M.M.	62	Malignant anaplastic tumor; histiocytic lymphoma?	3·0

The tumor data are summarized in Table 1, and corresponding photomicrographs are shown in Fig. 1. In one patient (case E.H.) fibroadenomas had been removed from the left breast at four occasions previously. However, this particular tumor showed abnormal growth and cytological characteristics to such an extent that the neoplastic condition had to be regarded as malignant. Mammary carcinomas of different degrees of differentiation were seen in two patients (cases M.N. and O.O., Fig. 1). Finally, in case M.M. the tumor apparently did not originate from mammary gland tissue. The histological features were those of a malignant anaplastic tumor, most likely a histiocytic lymphoma.

The material was processed as soon as possible or was stored in liquid nitrogen until use. All histopathologic diagnoses were confirmed by Dr. H. Henrikson, Department of Pathology, University of Lund, Sweden.

Poly(U)-DNA hybridization

The extraction and additional purification procedures of the DNAs which were used have been previously outlined [9, 12, 13]. Optimal annealing conditions of poly(U) to the (dA) regions in DNA have been reported [5–9]. Two classes of (dA) regions can be distinguished based on their ability to form ribonuclease A-resistant hybrids with poly(U); namely (dA) rich sequences and (dA) "pure" sequences. The (dA) rich sequences contain other deoxyribonucleotides which encourage mismatched base-pairing and render the (dA) rich-poly(U) hybrids very sensitive to ribonuclease A digestion. Most of the (dA) "pure" sequences also contain other deoxynucleotides but fewer than found in the (dA) rich sequences, and therefore, the more (dA) "pure" poly(U) hybrids are much more resistant to ribonuclease A treatment.

In the following experiments, the poly(U)-DNA hybridization was carried out in 2 × SSC (SSC = 0·15 M NaCl—0·015 M sodium citrate) at 25°C for 18 hr with the DNA immobilized on nitrocellulose filters. All DNA samples were hybridized at the same time in the same reaction vial. RNAase treatment was 5 μg/ml ribonuclease A in 2 × SSC at 25°C for 30 min (analysis of (dA) rich sequences) and for 6 hr (analysis of (dA) "pure" sequences). A detailed parameter study of the techniques involved in the poly(U)-human DNA hybridization procedure including the materials used, the determination of appropriate RNAase conditions, the DNA preparation and quantitative determination, and the factors affecting poly(U) hybridization on nitrocellulose filters have been presented elsewhere [9]. The *t*-test for measuring significance ($P < 0{\cdot}05$) between 2 means was applied for statistical comparison of normal and breast tumor DNAs.

Temperature Elution of DNA from hydroxylapatite

Native DNA (350 μg/10 ml 2 × SSC) was sheared by sonication with 10 10-sec pulses at a power supply setting of 6 on a Model S125 Branson sonifier equipped with a microtip.

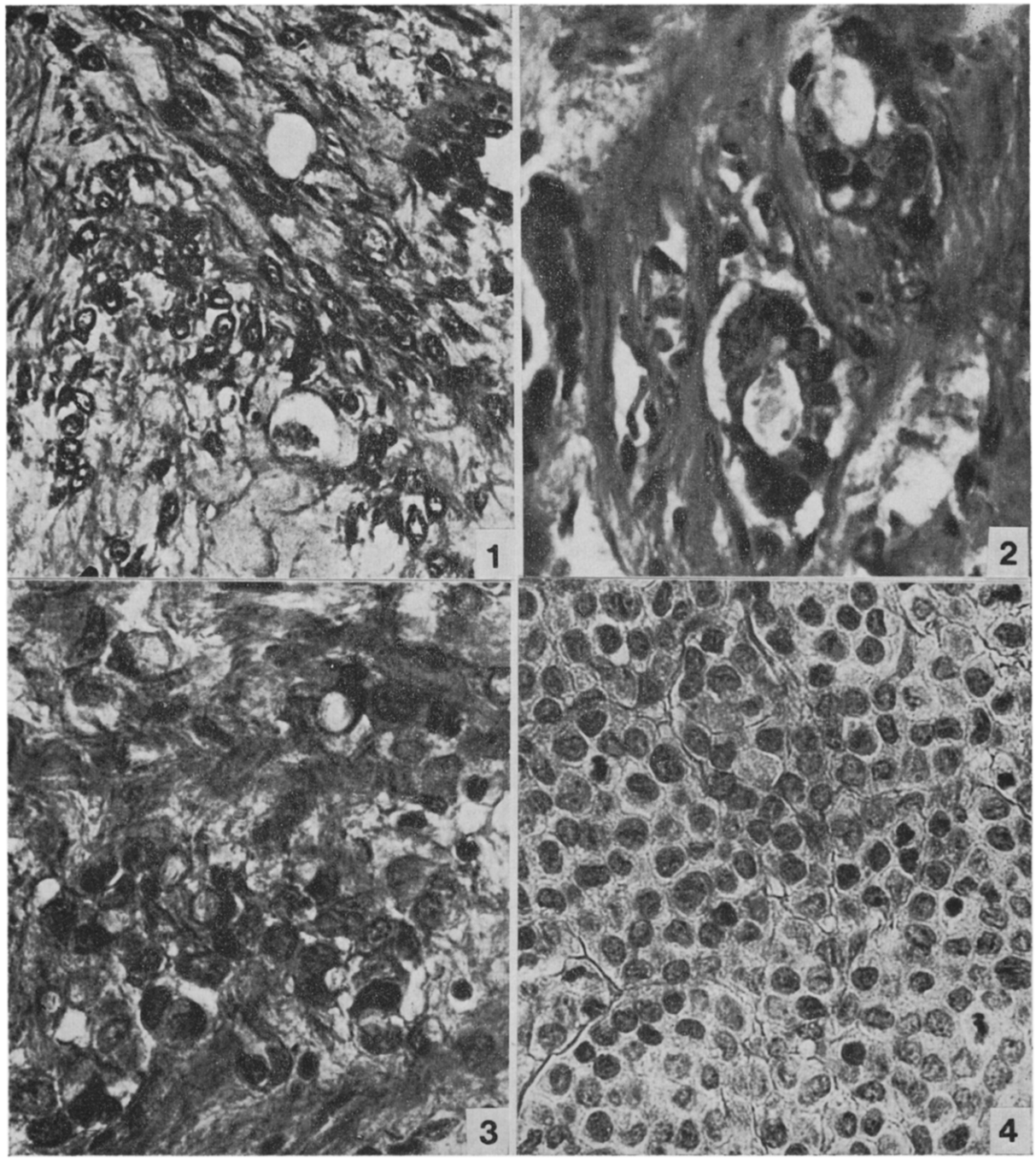


Fig. 1. *Photomicrographs of the tumors described in Table* 1. (1) *Case E.H., malignant cystosarcoma Phyllodes,* (2) *and* (3), *Cases M.N. and O.O. respectively, two mammary carcinomas with different degrees of differentiation,* (4) *Case M.M., malignant anaplastic tumor. Note the different degrees of differentiation of the tumors.*

(to face page 862)

Temperature controlled chromatographic columns were packed with hydroxylapatite (BioRad Laboratories, non-DNA grade) suspended in 0·12 M phosphate buffer (equimolar mixture of NaH_2PO_4 and Na_2HPO_4, pH 6·8). Approximately 200 μg DNA dissolved in 0·12 M phosphate buffer was bound to the hydroxylapatite (1 ml bed volume) by passage through the column at room temperature. The temperature of the column was raised to 88°C, and then eluted further with 0·12 M phosphate buffer. The T_M of human DNA, or the temperature by which 50% of double-stranded DNA has become single stranded, is 87°C in SSC [14]. In addition, the T_M profiles of DNA are dependent on base composition with adenine + thymine (A + T) rich DNA having a lower T_M than guanine + cytosine (G + C) rich DNA [15]. In our hands, temperature elutions up to 88°C resulted in 46% ± 3% of the native DNA eluting in an A + T rich fraction. The more G + C rich DNA fraction still bound to the hydroxylapatite column was eluted with 0·4 M phosphate buffer and represented 54% ± 3% of the native DNA. Native DNA from tumor cases E.H. and M.M. as well as from normal breast tissue was fractionated into A + T rich and G + C rich fractions, and then the level of (dA) regions in each fraction determined by hybridization to poly(U).

RESULTS

Examination of Fig. 2 and 3 reveals that the level of (dA) regions in human DNA remains

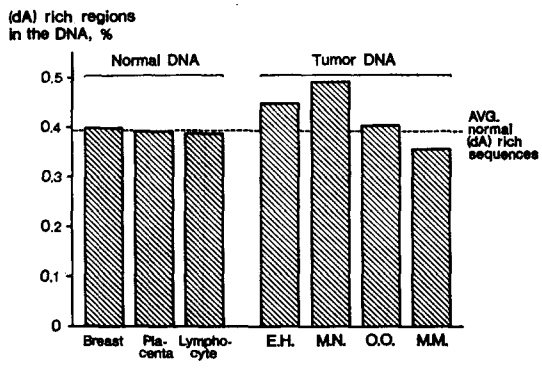


Fig. 2. The level of (dA) rich regions in the native DNA from normal human tissues and from four breast tumors. The (dA) rich regions were estimated from the ability of the DNAs to form stable hybrids with poly(U) after 30 min digestion with 5 μg/ml ribonuclease A. Case E.H., M.N., and M.M. tumor DNAs gave $P \leqq 0{\cdot}05$ when compared to the % (dA) rich regions in normal DNA.

constant from one cell type to another, since DNA from normal breast, placenta and lymphocyte cells had the same per cent of (dA) rich and (dA) pure sequences. However, when the DNAs from 4 histologically different neoplasms in the breast were quantitatively analyzed for (dA) regions, all four tumors

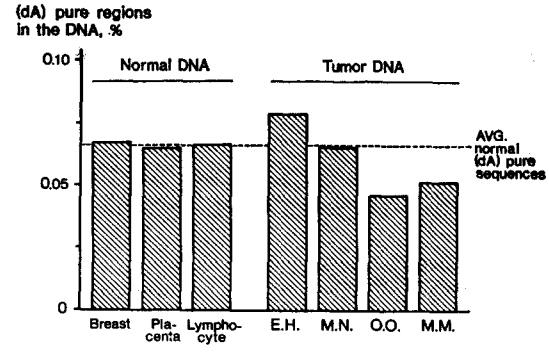


Fig. 3. The level of (dA) "pure" regions in the native DNA from normal human tissues and from four breast tumors. The (dA) "pure" regions were estimated from the ability of the DNAs to form stable hybrids with poly(U) after 6 hr digestion with 5 μg/ml ribonuclease A. Cases E.H., O.O., and M.M. tumor DNAs gave $P \leqq 0{\cdot}05$ when compared to the % (dA) pure regions in normal DNA.

showed distinct variations from the normal pattern. In addition, each tumor could be biochemically distinguished from the other tumors as well as from normal tissues based on the patterns of disturbances between the (dA) rich and (dA) "pure" regions of the different DNAs (Figs. 2–3). On the one hand, case E.H. DNA had increased levels of both (dA) rich and (dA) "pure" sequences when compared to normal DNA, while on the other hand case M.M. had significantly lower levels of both (dA) rich and (dA) "pure" sequences when compared to normal DNA. These biochemical differences between case E.H. (cystosarcoma Phyllodes) and case M.M. (anaplastic tumor) correlated well to the observed differences in their tumor histology such as degree of differentiation and apparent tissue of origin (Fig. 1).

The differences in (dA) regions were also rather striking between the 2 mammary carcinoma DNAs (Fig. 2–3). Case M.N. DNA had a completely normal level of (dA) "pure" sequences, but the (dA) rich sequences were increased over normal levels. Case O.O. DNA showed the opposite trend, having normal amounts of (dA) rich sequences, but a significant decrease in the level of (dA) "pure" sequences. Again the (dA) disturbances between these tumor DNAs were correlated to the differences in their histology (Fig. 1). These results suggest that, at least, some breast tumors can be distinguished or identified on the basis of their genetic material by analysis of (dA) regions in DNA.

The distribution of (dA) alterations in the DNA of case E.H. and M.M. tumors was also studied by fractionation of the native DNAs into A+T rich and G+C rich fractions using thermal elution chromatography at 88°C. These particular 2 cases, since they represented the opposite but most extreme (dA) alterations from normal patterns, were considered good models for further analyzing the distribution of (dA) alterations within neoplastic DNA. The G+C rich fraction of normal DNA contained about 2 times the level of both (dA) rich and (dA) "pure" sequences when compared to the

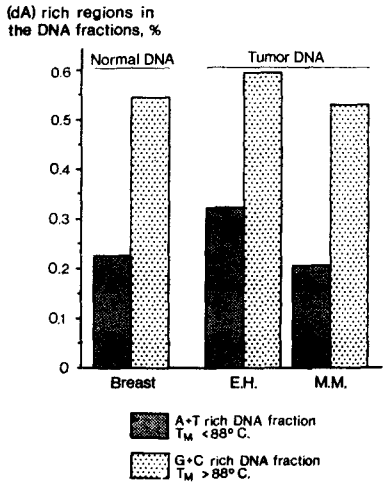


Fig. 4. The level of (dA) rich regions in the DNA from normal and tumor tissues of the breast when the native DNA has been fractionated into A+T rich and G+C rich fractions. The (dA) rich regions were estimated from the ability of the A+T rich and G+C rich DNA fractions to form stable hybrids with poly(U) after 30 min digestion with 5 μg/ml ribonuclease A. Only case E.H. A+T rich and G+C rich DNA fractions gave $P \leqq 0{\cdot}05$ when compared to the % (dA) rich regions in the corresponding normal DNA fractions.

A+T rich fraction (Fig. 4–5). This distribution pattern for normal DNA was repeated for case E.H. DNA but not for case M.M. DNA.

Although the G+C rich fraction of case M.M. DNA contained nearly twice as many (dA) rich sequences than did the A+T rich fraction, the (dA) "pure" sequences were distributed in exactly the reverse order, so that the level of (dA) "pure" regions in the G+C rich fraction was one half the level found in the A+T rich DNA.

The original (dA) alterations observed previously when these tumor DNAs were compared to normal native DNA (Fig. 2–3), were not confined to either the A+T rich or the G+C rich DNA fractions but were found distributed into both fractions when they were compared to the (dA) levels in normal A+T rich and G+C rich fractions (Fig. 4–5). These

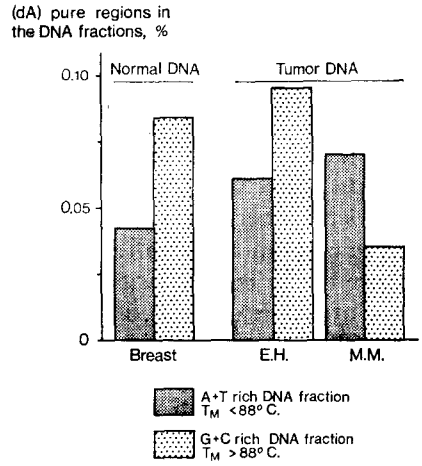


Fig. 5. The level of (dA) "pure" regions in the DNA from normal and tumor tissues of the breast when the native DNA has been fractionated into A+T rich and G+C rich fractions. The (dA) "pure" regions were estimated from the ability of the A+T rich and G+C rich DNA fractions to form stable hybrids with poly(U) after 6 hr digestion with 5 μg/ml ribonuclease A. Cases E.H. and M.M. tumor DNA fractions gave $P \leqq 0{\cdot}05$ when compared to the % (dA) pure regions in normal A+T rich and G+C rich DNA.

results demonstrate that (dA) alterations found in native tumor DNA can be further studied by fractionation into classes; *i.e.*, the (dA) alterations associated with the A+T rich DNA and those associated with the G+C rich DNA.

DISCUSSION

Our results have shown that alterations in the (dA) regions of DNA from human breast tumors can be detected and measured in native DNA or in both A+T rich and G+C rich fractions of the DNA. Separation of the (dA) alterations into different classes by quantitative comparison to normal (dA) levels corresponded to associated histologic differences between the tumor types. We have seen similar correlations with experimental rat tumors [7] and with lymphocytes from CLL patients (to be published). The (dA) alterations in primary DMBA-induced rat tumor DNAs were increased by transplanting the tumor. In CLL lymphocyte DNA, low WBC patients had increased (dA) levels while high WBC patients had reduced levels. Together these results are suggestive of a correlation between the degree of cell malignancy, often seen in terms of neoplastic differentiation, and the type of associated (dA) alterations. In all cases the most malignant condition evaluated histologically had DNA with a reduced level of (dA) "pure" regions, *i.e.*, CLL patients with high WBC, transplanted DMBA rat tumors, and case M.M. anaplastic tumor.

The etiology of breast cancer is poorly understood basically because of the difficulty in identifying and classifying the etiologic agents involved [1, 2]. We propose that analysis of (dA) regions in breast tumor DNA may be a useful tool by which the relationship between oncogen and breast cancer epidemiology can now be studied.

REFERENCES

1. D. V. Vakil and R. W. Morgan, Etiology of breast cancer I. Genetic aspects. *Canad. med. Assoc. J.* **109,** 29 (1973).
2. D. V. Vakil and R. W. Morgan, Etiology of breast cancer II. Epidemiologic aspects. *Canad. med. Assoc. J.* **109,** 201 (1973).
3. D. Colcher, S. Spiegelman and J. Schlom, Sequence homology between the RNA of Mason–Pfizer monkey virus and the RNA of human malignant breast tumors. *Proc. nat. Acad. Sci. (Wash.)* **71,** 4975 (1974).
4. A. B. Vaidya, M. M. Black, A. S. Dion and D. H. Moore, Homology between human breast tumor RNA and mouse mammary tumor virus genome. *Nature (Lond.)* **249,** 565 (1974).
5. A. Shenkin and R. N. Burdon, Deoxyadenylate-rich and deoxyguanylate-rich regions in mammalian DNA. *J. molec. Biol.* **85,** 19 (1974).
6. A. Shenkin and R. H. Burdon, Deoxyadenylate-rich sequences in mammalian DNA. *FEBS Lett.* **22,** 157 (1972).
7. R. W. Pero, T. Bryngelsson, F. Mitelman and G. Levan, Changes in the deoxyadenylate regions of rat DNA in sarcomas induced by 7,12-dimethylbenz[a]anthracene and Rous sarcoma virus. *Hereditas* **80,** 153 (1975).
8. J. O. Bishop, M. Rosbash and D. Evans, Polynucleotide sequences in eukaryotic DNA and RNA that form ribonuclease-resistant complexes with polyuridylic acid. *J. molec. Biol.* **85,** 75 (1974).
9. R. W. Pero, T. Bryngelsson, C. Bryngelsson, A. Deutsch, Å. Norden and A. Norgren, Hybridization of polyuridylic acid to human DNA immobilized onto nitrocellulose filters. *Nucleic Acids Res.* **2** (7), 1163–1176 (1975).
10. G. R. Molloy, W. L. Thomas and J. E. Darnell, Occurrence of uridylate-rich oligonucleotide regions in heterogenous nuclear RNA of Hela cells. *Proc. nat. Acad. Sci (Wash.)* **69,** 3684 (1972).
11. A. Bøyum, Separation of leucocytes from blood and bone marrow. *Scand. J. clin. lab. Invest.* **21** suppl., 97 (1968).
12. J. Marmur, A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. molec. Biol.* **3,** 208 (1961).
13. K. S. Kirby and E. A. Cook, Isolation of deoxyribonucleic acid from mammalian tissues. *Biochemistry J.* **104,** 254 (1967).
14. G. Corneo, P. Bianchi, E. Ginelli and E. Polli, Heterogeneity of base composition of DNA extracted from human leucocytes. *Europ. J. Cancer* **2,** 307 (1966).
15. J. Marmur and P. Doty, Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. molec. Biol.* **5,** 109 (1962).

Europ. J. Cancer Vol. 11, pp. 867–872. Pergamon Press 1975. Printed in Great Britain

Comparative Study of the Ultrastructure of Tracheal and Pulmonary Tumours Induced by Multiple Intratracheal Instillations of Diethylnitrosamine in Syrian Golden Hamsters*

B. J. SPIT and V. J. FERON

Central Institute for Nutrition and Food Research TNO, Zeist, The Netherlands

Abstract—*Tracheal and pulmonary tumours induced in Syrian golden hamsters by multiple intratracheal instillations of diethylnitrosamine (DENA) were studied by electron microscopy.*

In the epithelial part of the tracheal tumours, classified as papillomas, four main types of cells could be distinguished, (a) cells joined by complex interdigitations forming wide intercellular spaces, (b) cells with complex interdigitations and closely apposed plasma membranes between which a markedly electron-dense coat substance occurred, (c) ciliated cells and (d) cells resembling mucus-producing cells. These types of cells were found also in the pulmonary tumours with the exception of the ciliated cells. In addition in the peripheral zone of the lung tumours cells occurred which were considered to represent hyperplastic type II pneumocytes.

Comparison of the morphology of the tracheal tumours with that of the lung neoplasms produced insufficient evidence to answer definitely the question whether the peripheral tumours were primary lung tumours or were caused by transplantation of the tracheal tumours. Transplantation was nevertheless regarded as the most probable explanation for the presence of tumours in the bronchiolo-alveolar tissue.

INTRODUCTION

THE SIMULTANEOUS occurrence of tracheal papillomas and pulmonary adenomas in Syrian golden hamsters have been reported (a) following multiple intratracheal instillations of diethylnitrosamine (DENA) [1] and (b) after subcutaneous injection of DENA followed by intratracheal instillation of 0·9% NaCl solution alone [2] and with dusts like Fe_2O_3 [3], MgO, Al_2O_3 and carbon [2].

Accepted 18 August 1975.

*This work was supported in part by a grant from the research fund of the Scientific Advisory Committee Smoking and Health. This fund has been established by the Dutch Cigarette Industry Foundation.

The lung tumours in these animals might have originated from the bronchiolo-alveolar epithelium, but their presence has also been suggested to be due to transplantation of the tracheal papillomas into the lower respiratory tract as a consequence of the instillation procedure [1].

DENA-induced tracheal neoplasms have often been described as squamous cell tumours [4–7]. However, more recent studies on these tumours using semithin techniques did not reveal cornified or uncornified squamous cells [8]. In DENA-induced lung tumours examined by electron microscopy transformation to squamous cells was not observed either, but two other distinctive types of epithelial cells could

be identified, (a) small undifferentiated cells and (b) larger well-differentiated cells closely resembling type II pneumocytes. It was, however, not clear whether the larger cells were an essential part of the tumour or represented a cellular reaction to the presence of the true neoplastic cells in the lung tissue [9].

In an attempt to elucidate the origin of the lung tumours and to determine whether the type II pneumocyte-like cells present in these neoplasms were neoplastic or reactive in nature, a detailed comparative ultrastructural study was performed on tracheal and pulmonary tumours induced by multiple intratracheal instillations of DENA in Syrian golden hamsters.

MATERIAL AND METHODS

Tumour material was obtained from 1 male and 6 female Syrian golden hamsters, which had received intratracheal instillations of 0·2 ml 1·5% DENA (EGA-Chemie K.G., Keppler and Reif, Steinheim/Albuch, W.-Germany) in 0·9% NaCl solution (sterile, non-pyrogenic, Baxter Laboratories, A. Christiaens N.V., Brussels, Belgium) with or without 3 mg ferric oxide (Fe_2O_3, haematite, obtained from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.) once every week for a period of 15 weeks starting at an age of 8 weeks. The animals were 30–40 weeks old when they were killed in extremis. The origin of the hamsters, their housing and feeding were the same as described previously [1].

Following anaesthesia with Brietal Sodium (Lilly Co., Indianapolis, Ind., U.S.A.) injected i.p., the thoracic cavity was opened and the lungs with trachea were quickly excised and immersed in ice-cold fixative. The trachea (with tumours) was immediately removed and lengthwise cut into two halves. The lung tumours were cut into thin slices with a razor blade. Pieces were taken from both the peripheral and the more central parts of the tumours. The fixative used was the modified glutaraldehyde-osmium fixative described by De Bruijn [10], using $K_3Fe(CN)_6$ in the postfixation step. The main points of this fixation procedure are: primary fixative is 3% glutaraldehyde in 0·1 M sodium cacodylate, pH 7·4, to which 0·05 M $CaCl_2$ anhydride is added; the fixation period was 60 hr at 4°C. The tissues were stored in the buffer used for periods varying from two days to six weeks and postfixed in 1% OsO_4 in 0·1 M sodium cacodylate buffer to which 0·05 M $K_3Fe(CN)_6$ was added, pH 7·4 for 16 hr at 4°C.

Dehydration was accomplished in graded series of acetone dilutions at room temperature, followed by embedding in Epon 812. One micron sections were stained with toluidine blue and examined by light microscopy. Thin sections were stained with uranyl acetate in water and lead citrate [11] and viewed with a Philips EM 200 microscope at 60 kV.

Two tracheas were investigated by scanning electron microscopy.* A Cambridge Stereoscan microscope was used. The fixed tracheas were lengthwise cut into two halves and freeze dried in 100% acetone using the critical point method and mounted on a specimen holder with silver paste. The specimens, mounted on a rotating stage, were made electrically conductant by high vacuum evaporation of carbon and gold. Pictures were made at 5 and 20 kV.

RESULTS

Tracheal tumours

The tracheal neoplasms appeared to be papillomatous growth (Fig. 1), which often were multiple and filled almost completely the tracheal lumen. They were lined by stratified epithelium separated from a well-vascularized loosely arranged connective tissue by a thin basal membrane. In the stalk of the papillomatous growth the basal lamina was clearly seen to be continuous with that of the trachea.

Typical differences in morphology of the neoplastic epithelium existed between various tumours or even between various lobes within the same tumours. Ultrastructurally, four main types of epithelial cells could be distinguished:

1. *Cells joined by complex interdigitations forming wide intercellular spaces.* This type of cells (Fig. 2) was most frequently observed and often occurred in large areas extending from the basal lamina to the surface of the tumour. The cells contained a relatively large indented nucleus surrounded by a narrow zone of cytoplasm poor in organelles. The chromatin was condensed at the periphery of the nucleus.

The cytoplasm contained many free ribosomes, but mitochondria, endoplasmic reticulum and Golgi-system were rare. Strikingly electron lucent cells (light cells) occurred in the superficial cell layers. Their cytoplasm was inhomogeneous and flocky, and occasionally contained electron dense granules, scattered membranes and all kinds of vesicular structures. These cells were considered to be more or less degenerated or sometimes even necrotic (Fig.

*This work was performed in the "Centrum voor Submicroscopisch Onderzoek", State University at Utrecht (Head Dr. P. F. Elbers).



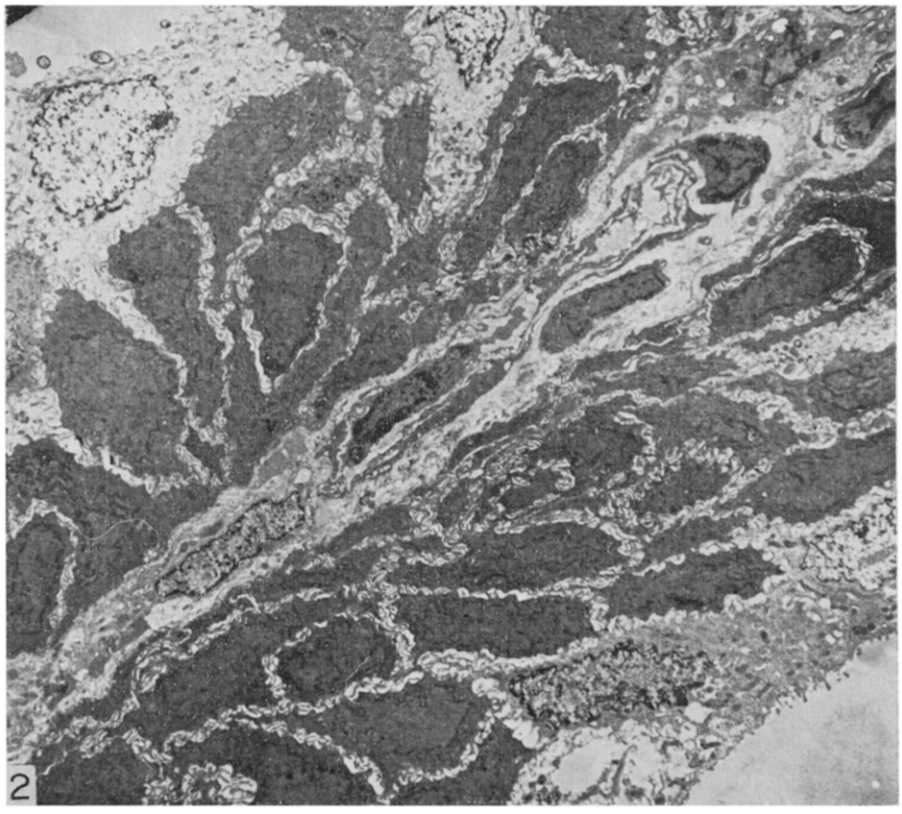
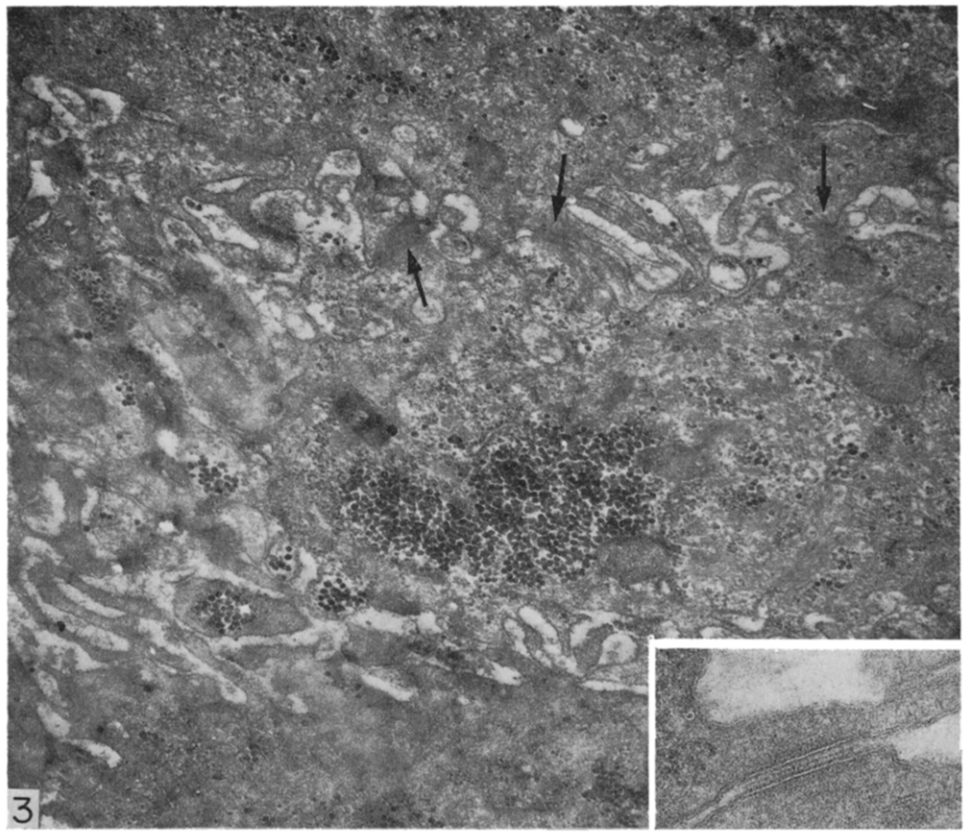


Fig. 1. *Scanning electron micrograph of tracheal papilloma. Freeze dried, C-Au evaporation* × 50.

Fig. 2. *Longitudinal section through a lobe of a tracheal tumour, showing the type of cells joined by complex interdigitations forming wide intercellular spaces. From the lower left corner to the upper right corner runs a strip of stroma. At the surface light cells more or less degenerated.* × 24,000.

(to face page 868)



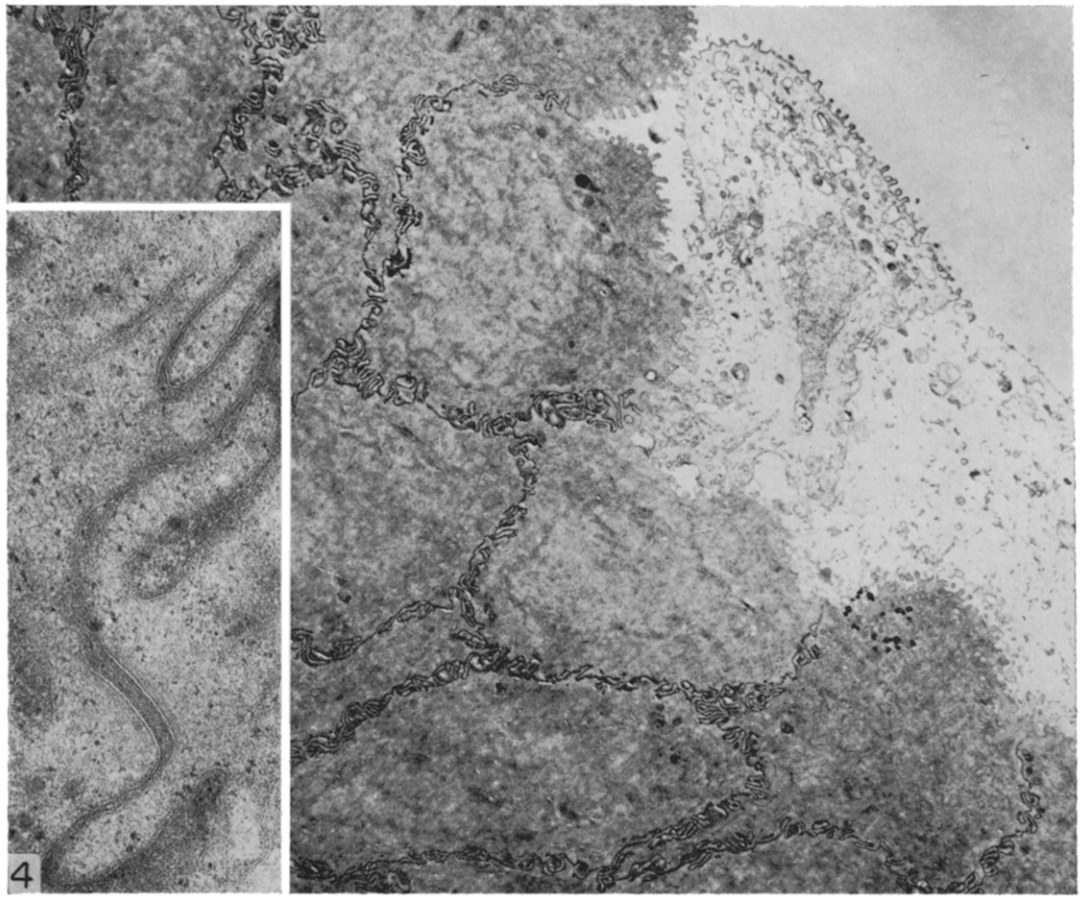
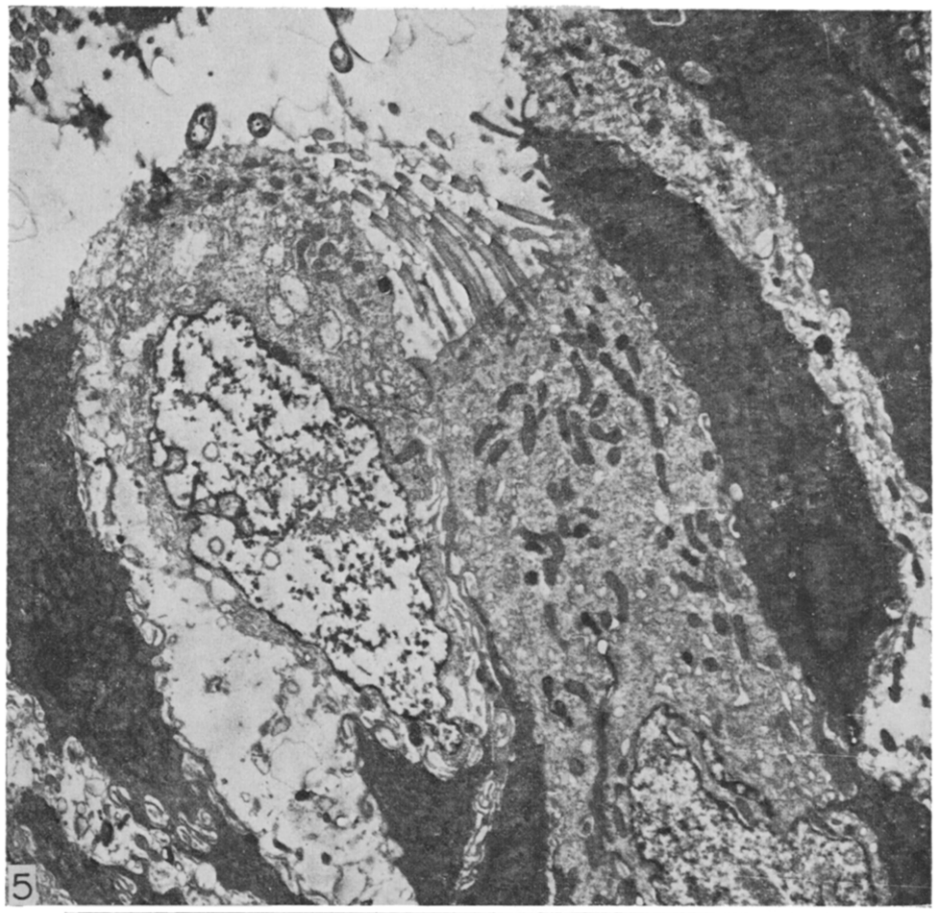


Fig. 3. Tracheal tumour cells. Complex interdigitations and wide intercellular spaces. Desmosomes are visible (arrow). β-Glycogen particles occur either in clusters or scattered in the cells and the intercellular spaces. × 22,000. *Inset: desmosome without tonofilaments.* × 99,000.

Fig. 4. Tracheal tumour cells with complex interdigitations and closely apposed cell membranes, between which an electron dense coat substance occurred (see inset). At the surface a necrotic epithelial cell. × 5200. *Inset:* × 74,500.



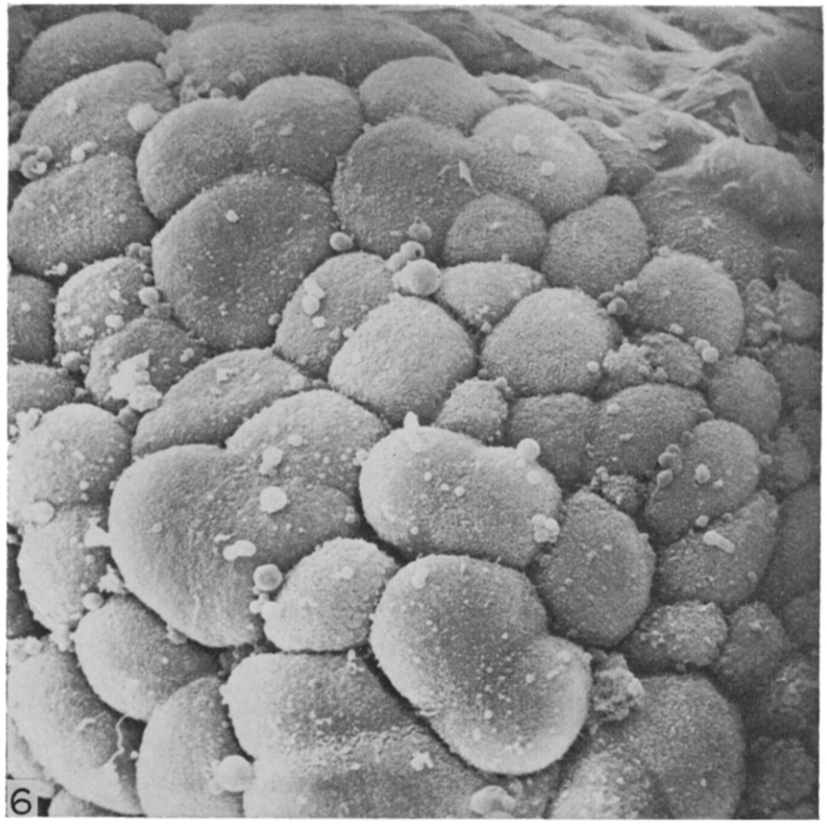
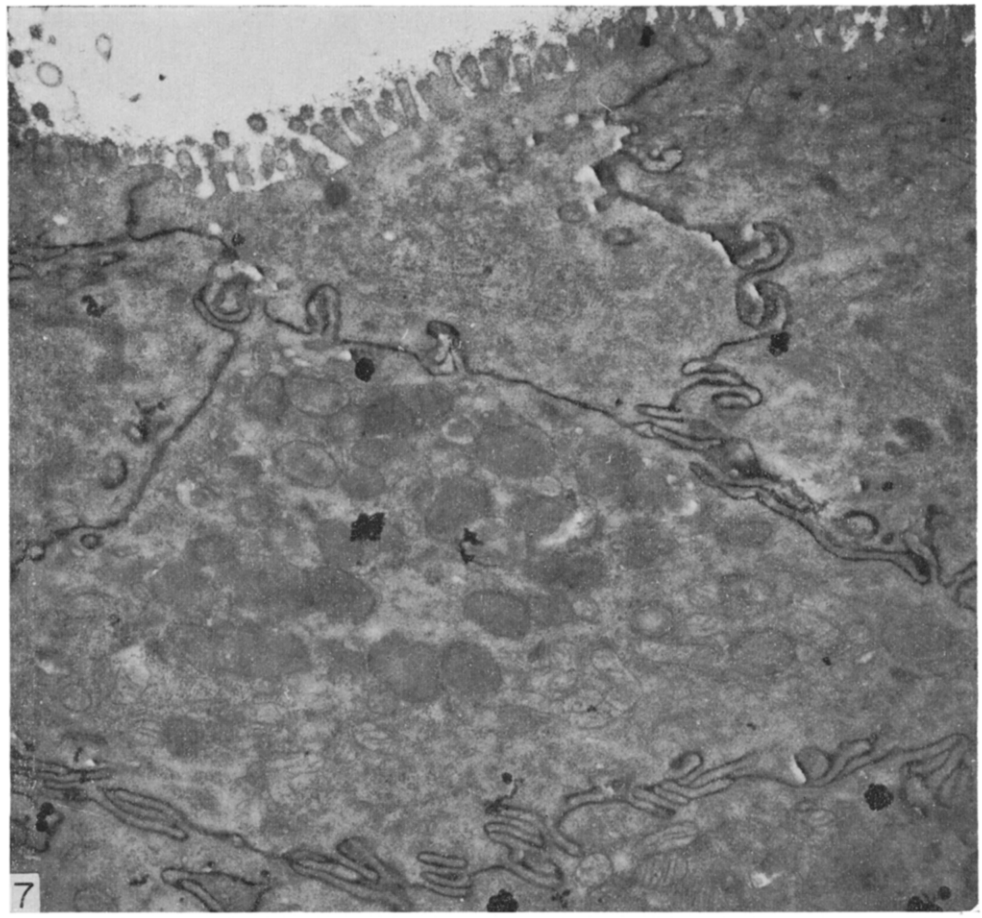


Fig. 5. *Ciliated cell in a tracheal papilloma surrounded by light and dark cells.* × 5000.

Fig. 6. *Scanning electron micrograph of the surface of cells in a tracheal neoplasm. Only cells bearing microvilli are visible.* × 2000.



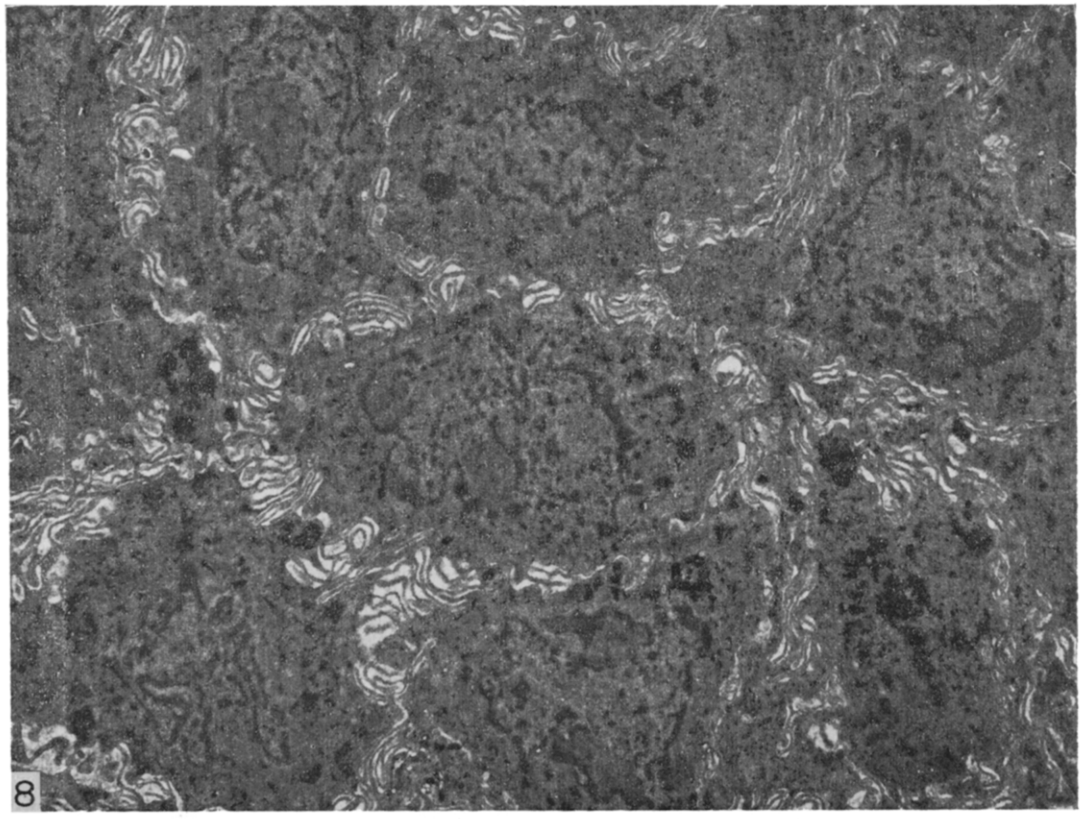
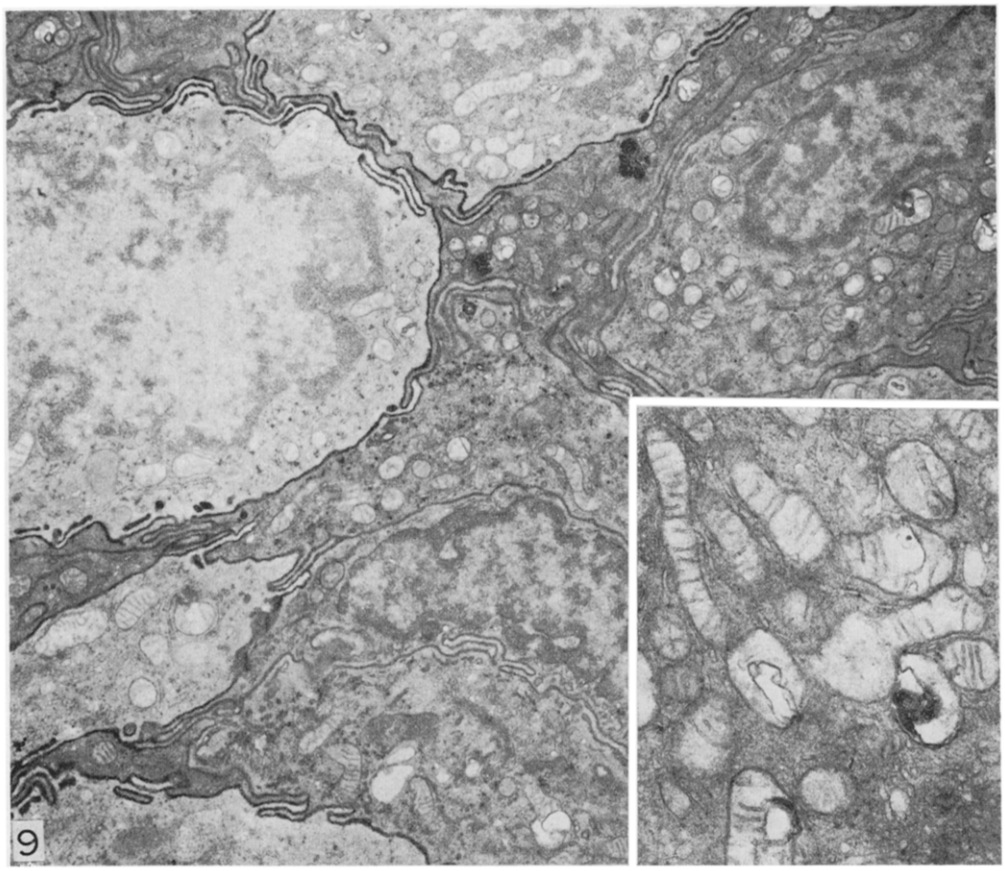


Fig. 7. Tracheal tumour cells of the "closed type". At the surface microvilli. In the centre of the picture a mucus-producing cell with membrane-bounded droplets. × 17,500.

Fig. 8. Lung tumour. Undifferentiated cells with complex interdigitations and wide intercellular spaces. The nucleus is surrounded by a small zone of cytoplasm in which clusters of β-glycogen particles occur. × 7900.



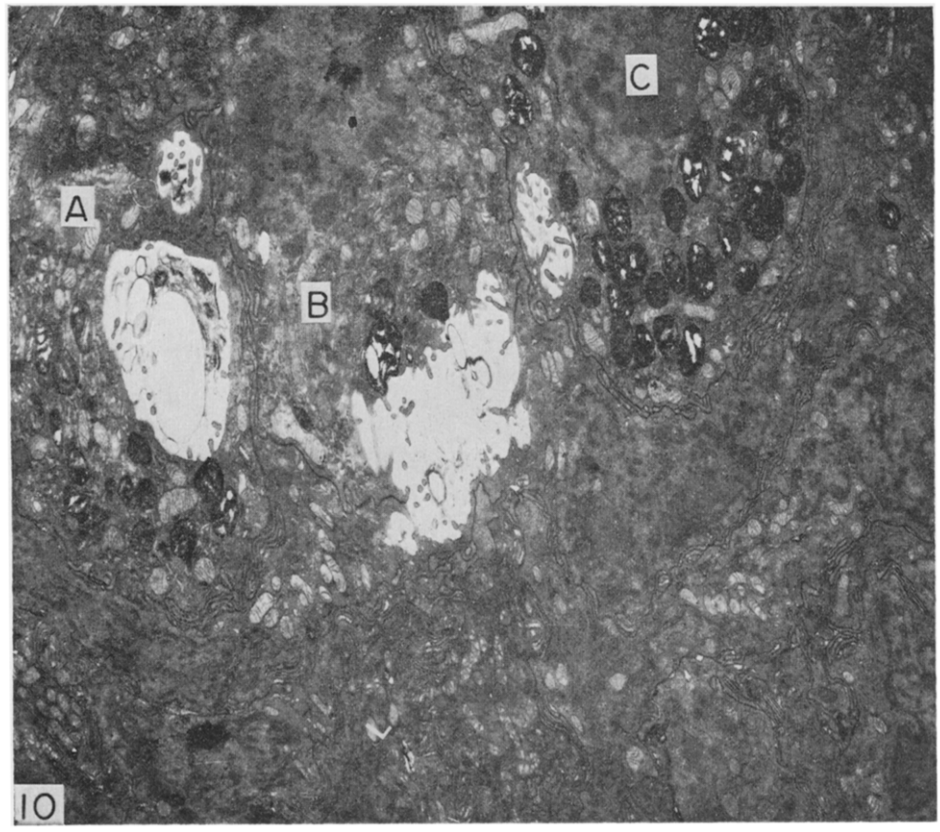


Fig. 9. *Lung tumour. Cells of the "closed type" with many cell organelles. The swollen mitochondria have inclusions.* × 13,000. *Inset:* 22,000.

Fig. 10. *Lung tumour. Type II pneumocytes (A, B, C) are surrounded by tumour cells of the "closed type". Characteristic lamellar bodies and microvilli are visible.* × 8100.

2). In general, numerous microvilli were visible at the free surface of these cells.

A characteristic feature of these neoplastic cells was their complex interdigitations which formed wide intercellular spaces measuring up to 1·5 μm in width. There were many desmosomes, but tonofilaments could not be discerned at all. Occasionally, myelin-like figures and electron dense particles occurred within the interspaces. Similar particles were also present in the cytoplasm either alone or in clusters (Fig. 3). Since they were visible neither before the lead-staining nor following treatment of the tumour tissue with diastase, they were considered to represent β-glycogen particles.

2. *Cells with complex interdigitations and closely apposed plasma membranes between which a markedly electron dense coat substance occurred.* A characteristic feature was the strongly interdigitating folds of adjacent cell surfaces which were highly conspicuous. As revealed at high magnifications the prominent appearance of the plasma membranes was actually caused by the presence of a thin layer of very electron dense material between the outer leaflets of the cell membranes (Fig. 4).

In general, the cytoplasm was rich in organelles. Especially mitochondria were present in large numbers. Many of them were partly swollen or contained granular or membranous inclusions. The cells contained many ribosomes occasionally attached to membranes. Beta-glycogen particles could not be discerned. Desmosomes were rare and tonofilaments were not seen at all.

Another striking feature was the occurrence of dark (electron dense) next to light (electron lucent) neoplastic cells. There were, however, no clear structural differences between dark and light cells. The latter occurred throughout the neoplastic epithelium and were, thus, not only located in the superficial cell layer as were those of the type of tumour cells described above.

Both the cells having wide intercellular spaces and those with closely apposed plasma membranes were found in the same tumour, but intermediate stages were not observed.

3. *Ciliated cells.* Ciliated cells were observed in the superficial cell layer of tracheal neoplasms upon light microscopical examination of semithin plastic sections as well as upon transmission electron microscopy (Fig. 5). The cells closely resembled the ciliated cells of the normal tracheal epithelium. Their incidence was extremely low as is illustrated by the fact that scanning electron microscopical examination of several tumours did not reveal the presence of ciliated cells (Fig. 6). The free surface of most of the superficial tumour cells appeared to be provided with microvilli (Fig. 4, 7).

4. *Mucus-producing cells.* Like ciliated cells mucus-producing cells were only sporadically seen in the superficial cell layer of the tracheal neoplasms (Fig. 7). They most often occurred among the cells with closely apposed plasma membranes. The mucous material was not found in the form of closely packed droplets as is seen in goblet cells, but was always encountered as separate droplets encased by a membrane and located in the apical part of the cells where the cytoplasm was poor in other organelles.

Lung tumours

For a description of the gross and light microscopical appearance of the neoplasms found in the bronchiolo-alveolar region the reader is referred to previous reports [1, 2, 9].

Electron microscopical examinations showed that three of the four types of epithelial cells encountered in the tracheal papillomas and described above were also present in the neoplasms located in the pulmonary tissue (Fig. 8, 9). Ciliated cells were the type of cells that could not be discovered in the lung tumours.

The epithelial cells of the pulmonary neoplasms were ultrastructurally very similar to those of the tracheal tumours. Hence, their morphology needs no detailed description. Yet, two minor but consistent differences in cellular morphology existed between the tracheal and pulmonary tumours: (1) The cells with closely apposed plasma membranes found in the lung tumours contained much more partly swollen mitochondria with interrupted cristae than did the corresponding type of cells in the tracheal tumours. (2) The difference in density between "light" and "dark" cells of the type of cells with closely joined plasma membranes was much more pronounced in the tumours of the lung than in those of the trachea. In addition, the light cells of the lung tumours contained a larger number of cisternae of the rough endoplasmic reticulum than did the light cells found in the tracheal tumours.

In the lung tumours, cells closely resembling or being type II pneumocytes were sporadically observed either solitarily or in small groups (Fig. 10). These cells were invariably located in the outer zone of the neoplastic pulmonary nodules.

DISCUSSION

In the earliest reports on DENA-induced tracheal tumours in Syrian golden hamsters,

the authors invariably referred to squamous cell tumours [4–6]. Montesano and Saffiotti [7] distinguished between papillomas showing squamous epithelium and polyps containing mucus-producing epithelium. Mohr [12] proposed to classify the DENA-induced tracheal neoplasms in hamsters as papillomas (squamous epithelium), polyps (mucus-secreting epithelium) and papillary polyps (squamous and mucus-secreting epithelium). More recent studies on this type of tumours by Althoff *et al.* [8] using semithin techniques revealed the presence of mucus-producing and ciliated cells and precursors of these types of cells, but no evidence was obtained for transformation to squamous epithelium. The absence of squamous cell differentiation was confirmed by the results of the present ultrastructural study, which also confirmed the occurrence of ciliated and mucus-producing cells, though their incidence was much lower than in the tumours examined by Althoff *et al.* [8].

Two types of slightly differentiated cells constituted the great majority of the epithelial cells of the tracheal tumours examined in the present study. The exact relationship between the two types of cells was not clear. Intermediate stages of the two types of cells were not observed. In addition they generally occurred independently from each other in large areas extending from the basal lamina up to the surface of the lobes of the tumours. Therefore both types of cells are regarded as neoplastic cells more or less independent from one another and each originating directly from DENA-exposed basal cells of the tracheal epithelium.

De Bruijn [10, 13] and Dvorak *et al.*. [14] have described a method to enhance the contrast for β-glycogen particles and the cell coat by using iron-ions in the postfixation step. In agreement with the findings of these authors an excellent visualization was reached of both the glycogen particles in the cells with wide intercellular spaces and the surface coat joining the cells of the closed type.

Considerable differences in morphology between tracheal tumours found in hamsters following treatment with DENA emerged from the various studies mentioned above. These differences may at least partly be ascribed to inaccurate identification of the tumour cells, because light microscopy on paraffin sections as used in most of the earlier studies, insufficiently warrants a correct typification of cells [8, 9]. Differences in breeding, feeding and housing of the animals used in the various studies have also been mentioned as possible causes for the different results [12]. In addition, the total dose of DENA administered may be of significance for the type and degree of differentiation.

The above information allows the conclusion that administration of sufficiently high doses of DENA to Syrian golden hamsters results in papillary tumours of the trachea containing epithelium of which the type and degree of differentiation seems to depend on secondary factors. A judgement of the cell types involved should be based on unambiguous observation of characteristic features such as cilia, mucous droplets, tonofilaments or keratin granules.

Comparison of the morphology of the tracheal tumours with that of the pulmonary tumours yielded the following points of correspondence and difference:

Points of correspondence. (1) The epithelium was stratified, (2) the vast majority of the epithelial cells belonged to two types of slightly differentiated cells and (3) mucus-producing cells occurred only sporadically.

Points of difference. (1) The architecture of the tracheal tumours was predominantly papillomatous, that of the lung tumours adenomatous, (2) ciliated cells were found only in the tracheal neoplasms and (3) the peripheral part of the pulmonary tumours contained cells closely resembling or being type II pneumocytes.

On closer consideration none of the points of correspondence or difference appeared to be sufficiently marked to give a definite answer to the question whether the peripheral tumours were primary lung tumours or were caused by transplantation of the tracheal neoplasms.

Ultrastructurally, the tumours in the lung were markedly similar to those of the trachea. Because of this striking morphological resemblance we are inclined to prefer the "transplantation hypothesis". On the other hand this morphological similarity can be rebutted as an argument in favour of the "transplantation hypothesis" because the bronchiolo-alveolar and tracheal epithelium might produce the same type of tumours when exposed to a certain carcinogenic factor. This assumption is based on the fact that the respiratory epithelium, from larynx through alveoli, originates from the same tissue, namely the pharynx endoderm [15].

The adenomatous appearance of the lung tumours does not exclude these tumours actually being derived from tracheal papillomas, as it is likely that the growth pattern of the transplanted cells is an imitation of the alveolar architecture, especially in the initial phase of the growth. Moreover the tracheal tumours occasionally showed a histological appearance more suggestive of an adenomatous than a

papillomatous growth pattern. Therefore, the difference in histological picture is no longer a solid argument against the "transplantation hypothesis". This also holds for the absence of ciliated cells in the pulmonary neoplasms because differentiation to ciliated cells may take place in the primary but not in the secondary (transplanted) tumour.

The type II pneumocytes (or cells resembling them) found in the lung tumours may represent either an essential part of the tumour or a cellular reaction of the pulmonary tissue to the presence of the true neoplastic cells [9]. In the present study type II pneumocytes were found only in the peripheral zone of the nodules which is in accordance with the idea of a cellular reaction, because by penetrating the alveolar tissue the tumour may surround, among others, type II pneumocytes which at first increase in number and survive, but get oppressed in a later stage and finally disappear. If these cells indeed are hyperplastic instead of neoplastic, their presence in the lung tumours is of no significance for establishing the origin of the peripheral tumours. The idea of a cellular reaction also fits in with the doubts expressed by Kuhn [16] regarding the neoplastic nature of the cells depicted by Adamson *et al.* [17] and Coalson *et al.* [18] to demonstrate the origin from type II pneumocytes of the tumours studied.

The "transplantation hypothesis" is strongly supported by recent observations of Stenbäck *et al.* [2], who found that subcutaneous injection of DENA followed by repeated intratracheal instillations of 0·9% NaCl solution caused lung tumours in as many as 18 out of 33 hamsters, whereas the DENA-treatment alone induced a lung tumour in only one out of a group of 35 hamsters. Rejection of the "transplantation hypothesis" would imply that a 0·9% NaCl solution is co-carcinogenic to the hamster lung, which is possible but improbable.

Acknowledgement—The authors wish to thank Mr. Fred van Welie for his technical assistance, Dr. J. G. Bluemink of the Hubrecht Laboratory of the Royal Dutch Academy of Science at Utrecht for carrying out the freeze drying and Dr. A. P. de Groot for critical reviewing the manuscript.

REFERENCES

1. V. J. Feron, P. Emmelot and T. Vossenaar, Lower respiratory tract tumours in Syrian golden hamsters after intratracheal instillations of diethylnitrosamine alone and with ferric oxide. *Europ. J. Cancer* **8,** 445 (1972).
2. F. G. Stenbäck, A. Ferrero and P. Shubik, Synergistic effects of diethylnitrosamine and different dusts on respiratory carcinogenesis in hamsters. *Cancer Res.* **33,** 2209 (1973).
3. R. Montesano, U. Saffiotti and P. Shubik, The role of topical and systemic factors in experimental respiratory carcinogenesis. In: *Inhalation Carcinogenesis* (edited by M. G. Hanna, P. Nettesheim and J. R. Gilbert). Proc. Biol. Div., Oak Ridge Natl Lab., U.S. Atomic Energy Comm., Conf. 691001, p. 353, Gatlinburg (1970).
4. W. Dontenwill and U. Mohr, Carcinome des Respirationstractus nach Behandlung von Goldhamstern mit Diäthylnitrosamin. *Z. Krebsforsch.* **64,** 305 (1961).
5. K. M. Herrold and L. J. Dunham, Induction of tumors in the Syrian hamster with diethylnitrosamine (N-nitrosodiethylamine). *Cancer Res.* **23,** 773 (1963).
6. K. M. Herrold, Effect of route of administration on the carcinogenic action of diethylnitrosamine (N-nitrosodiethylamine). *Brit. J. Cancer* **18,** 763 (1964).
7. R. Montesano and U. Saffiotti, Carcinogenic response of the respiratory tract of Syrian golden hamsters to different doses of diethylnitrosamine. *Cancer Res.* **28,** 2197 (1968).
8. J. Althoff, R. Wilson and U. Mohr, Diethylnitrosamine-induced alterations in the tracheobronchial system of Syrian golden hamsters. *J. nat. Cancer Inst.* **46,** 1067 (1971).
9. W. Straks and V. J. Feron, Ultrastructure of pulmonary adenomas induced by intratracheal instillation of diethylnitrosamine in Syrian golden hamsters. *Europ. J. Cancer* **9,** 359 (1973).
10. W. C. de Bruijn, Glycogen, its chemistry and morphologic appearance in the electron microscope I, A modified OsO_4 fixative which selectively contrasts glycogen. *J. Ultrastruct. Res.* **42,** 29 (1973).

11. J. H. Venable and R. Coggeshall, Simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25,** 407 (1965).
12. U. Mohr, Effect of diethylnitrosamine in the respiratory system of Syrian golden hamsters. In: *Morphology of Experimental Respiratory Carcinogenesis,* Atomic Energy Commission Symposium Series 21 (edited P. Nettesheim, M. G. Hanna and J. W. Deatherage) p. 255, Oak Ridge, Tennessee, U.S. Atomic Energy Comm. Div. Techn. Inform. Extension (1970).
13. W. C. de Bruijn, A modified OsO_4- (double) fixation procedure which selectively contrasts glycogen. Electron microscopy, *Fourth European Regional Conf. on Electron Microscopy.* Vol. II, p. 65, Typografia Poliglotta Vaticana. Roma (1968).
14. A. M. Dvorak, M. E. Hammond, H. F. Dvorak and M. J. Karnovsky, Loss of cell surface material from peritoneal exudate cells associated with lymphocyte-mediated inhibition of macrophage migration from capillary tubes. *Lab. Invest.* **27,** 561 (1972).
15. S. P. Sorokin, The cells of the lungs. In: *Morphology of experimental Respiratory Carcinogenesis,* Atomic Energy Commission Symposium Series 21 (edited P. Nettesheim, M. G. Hanna and J. W. Deatherage) p. 3, Oak Ridge, Tennessee, U.S. Atomic Energy Commission Div. Techn. Inform. Extension (1970).
16. C. Kuhn, Fine structure of bronchiolo-alveolar cell carcinoma, *Cancer* **30,** 1107 (1972).
17. J. S. Adamson, R. M. Senior and T. Merrill, Alveolar cell carcinoma. An electron microscopic study. *Amer. Rev. resp. Dis.* **100,** 550 (1969).
18. J. J. Coalson, J. A. Mohr, J. K. Pirtle, A. L. Dee and E. R. Rhoades, Electron microscopy of neoplasms in the lung with special emphasis on the alveolar cell carcinoma. *Amer. Rev. resp. Dis.* **101,** 181 (1970).

Europ. J. Cancer Vol. 11, pp. 873–877. Pergamon Press 1975. Printed in Great Britain

Urinary and Plasma Androgens and Their Relation to Familial Risk of Breast Cancer

D. Y. WANG, R. D. BULBROOK and J. L. HAYWARD*
Department of Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX

Abstract—*The urinary excretion of androsterone, aetiocholanolone and dehydroepiandrosterone has been measured in 87 daughters and 39 sisters of women with breast cancer. Also 175 women who had no known family history of breast cancer were studied. Similarly, plasma dehydroepiandrosterone sulphate (DS) and androsterone sulphate (AS) were determined in 52 sisters and 55 daughters of women with breast cancer. There were 290 control women.*

The urinary excretion of aetiocholanolone was significantly lower ($P < 0.05$) in young sisters (30–40 years) compared to controls. Similarly, the age-adjusted geometric mean level of androsterone was lower ($P < 0.05$) in unaffected sisters compared to controls. No significant differences were found between daughters of breast cancer patients and controls for any of these urinary steroids. Neither was the general level of dehydroepiandrosterone different for any of the groups studied.

The results for plasma DS and AS were similar to those for androsterone and aetiocholanolone. Thus the age-adjusted geometric mean level of DS and AS for sisters of breast cancer patients was significantly lower than that for controls ($P < 0.025$ and $P < 0.005$, respectively). There were no significant differences in the concentration of these steroids between daughters and controls. The main difference between unaffected sisters and controls appears to occur in the youngest age group studied (30–40 years).

These results demonstrate an association between increased risk of breast cancer due to familial history and altered endocrine function.

RETROSPECTIVE studies up to 1967 generally showed that breast cancer occurred more often than expected in relatives of women with the disease [1] but these investigations were based on the premise that a single disease was under consideration [2]. More recent work has indicated that there may be several categories of breast cancer and that a strong familial link is only found in some of these [3].

Among other risk factors in breast cancer such as reproductive history, weight and height, age at menarche and age at menopause [4, 5], androgen status also appears to be a major determinant [6, 7]. It was possible that familial risk might also be related to abnormalities in endocrine function since the latter have been shown to be genetically determined in some instances [8–10].

We have described previously a prospective study of the relation between androgen and corticosteroid metabolite excretion and risk of breast cancer in a large normal population in Guernsey [6, 7]. It seemed to us that it might be possible to carry out a pilot investigation of endocrine function in women with a family history of breast cancer and in appropriate controls in this population. The urinary excretion of these steroid metabilites has been measured, therefore, in the kindred of women with breast cancer in Guernsey. A second prospective study was started in Guernsey in 1968 in which blood was obtained from 4600 normal women aged 30–60 yr. Plasma androsterone and

Accepted 12 September 1975.
*Breast Unit, Guy's Hospital, London, SE1 9RT.

dehydroepiandrosterone sulphates have been assayed in plasma from the relatives of women with breast cancer and in controls with no known family history.

METHODS

Subjects

The women involved in these studies were ostensibly healthy volunteers living on the Island of Guernsey. In the first investigation started in 1961, 5230 women supplied a 24 hr urine specimen. Subsequently, 50 of these women developed breast cancer and 13 of their unaffected sisters (who had also supplied urine specimens) were traced. In addition, 113 unaffected volunteers had stated in a questionnaire that their mothers, and/or sisters had breast cancer. This gave a total of 87 daughters and 39 sisters. Age-matched controls (175) with no stated family history were selected at random.

In the second prospective study, plasma steroids were measured in 52 women whose sisters had breast cancer and in 55 women whose mothers had the disease. There were 290 controls.

The statements concerning family history had to be taken at face value and it was not possible to verify the accuracy of the history.

Urine and blood specimens were collected, stored and analysed as previously described [6, 11].

RESULTS

1. *Urinary steroid metabolites*

The mean amount of this steroid excreted by the sisters aged 30–40 yr is marginally lower than that in controls ($t = 2{\cdot}18 : P < 0{\cdot}05$: log steroid values used). Unaffected sisters in the two older age groups excreted amounts of aetiocholanolone which did not differ from those found in controls. This urinary excretion of aetiocholanolone in the control group and in unaffected sisters, in three decades from 30 to 60 yr is shown in Fig. 1. The excretion of androsterone was lower in sisters than in controls in all three decades but the differences were not significant (Table 1).

The excretion of androsterone and aetiocholanolone by unaffected daughters of women with breast cancer did not differ from that found in controls (Table 1).

The amount of androgen metabolites found in the urine of normal women are negatively correlated with age [12] and the data in Table 1 were re-analysed by calculating the regression of the log steroid values on age.

As far as aetiocholanolone is concerned, a significant association between steroid values and age was not found in this series and there was no difference between the slopes of the regression lines or the general level for sisters or controls. However, the amounts of androsterone excreted by both unaffected sisters and controls were significantly correlated with age ($r =$

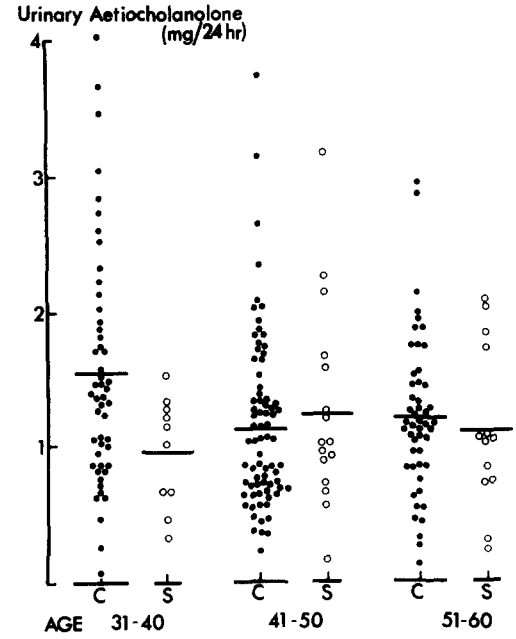


Fig. 1. Comparison of amounts of urinary aeticholanolone excreted by normal controls (C) and sisters (S) of women with breast cancer. The horizontal bars show the arithmetic means.

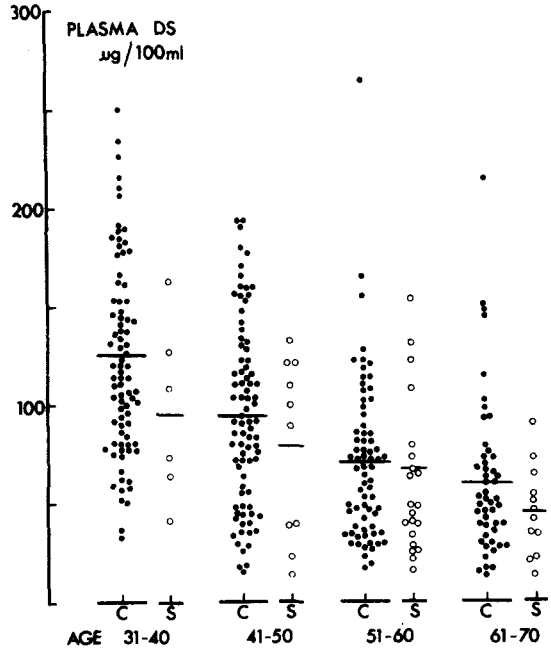


Fig. 2. Comparison of dehydroepiandrosterone sulphate levels in normal controls (C) and sisters (S) of women with breast cancer. The horizontal bars show the arithmetic means.

Table 1. *Urinary creatinine, urinary and plasma steroids in normal women and in women with a family history of breast cancer. C = Controls: S = Sisters: D = Daughters: CR = creatinine (mg/24 hr); 17-OHCS = 17-hydroxycorticosteroids (mg/24 hr); D = dehydroepiandrosterone (μg/24 hr); A = androsterone (μg/24 hr); AE = aetiocholanolone (μg/24 hr); DS = dehydroepiandrosterone sulphate (μg/100 ml plasma); AS = androsterone sulphate (μg/100 ml plasma).*

	30 - 40			41 - 50			51 - 60			61 - 70		
	C	S	D	C	S	D	C	S	D	C	S	D
CR	1.066 ±0.214 (50)	1.160 ±0.201 (10)	1.13 ±0.236 (39)	1.033 ±0.194 (75)	1.019 ±0.220 (16)	0.955 ±0.170 (29)	0.962 ±0.181 (50)	0.931 ±0.243 (13)	1.006 ±0.208 (16)	-	-	-
17-OHCS	0.85 ±0.13 (49)	0.876 ±0.12 (10)	0.895 ±0.15 (39)	0.828 ±0.12 (75)	0.827 ±0.22 (16)	0.819 ±0.17 (29)	0.811 ±0.15 (50)	0.757 ±0.16 (13)	0.830 ±0.136 (16)	-	-	-
D	2.57 ±0.62 (50)	2.57 ±0.45 (10)	2.554 ±0.58 (39)	2.32 ±0.49 (75)	2.42 ±0.50 (16)	2.29 ±0.56 (29)	2.33 ±0.49 (50)	2.21 ±0.52 (13)	2.49 ±0.53 (16)	-	-	-
A	3.18 ±0.27 (50)	3.10 ±0.24 (10)	3.24 ±0.26 (39)	3.01 ±0.24 (75)	2.945 ±0.110 (16)	3.05 ±0.23 (28)	3.04 ±0.21 (50)	2.94 ±0.24 (13)	3.00 ±0.23 (16)	-	-	-
AE	3.12 ±0.29 (50)	2.95* ±0.220 (10)	3.21 ±0.25 (40)	3.00 ±0.24 (75)	3.02 ±0.29 (16)	3.03 ±0.27 (31)	3.03 ±0.25 (50)	2.98 ±0.28 (13)	3.06 ±0.29 (16)	-	-	-
DS	2.06 ±0.19 (82)	1.94 ±0.22 (6)	2.05 ±0.24 (13)	1.90 ±0.27 (85)	1.79 ±0.36 (10)	1.99 ±0.30 (21)	1.78 ±0.24 (71)	1.75 ±0.30 (23)	1.79 ±0.20 (15)	1.70 ±0.27 (52)	1.58 ±0.26 (12)	1.57 ±0.17 (6)
AS	1.53 ±0.24 (82)	1.48 ±0.24 (6)	1.43 ±0.34 (13)	1.44 ±0.36 (85)	1.17* ±0.36 (10)	1.41 ±0.57 (21)	1.36 ±0.32 (71)	1.26 ±0.40 (24)	1.25 ±0.34 (15)	1.32 ±0.29 (52)	1.14 ±0.46 (12)	1.31 ±0.13 (6)

The means given are those of the logarithms to the base 10 of the steroid values ± standard deviation. Figures in brackets refer to the number of subjects in each category. *refer to significant differences ($P < 0.05$) from the normal mean.

-0.32, $P = < 0.05$ and $r = -0.26$, $P = < 0.01$, respectively). While the slopes of the regression lines were the same in the two groups, the general level of androsterone excretion was significantly, although marginally, lower in sisters than in controls ($P < 0.05$).

There was no significant difference between the level of DHA in controls compared to unaffected sisters or daughters. This was true whether the data were analysed by t test or if age-adjusted means were compared.

2. *Plasma DS and AS*

The plasma DS levels in sisters of women with breast cancer and in controls are shown in Fig. 2. The mean DS levels are consistently lower in the sisters than in controls in the four decades studied but the differences are not significant. However, the greatest difference between these means occurs in the youngest age group, and this is illustrated in Fig. 2. The results for AS are very similar except that the level for sisters was significantly lower than that of the controls in the fifth decade ($t = 2.3$, $P = < 0.05$; log AS values used).

Plasma DS (expressed logarithmically) is negatively correlated with age for both sisters and controls ($r = -0.37$, $P = <0.01$; $r = -0.53$, $P = <0.001$, respectively). While the slopes of the regression lines are identical for both groups, the sisters have a significantly lower concentration of DS in their blood ($P < 0.025$). Since the regression lines were calculated using the logarithms of the steroid values, the greatest difference in absolute terms is found in the younger age group.

The regression of AS on age gives negative slopes for both sisters and controls, but the correlation is statistically significant only for controls ($r = -0.28$, $P < 0.01$). Nevertheless, the general level of plasma AS in sisters is significantly subnormal ($P < 0.005$).

The mean plasma DS and AS levels in unaffected daughters was the same as that of

controls, whether the data were examined by decade age groups or by the comparing regression of steroid levels on age.

DISCUSSION

The results of this study show that unaffected sisters of women with breast cancer have significantly lower plasma DS and AS levels and excrete subnormal amounts of androsterone when compared with women with no family history of the disease. The amounts of urinary aetiocholanolone are also subnormal in sisters aged 30–40 yr. In contrast, no significant abnormalities in plasma or urinary steroid concentrations were found in daughters of women with breast cancer, and both urinary creatinine and 17-OHCS values were almost identical in all groups studied.

The difference between the androgenic status of sisters and controls is small but the fact that similar results were obtained in two quite separate prospective experiments indicates that the difference is a real one. The case is strengthened by the remarkable agreement between the plasma and urinary steroid levels in unaffected daughters and controls. Such reassurance is necessary in a study of this sort where there are so many possible sources of inaccuracy and bias [13].

Pre-menopausal sisters with a familial history of breast cancer have a higher degree of risk than post-menopausal sisters of such cases [3]. This difference in risk is precisely reflected by the results of the androgen assays in that the greatest absolute differences between sisters and controls is found in the younger age groups. It is also not surprising that the overall difference in urinary and plasma steroid values is small since the relative risk of the generality of unaffected sisters is not great [1, 3]. However, certain categories of women are at very high risk. For example, women whose sisters developed bilateral breast cancer at an early age and whose mothers also had the disease may have 20 times the risk of developing breast cancer compared to that for women without a family history [14]. Such cases are rare and while they may be found if studies are based on records from a large hospital, none were discovered in the present investigation which was based on a normal population.

The increased risk of breast cancer in relatives could be attributed to either environmental or genetic factors (or both). Attempts have been made to clarify this point by studying cancer incidence in twins. Macklin [15] reported that of 12 monozygotic twins, 7 were concordant (i.e., breast cancer occurred in both) compared with only 1 of 6 in dizygotic twins. Other evidence for genetic risk in breast cancer is summarized by Oliver [13] and Heston [16].

The results of our study suggest that familial risk in women may be associated with the inheritance of a particular pattern of endocrine function. There is evidence that some aspects of endocrine function are genetically determined, such as plasma cholesterol levels [8], thyroxine binding [9], and oestrogen metabolism [17] (see Shire [10]). There are several reports suggesting that age at puberty is genetically determined (see Reame and Hafez [18]). Kwa and his colleagues [19] have reported recently that plasma prolactin levels are normal in the majority of patients with breast cancer but that high values are found in patients with a family history of cancer.

Obviously, environmental factors affect endocrine function, and even if patterns of hormone production leading to various risk-levels were genetically determined they could be modified considerably by extraneous factors.

The finding of sub-normal androgen levels in sisters, who have a higher risk of breast cancer than that of normal women is in accord with similar findings in the general population [7], in women with benign breast disease [20] and in nuns [21]. Patients with Klinefelter's syndrome have a 60-fold increase in the frequency of breast cancer compared with normal men and, again, such patients excrete sub-normal amounts of urinary androgen metabolites [22]. However, the general association between diminished androgen metabolite excretion and enhanced risk does not hold for normal Japanese women whose risk of breast cancer is lower than that for Caucasians but whose androgen excretion is low [23, 24].

The results of the present study suggest that it would be useful to investigate the genetic element involved in familial risk in terms of hereditability of endocrine function. It would be an attractive hypothesis that the mechanism of genetic risk of breast cancer is mediated through the inheritance of an unfavourable endocrine environment.

Acknowledgements—The authors wish to thank Mr. John Moore and his staff and Miss Marenka Herian for their skilled technical assistance.

REFERENCES

1. ANNOTATION, Breast cancer and heredity. *Lancet* **i,** 935 (1967).
2. D. E. ANDERSON, Familial factors in breast cancer and their implications. *Hosp. Pract.* June, 107 (1972).
3. D. E. ANDERSON, A high-risk group for breast cancer. *Cancer Bull.* (*Tex.*) **25,** 23 (1973).
4. B. MACMAHON, P. COLE and J. B. BROWN, Etiology of human breast cancer. *J. nat. Cancer Inst.* **50,** 21 (1973).
5. F. DE WAARD and E. A. BAANDERS-VAN HALEWIJN, A prospective study in general practice on breast cancer risk in post-menopausal women. *Int. J. Cancer* **14,** 153 (1974).
6. R. D. BULBROOK and J. L. HAYWARD, Abnormal urinary steroid excretion and subsequent breast cancer. *Lancet* **i,** 519 (1967).
7. R. D. BULBROOK, J. L. HAYWARD and C. C. SPICER, Relation between urinary androgen and corticoid excretion and subsequent breast cancer. *Lancet* **ii,** 395 (1971).
8. O. MAYO, S. L. WIESENFELD, G. STAMATOYANNOPOULOS and G. R. FRASER, Genetical influences on serum-cholesterol level. *Lancet* **ii,** 554 (1971).
9. P. J. FIALKOW, E. R. GIBLETT and B. MUSA, Increased serum thyroxine-binding globulin capacity: Inheritance and linkage relationships. *J. clin. Endocr.* **30,** 66 (1970).
10. J. G. M. SHIRE, Endocrine genetics of the adrenal gland. *J. Endocr.* **62,** 173 (1974).
11. D. Y. WANG, R. D. BULBROOK, MARENKA HERIAN and J. L. HAYWARD, Studies on the sulphate esters of dehydroepiandrosterone and androsterone in the blood of women with breast cancer. *Europ. J. Cancer* **10,** 477 (1974).
12. R. D. BULBROOK, J. L. HAYWARD, C. C. SPICER and B. S. THOMAS, A comparison between the urinary excretion of normal women and women with advanced breast cancer. *Lancet* **ii,** 1235 (1962).
13. C. P. OLIVER, Genetic factors in breast cancer. *Proc. nat. Cancer Conf.* **5,** 133 (1964).
14. D. E. ANDERSON, Genetic study of breast cancer: Identification of a high-risk group. *Cancer* (*Phil.*) **34,** 1090 (1974).
15. MADGE T. MACKLIN, An analysis of tumors in monozygous and dizygous twins. *J. Hered.* **31,** 277 (1940).
16. W. E. HESTON, Genetics of mammary cancer. In *Methods in Cancer Research.* Edited by HARRIS BUSCH, Vol. VII, p. 115. Academic Press, New York and London (1973).
17. H. M. LEMON, Genetic predisposition to carcinoma of the breast: Multiple human genotypes for estrogen 16 alpha hydroxylase activity in caucasians. *J. surg. Oncology* **4,** 255 (1972).
18. N. E. REAME and E. S. E. HAFEZ, Hereditary defects affecting fertility. *New Engl. J. Med.* **292,** 675 (1975).
19. H. G. KWA, M. DE JONG-BAKKER, E. ENGELSMAN and F. J. CLETON, Plasma-prolactin in human breast cancer. *Lancet* **i,** 433 (1974).
20. M. J. BRENNAN, R. D. BULBROOK, N. DESHPANDE, D. Y. WANG and J. L. HAYWARD, Urinary and plasma androgens in benign breast disease: Possible relation to breast cancer. *Lancet* **i,** 1076 (1973).
21. P. PITT and G. SARFATY, Androgen metabolites in the urine of average women, nuns and women with regional and metastatic breast cancer. *Cancer Forum* **1,** 27 (1974).
22. O. G. DODGE, A. W. JACKSON and S. MULDAL, Breast cancer and interstitial-cell tumor in a patient with Klinefelter's syndrome. *Cancer* (*Philad.*) **24,** 1027 (1969).
23. R. D. BULBROOK, B. S. THOMAS, J. UTSUNOMIYA and E. HAMAGUCHI, The urinary excretion of 11-deoxy-17-oxosteroids and 17-hydroxycorticosteroids by normal Japanese and British women. *J. Endocr.* **38,** 401 (1967).
24. D. Y. WANG, R. D. BULBROOK, J. L. HAYWARD, S. KUMAOKA, O. ABE and J. UTSUNOMIYA, Blood and urinary ketosteroids and the aetiology of breast cancer in pre-menopausal Japanese and British women. *XIth Int. Cancer Cong.* Vol. 2, p. 159, U.I.C.C. Florence (1974).

Europ. J. Cancer Vol. 11, pp. 879–881. Pergamon Press 1975. Printed in Great Britain

The PHA Response in Hodgkin's Disease

JUDITH BRAEMAN and PAULINE COTTAM
Tenovus Laboratories, Velindre Hospital, Cardiff, Great Britain

Abstract—*The lymphocyte response of 94 patients with Hodgkin's disease has been studied using phytohaemagglutinin (PHA). Fifty-one patients were tested before treatment; 43 patients were tested after radiotherapy and/or chemotherapy; 15 patients were studied both before and after treatment; 28 patients were studied when in remission after treatment.*

The PHA response of the patients was significantly lower than that of the controls, $P \ll 0{\cdot}001$. There was no significant difference between the PHA results when correlated with the clinical stage of the disease. There was a lower result in those patients with systemic symptoms compared with those without systemic symptoms. This result was significant, $P = 0{\cdot}05$.

INTRODUCTION

OTHER workers [1] agree that the PHA response is significantly reduced in Hodgkin's disease and found a wide variation in results in both the patients and the controls. Winkelstein *et al.* [2] also found the PHA response impaired in untreated Hodgkin's disease and the response was reduced when the patients were in relapse. Han [3] found that the PHA response varied greatly among patients with active Hodgkin's disease and was affected by a variety of factors related to the disease and the treatment. Han found that the PHA response was normal among patients in remission, except where depression could be attributed to antecedent therapy. However, Levy and Kaplan [4] concluded that there was an impaired response in treated patients in remission and the PHA response of patients was lower than that of controls. These authors, however, found that the later stages of the disease had lower results, but there was no correlation between the PHA response and the presence of "B" symptoms, i.e. fever, weight loss, night sweats, etc. Winkelstein *et al.* [2] found a lower response only in Stage IV disease and their results were not altered by the presence of systemic symptoms, histology, or lymphocyte count. The present study agrees with that of Corder *et al.* [5] who found no correlation between PHA transformation at the time of diagnosis and the clinical course of the disease. They did, however, find that Stage IV patients had a lower result than Stage I, II and III. In the present study the authors have found that there is no significant difference between the PHA response of the patients with various stages of Hodgkin's disease, although patients with systemic symptoms have a lower response than those without systemic symptoms. The response of the patients was significantly lower than that of the controls. Patients were staged by pathological criteria.

Accepted 10 September 1975.

MATERIAL AND METHODS

The method of lymphocyte transformation was as described in Braeman and Deeley [6]. 0·25 ml PHA (Wellcome reagent grade) was added to each culture and a duplicate culture was set up without PHA. Results were assessed morphologically and the number of transformed cells and mitoses expressed as a percentage of the total number of lymphoid cells counted.

RESULTS

Results are shown in Table 1. Controls were healthy hospital staff with an average age of 30 yr. The average age of the patients was 43 yr. The sex distribution of the patients and controls was identical. The difference between the PHA

result of the controls and the untreated patients was significant, $t = 7{\cdot}1$, $P \ll 0{\cdot}001$. The difference between the controls and all cases of Hodgkin's disease, both treated and untreated, was also significant, $t = 8{\cdot}1$, $P \ll 0{\cdot}001$. There was no significant difference between the PHA response of the treated and the untreated cases $t = 1{\cdot}2$, $P = 0{\cdot}1$.

Twenty-eight patients were studied in remission when having no treatment, but these patients did not have a significantly higher result than those with active disease, $t = 1{\cdot}4$, $P = 0{\cdot}1$.

Since there was no significant difference between the stages of the disease the patients were grouped according to whether they had systemic symptoms or not. The difference between the PHA response of the "A" and "B" patients was significant, $t = 2{\cdot}0$, $P = 0{\cdot}05$.

No other correlations could be found, e.g. between PHA response and age, sex, histology of the disease, extralymphatic involvement, or type of treatment.

Table 1. *PHA response in Hodgkin's disease*

	Mean PHA response	S.E.M.	Range	Number of patients
Controls	68.4%	1.6	51.2–92.8%	34
Untreated Hodgkin's disease	37.6%	3.3	nil–80.8%	51
Treated Hodgkin's disease	32.0%	3.4	1.5–78.7%	43
Treated and untreated Hodgkin's disease	35.3%	2.4	nil–80.8%	94
Stage I untreated	35.2%	7.2	4.1–62.7%	12
Stage II untreated	41.8%	6.6	7.6–74.1%	12
Stage III untreated	40.4%	6.9	4.7–77.0%	13
Stage IV untreated	39.3%	6.4	6.4–70.8%	12
All stages "A"	42.8%	4.0	3.3–79.5%	31
All stages "B"	29.6%	5.5	nil–80.8%	20
In remission	35.0%	4.0	3.3–78.7%	28

DISCUSSION

These results agree with other workers (see Introduction) who claim that there is an impairment of the lymphocyte response in Hodgkin's disease. Han and Sokal [7] found that treatment of the disease either by radiotherapy or by chemotherapy lowered the PHA response.

In the present study there was no correlation between the stage of the disease and the PHA response, but patients with systemic symptoms had a lower response regardless of their clinical staging. However, the range of response in each sub-group was so great that for an individual patient a particular response was of no predictive value. The observation applies to the group as a whole and is not necessarily patient specific.

No transformation was observed in cultures without PHA either in controls or in patients. However, the method used here does not take into account the possible inhibitory effect of the patient's plasma and whether this plasma inhibitory factor appears and disappears during the course of the disease.

Although PHA transformation is reduced in Hodgkin's disease it is not useful as a prognostic indicator. This agrees with Corder *et al.* [5] who found that PHA transformation appears to be a general index of immunological status, but is not of proven value as a prognostic sign in Hodgkin's disease.

Acknowledgement—The authors wish to thank the Tenovus Organisation for financial support.

REFERENCES

1. J. M. LANG, F. OBERLING, P. BIGEL, S. MAYER and R. WAITZ, Lymphocyte reactivity to phytohaemagglutinin. *Biomed. Express* **21,** 372 (1974).
2. A. WINKELSTEIN, J. M. MIKULLA, G. P. SARTIANO and L. D. ELLIS, Cellular immunity in Hodgkin's disease. *Cancer (Philad.)* **34,** 549 (1974).
3. T. HAN, *In vitro* lymphocyte blastogenesis. *J. Surg. Oncology* **5,** 567 (1973).
4. R. LEVY and H. S. KAPLAN, Impaired lymphocyte function in untreated Hodgkin's disease. *New Engl. J. Med.* **290,** 181 (1974).
5. M. P. CORDER, R. C. YOUNG, R. S. BROWN and V. T. DEVITA, Phytohaemagglutinin-induced lymphocyte transformation: The relationship to prognosis of Hodgkin's disease. *Blood* **39,** 595 (1972).
6. J. BRAEMAN and T. J. DEELEY, Radiotherapy and the immune response in cancer of the lung. *Brit. J. Radiol.* **46,** 446 (1973).
7. T. HAN and J. E. SOKAL, Lymphocyte response to PHA in Hodgkin's disease. *Amer. J. Med.* **48,** 728 (1970).

Europ. J. Cancer Vol. 11, pp. 883–889. Pergamon Press 1975. Printed in Great Britain

Liposomes Containing ^{3}H-Actinomycin D. Differential Tissue Distribution by Varying the Mode of Drug Incorporation*

YUEH-ERH RAHMAN, WALTER E. KISIELESKI, EVELYN M. BUESS and ELIZABETH A. CERNY

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

Abstract—*Actinomycin D was encapsulated within liposomes in two different ways. When the drug was incorporated in the lipid phase, as part of the membrane bi-layers of the liposomes, they were called "lipid phase liposomes" (LPL); when the drug was incorporated in the aqueous solution in the center and between the lipid bi-layers of the liposomes, they were called "aqueous phase liposomes" (APL). Distributions of ^{3}H-actinomycin D in tissues of mice were determined from 15 minutes to 48 hours after a single intravenous injection of either LPL, APL, or nonencapsulated actinomycin D. Marked differences in tissue distribution were shown. ^{3}H-actinomycin D incorporated in LPL showed high concentrations in the lungs and low concentrations in the intestinal wall; whereas the reverse was found with APL. The spleen and bone marrow of mice receiving LPL showed an increase in ^{3}H-radioactivity between 3 and 24 hr after injection that was closely correlated with a concomitant decrease in activity in the liver and lungs. Mice receiving either APL or nonencapsulated actinomycin D had higher levels of the drug in the blood, kidneys and intestinal wall than did mice receiving LPL. In vitro studies showed that ^{3}H-actinomycin D leaked out from APL significantly faster than that from LPL. We have demonstrated that the tissue distribution of a drug can be modified not only by liposome encapsulation, but also by varying the way of incorporating the drug within liposomes, and thereby altering the surface properties. The two forms of incorporating drugs within liposomes are potentially useful to direct anti-tumor agents to specific tumor bearing tissues.*

INTRODUCTION

Most antitumor agents are highly toxic to animals. This toxicity could be alleviated if selected tissue distribution of the agent could be obtained. In earlier studies with chelating agents encapsulated within liposomes, i.e., multi-layered lipid spherules, we found that partial selective tissue uptake of the chelating agent can be obtained, depending on the lipid constituents of the liposomes [1, 2]. Therefore, we have attempted to direct the tissue deposition of antitumor agents by liposome encapsulation.

Actinomycin D, an antibiotic with known inhibitory effects on certain tumors [3–6], has been encapsulated within liposomes. Liposome-encapsulated actinomycin D, when given either i.v. or i.p. to mice, was found to be much less toxic than the nonencapsulated, free form [7]. It was also shown that mice inoculated with Ehrlich ascites tumor cells survived significantly longer when given single or multiple injections of liposome-encapsulated actinomycin D [7].

Since actinomycin D is soluble both in aqueous media and in lipid solvents, it can be incorporated in either the aqueous or the lipid phase of the liposomes. In the first case, in

Accepted 29 August 1975.

*Work supported by the U.S. Energy Research and Development Administration.

which the actinomycin D is in the aqueous solution in the center and between the lipid bi-layers of the liposomes, the liposomes are designated "aqueous phase liposomes" (APL). When the actinomycin D is incorporated as part of the lipid bi-layers [8] which form the outer shells of the liposomes, they are called "lipid phase liposomes" (LPL). These two kinds of liposomes containing actinomycin D are different in their surface properties. The present report concerns a comparative study of APL, LPL and non-encapsulated actinomycin D, with emphasis on their pattern of distribution in various mouse tisses after i.v. injections.

MATERIAL AND METHODS

Phosphatidylcholine (PC) and cholesterol were respectively obtained from Sigma Chem. Co., St. Louis, Mo. and Applied Sciences Laboratories, Inc., State College, Pa. Actinomycin D (Cosmegen) was from Merck, Sharp and Dohme, West Point, Pa. Radioactive ^{3}H-actinomycin D, with a specific activity of 3·0 Ci/mmole, was from Amersham/Searle Corp., Arlington Hts., Ill.

Preparation of actinomycin D liposomes

APL were prepared with a mixture of 3·0 mg of PC and 3·0 mg of cholesterol dissolved in chloroform and dried in a thin film inside of a round bottom flask. Actinomycin D, dissolved in 8 mM $CaCl_2$ at a concentration of 0·5 mg in 1 ml, was slowly added to the dried film of lecithin–cholesterol mixture with immediate and constant stirring. LPL were prepared by dissolving a mixture of 4·0 mg of PC, 2·0 mg of cholesterol and 0·5 mg of actinomycin D in chloroform. After being dried to a deep yellow film, an 8 mM $CaCl_2$ solution was then slowly added, and liposomes were formed by immediate stirring. The subsequent steps in the preparation of both forms of liposomes are essentially the same as described previously [1, 9]. For both APL and LPL, a suitable amount of radioactive ^{3}H-actinomycin D was added to the actinomycin D solution so that each mouse received about $2{\cdot}0 \times 10^5$ dis/min of radioactivity together with a nonlethal dose of actinomycin D of approximately 350 μg/kg. We should emphasize that high purity of PC is essential for successful liposome preparations. Liposomes made of either partially degraded or oxidized PC tend to aggregate into small clumps that render the preparations unsuitable for injection into animals [1].

Injection of ^{3}H-actinomycin D in liposome-encapsulated or nonencapsulated forms

Groups of 24 female Carworth Farm (CF 1) mice, 3 months of age, and weighing between 25–30 g were used. ^{3}H-actinomycin D was given in one of the following forms: (1) actinomycin D in nonencapsulated, free form; (2) actinomycin D in APL; (3) actinomycin D in LPL. Each mouse received a single intravenous injection of ^{3}H-actinomycin D in a volume of 0·40 ml; and 8 mM $CaCl_2$ solution, was used as injection vehicle for all the three forms of actinomycin D. Three groups of 4 mice each were killed at the following time intervals after the injections: 15 min, 0·5, 1, 3, 6, 24 and 48 hr. Mice were killed by an intravenous injection of an anesthetic dose of sodium pentobarbital (0·04 ml/mouse), followed by exsanguination. Samples of blood and of various tissues were removed immediately for ^{3}H-radioactivity determinations.

Preparation of mouse tissues

Blood samples, lungs, spleen, liver, kidneys, intestinal samples and a standard bone marrow sample from both tibias [10] were taken for ^{3}H analysis. At 15 min after injection, the following additional tissues were also taken for analysis: thymus, brain, urinary bladder including the urine, and sample of muscle and skin. Blood from a jugular vein was collected with a capillary micropipette. Duplicate samples of 100 μl each were absorbed into a small ball of cotton and allowed to air dry before being wrapped in a filter paper (Whatman No. 1, 4·25 cm dia). After removal, the spleen, lungs, brain, thymus, urinary bladder, each kidney, samples of muscle and skin each weighing between 100 and 150 mg were removed, weighed, wrapped in a filter paper, and air dried. And three 150–200 mg slices of liver were also wrapped separately for analysis. The total liver radioactivity was calculated as the mean counts per mg from the slices, times the total liver weight. The slices were always taken from the standard locations which preliminary determinations had shown to be representative of the whole. Three segments of the small intestine were excised and weighed after the contents had been extruded. Each segment was wrapped separately in a filter paper for radioactivity analysis. The bone marrow samples were washed out with 0·25 ml of water directly onto a small ball of cotton and then treated in the same manner as that described for the blood samples. The amount of ^{3}H-radioactivity in the total marrow was estimated by multiplying the activity in the two tibial segments by a

factor of 44 [11]. The absorbent paper used to collect urine samples was cut out, folded, and then wrapped in filter paper. The radioactivity in the total blood was calculated from two 100 μl samples and the estimated mouse blood volume of 2 cm^3.

Analysis of ^3H-radioactivity

Each air dried biological sample, all wrapped in Whatman No. 1 filter paper, was pressed in a pellet press (Parr Instruments Co., Moline, Ill.). After automatic combustion in a Packard 305 sample oxidizer and addition of scintillation fluid, each sample was analysed for ^3H-radioactivity in a Beckman liquid scintillation counter, Model LS-200B. The oxidation of each tissue sample was followed at least once by combustion of an empty filter paper to remove the residual activity in the system. In the case of tissues known to contain high levels of radioactivity, several successive empty filter papers were oxidized. The radioactivity obtained from these filter papers was added to that of the preceding tissue sample. Calculation of the amount of radioactivity injected was based on analysis of triplicate samples of 0·40 ml, measured with the same syringe that was used for injection. These samples were prepared for radioanalysis in the same manner as the blood and bone marrow.

In vitro *studies of release of ^3H-actinomycin D from APL and LPL*

Preparations of APL or LPL containing ^3H-actinomycin D, suspended either in 8 mM $CaCl_2$ or in culture medium (Eagle's medium containing 15% foetal calf serum), were incubated at 37°C in a water bath, with constant slow shaking. One ml aliquots from each preparation were then taken at hourly intervals. They were centrifuged in a Sorvall GLC-1 centrifuge at 2000 rev/min for 5 min. The release of ^3H-actinomycin D from the liposome was calculated from the ^3H-radioactivity found in the supernatant compared to the total radioactivity in the sample before centrifugation.

RESULTS

^3H-actinomycin D distribution in mouse tissues

Figure 1 shows the distribution in blood, lungs, spleen, liver, kidneys, and bone marrow of ^3H-actinomycin D, administered either in LPL, APL, or in nonencapsulated form. The disappearance of ^3H-actinomycin D from the blood was very rapid (Fig. 1(a)). Half of an hour after injection, the highest blood level (almost 2% of the injected radioactivity) was found in APL-injected mice, and the lowest level (about 0·5%) was found in the LPL-injected mice. A small but significant rise in the blood level of ^3H-actinomycin D in LPL-injected mice occurred between 24 and 48 hr after the injection (Fig. 1(a)).

There was a striking difference in the lung uptake of the different forms of ^3H-actinomycin D (Fig. 1(b)). In mice receiving the LPL injection, about 40% of the total injected dose was found in the lungs half of an hour after the injection, and the high concentration was maintained at least up to 6 hr; 24 hr after the injection, 10% of the total injected radioactivity was still retained in the lungs. In contrast, mice injected either with APL or with nonencapsulated ^3H-actinomycin D had very low concentrations of the drug in the lungs (Fig. 1(b)), the mice receiving the nonencapsulated form consistently had the lowest levels.

The initial uptake of ^3H-actinomycin D in the spleen was low in all three groups of mice. However, the spleens of LPL-injected mice showed a significant increase in ^3H-actinomycin D concentration at 3 hr and at 24 hr reached a peak concentration of about 20% of the total injected dose. The groups of mice receiving APL or free actinomycin D had very low concentrations of the drug in the spleen throughout the 48 hr after the injection.

The LPL-injected mice also had a higher uptake of ^3H-actinomycin D in the liver than mice injected either with APL or free actinomycin D (Fig. 1(d)). In LPL-injected mice, the level of the drug in the liver was maintained at least up to 6 hr, but then decreased.

In the kidneys the highest concentration of ^3H-actinomycin D occurred in mice injected with APL or free actinomycin D. The peak concentration was found at one half of an hour. The LPL-injected mice had very low concentrations of ^3H-actinomycin D in the kidneys throughout the experimental period (Fig. 1(e)).

Although the initial uptake of ^3H-actinomycin D in bone marrow of all three groups of mice was similar, the uptake in the LPL-injected mice continued to increase for 24 hr reaching a peak concentration of about 10%. In free actinomycin D and LPL-injected mice, there was a significant decrease of radioactivity in the bone marrow between 24 and 48 hr; while a constant level was maintained in APL-injected mice (Fig. 1(f)).

In the intestinal wall, mice injected either with APL or with nonencapsulated actinomycin D have an initial concentration (one-

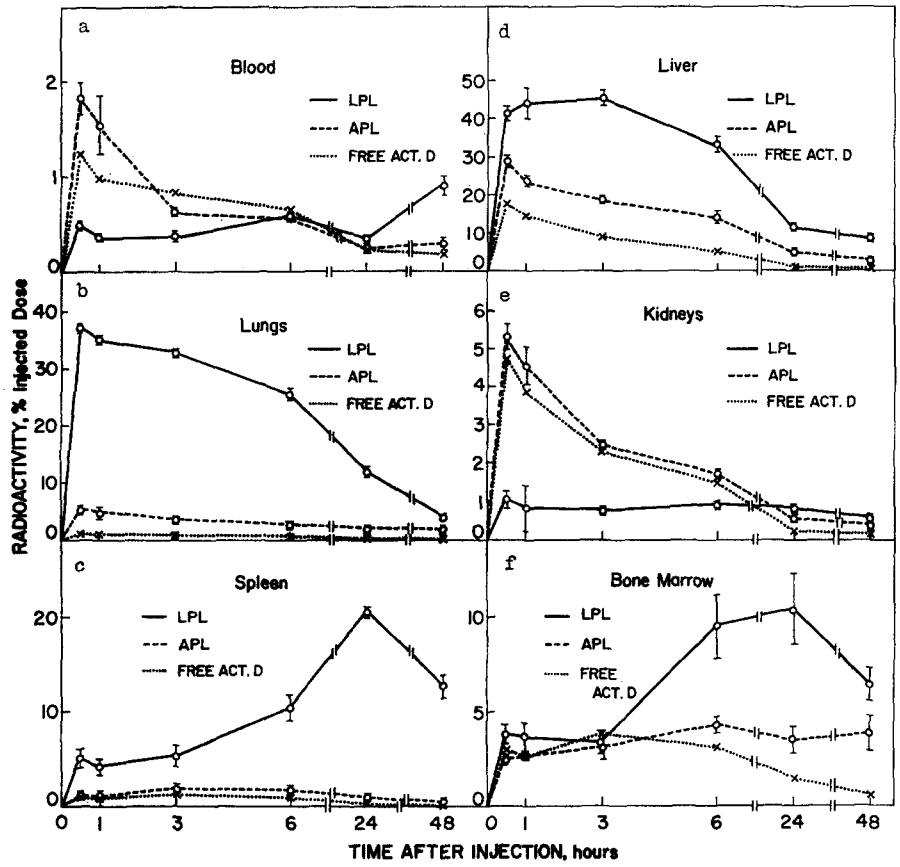


Fig. 1. Percent injected dose of ^{3}H-actinomycin D in: (a) *blood,* (b) *lungs,* (c) *spleen,* (d) *liver,* (e) *kidneys and* (f) *bone marrow. A single intravenous injection of one of the following: LPL (lipid phase liposomes), APL (aqueous phase liposomes), or free act. D was given to each mouse. The radioactivity in the blood (total) was calculated from two* 100 *μl samples and the estimated mouse blood volume of* 2 *cm^3. The level in the bone marrow (total) was estimated by multiplying the radioactivity in the two tibial segments by a factor of* 44 [11]. *Each point is the mean value from* 4 *mice. Vertical bars represent* 2 *standard errors of the mean.*

half to one hour) about five-times that in LPL-injected mice (Fig. 2). The radioactivity then decreases so that between 24 and 48 hr post injection, the levels of radioactivity in all three groups of mice are not significantly different (Fig. 2).

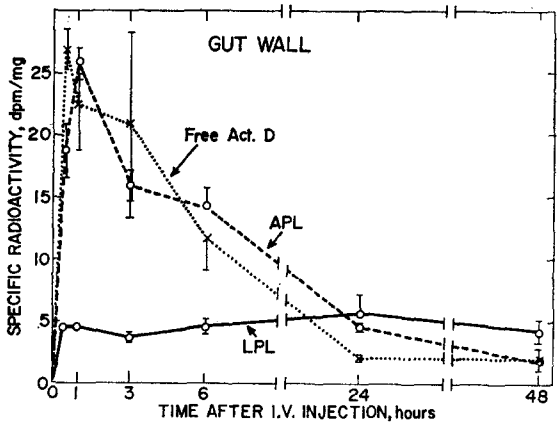


Fig. 2. Concentration of ^{3}H-actinomycin D (dis/min per mg) in the gut wall. Each point is the mean value from 4 *mice. Vertical bars represent* 2 *standard errors of the mean.*

Table 1 shows the ^{3}H-actinomycin D content of a few additional tissues, taken 15 min after injection. In all three groups of mice, low concentrations of ^{3}H-radioactivity were found in thymus, brain, urinary bladder, muscle and skin. However, the mice injected with non-encapsulated, free actinomycin D had higher concentrations of ^{3}H-radioactivity in the

Table 1. Tissue content of ^{3}H-actinomycin D (% injected dose, 15 min after injection) The data represent the averages obtained from four animals in each group

TISSUE	FREE ACT. D.	APL	LPL
Thymus	0.05	0.08	0.01
Brain	0.03	0.07	0.20
Urinary Bladder	0.05	0.31	0.03
Muscle*	12.75	5.02	5.37
Skin*	8.38	3.35	3.94

* For muscle and skin, the data represent dis/min per mg of tissue.

samples of muscle and skin compared to those of mice injected with either APL or LPL.

In vitro *release of ^{3}H-actinomycin D from liposomes*

The different rates of ^{3}H-actinomycin D release from LPL and APL are shown in Fig. 3. The liposomes were incubated at 37°C, with slow shaking. When incubated in 8 mM $CaCl_2$, LPL released negligible radioactivity up to 4 hr, and no significant difference was found when incubated in culture medium (Eagle's medium containing 15% foetal calf serum). In contrast, APL released more than 60% of the total radioactivity within an hour. When APL were incubated in culture medium, there was slightly but significantly less release of radioactivity. The amount of radioactivity released from APL at 0 time (Fig. 3) was partially due to the additional centrifugation and manipulation inflected during the experimental procedure.

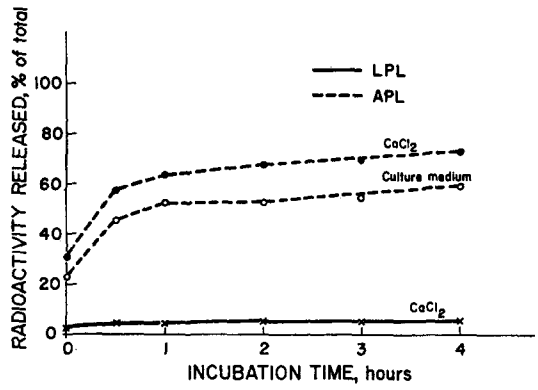


Fig. 3. In vitro *release of ^{3}H-actinomycin D (% of total) from LPL and APL. The liposomes were incubated at 37°C with slow shaking.* ●---, *APL incubated in 8 mM $CaCl_2$;* ○---, *APL incubated in culture medium (Eagle's medium containing 15% foetal calf serum);* X——, *LPL incubated in 8 mM $CaCl_2$.*

DISCUSSION

The tissue distribution of a given drug can be modified by encapsulating it within liposomes. This modification has been demonstrated by studies on a liposome-encapsulated chelating agent, namely ethylenediaminetetraacetic acid [1]. The present report provides evidence that liposome encapsulation can be used to modify tissue distribution of other drugs, such as the anti-tumor agent actinomycin D.

We have further demonstrated that by varying the way of incorporating the drug within liposomes, i.e., either in the aqueous or the lipid phase of the liposomes, marked differences in tissue distribution can be obtained. However, in comparison to results obtained with nonencapsulated, free actinomycin D, the incorporation of this drug in the aqueous phase of the liposomes gave a quantitative difference only in their tissue distribution. Higher actinomycin D concentrations were generally found in tissues of mice receiving APL injections.

Actinomycin D incorporated in the lipid phase shows high concentrations in the lungs, and low concentrations in the intestinal wall; whereas the reverse is true when it is in the aqueous phase. The different uptake in the lungs is not due to a size difference in the two forms of liposomes; the sizes are comparable when determined by a previously described method [1]. Studies on actinomycin D concentrations in mouse tissues by Schwartz, Sodergren and Ambaye [12] had shown a moderately high initial concentration in the small intestine, which was maintained only up to 3 hr after the injection of actinomycin D. Our present results are similar to theirs. The rapidly released ^{3}H-actinomycin D from within the APL would presumably be distributed in a similar manner as the nonencapsulated, free form of actinomycin D. Therefore, it is not surprising to find relatively high concentrations of radioactivity in the intestinal wall of mice injected with APL. What seems surprising, though, are the very low concentrations of ^{3}H-actinomycin D in the intestines of mice injected with LPL, and the very high concentrations in the lungs. In LPL, actinomycin D is incorporated within the lipid membranes which form the liposomes, so that one can assume that under such conditions, the surface properties of the actinomycin D molecule has been altered due to its bindings to the liposomal lipids. Consequently, the affinity between this drug and the tissues may also be modified. However, the chemical nature of the binding between actinomycin D and the liposomal lipids is not known.

The tissue distribution is quite similar in mice injected either with nonencapsulated, free actinomycin D or APL, while the levels of ^{3}H-actinomycin D concentration are generally higher in tissues of mice injected with APL (Fig. 1 and Table 1). Again, the rapid release of actinomycin D from APL, as shown by *in vitro* studies (Fig. 3), would readily explain the similar distribution patterns in tissues of mice receiving either free actinomycin D or APL.

The relatively high initial amounts of ^{3}H-actinomycin D found in kidneys of mice receiving either free actinomycin D or APL are due to the rapid excretion of the drug in its free form.

The spleen and bone marrow of mice receiving LPL show an increasing concentration of ^{3}H-radioactivity between 3 and 24 hr after injection (Fig. 1(c)). This increase is closely correlated with a concomitant decrease in actinomycin D concentration in the liver and lungs. Therefore, the increased ^{3}H-radioactivity observed in the spleen and bone marrow, within this interval, seems to be the result of a translocation phenomenon between the liver and lungs on one hand and the spleen and marrow on the other. Actinomycin D is not being metabolized in animal tissues at least up to 24 hr after injection [12, 13], the ^{3}H-radioactivity found between 3 and 24 hr in the spleen and bone marrow therefore could still represent intact molecules of actinomycin D. Liposome encapsulation of the drug could further protect it from being degraded within cells.

In all three groups of mice, the liver has the highest ^{3}H-actinomycin D activity among the tissues examined. In mice receiving either free actinomycin D or APL, the peak concentration in liver is at half an hour after injection; while the mice receiving LPL have the highest activity between one and three hours. Electron microscopic studies have shown that Kupffer cells as well as hepatocytes can rapidly take up liposomes containing chelating agents [14]. Preliminary results have indicated that both of these two cell types can also take up APL and LPL.

The *in vitro* study of ^{3}H-actinomycin D release from liposomes (Fig. 3) shows that the release from APL is very rapid compared to that from LPL. Even when APL were incubated in a culture medium more closely approximating the conditions in the blood, more than 40% of the total ^{3}H-actinomycin D leaks out of the liposomes after only one-half hour of incubation (Fig. 3). The binding between actinomycin D and the liposomal lipids in LPL apparently retards the release of the drug from liposomes, thus preventing the drug from leaking out from liposomes at least during their passage in the blood stream. This phenomenon of slow drug release from LPL could be useful in future clinical applications.

One of the most challenging problems in chemotherapy is to deliver a given drug specifically to a cell type within the target tissue. We have evidence that liposome uptake in tissues is not limited to phagocytic cells. As we have reported, parenchymal cells as well as Kupffer cells of the liver readily take up liposomes [14]. In addition, preliminary electron microscopic observations of mouse lung, after APL or LPL injections, indicate that several cell types incorporate liposomes. Therefore, higher tissue concentrations of a given drug obtained by liposome encapsulation, increase the potential for a higher drug concentration in any specific cell type, including tumor cells, within that tissue.

Another aspect of the present study is that liposome encapsulation can be used as a tool to unravel problems related to tissue specificity, e.g., to learn the specific binding sites of a given tissue to liposomes. It should be emphasized, however, that before the problems of tissue specificity can be approached, the surface properties of APL and LPL, as well as the nature of the chemical binding between actinomycin D and the liposomal lipids, must be first understood. Furthermore, the quantitative uptake of liposomes by different cell types within a given tissue should be determined. We are currently investigating such problems.

Acknowledgement—The authors wish to thank Ms. Gloria A. Kokaisl for her valuable assistance in the early phase of this work.

REFERENCES

1. Y. E. Rahman, M. W. Rosenthal, E. A. Cerny and E. S. Moretti, Preparation and prolonged tissue retention of liposome-encapsulated chelating agents. *J. lab. clin. Med.* **83,** 640 (1974).
2. M. M. Jonah, E. A. Cerny and Y. E. Rahman, Tissue distribution of EDTA encapsulated within liposomes of varying surface properties. *Biochim. biophys. Acta* **401,** 366 (1975).
3. S. Farber, Clinical and biological studies with actinomycins. *Ciba Found. Symp. Amino Acids Peptides Antimetab. Activity*, pp. 138–148 (1959).
4. G. T. Ross, L. L. Stolbach and R. Hertz, Actinomycin D in the treatment of methotrexate resistant trophoblastic disease in women. *Cancer Res.* **22,** 1015 (1962).
5. H. R. Oettgen, P. Clifford and D. Burkitt, Malignant lymphoma involving the jaw in African children: Treatment with alkylating agents and actinomycin D. *Cancer Chemother. Rept.* **28,** 25 (1963).

6. J. H. COLEBATCH, R. HOWARD, A. L. WILLIAMS, G. R. KURRLE and A. C. L. Clark, Results of actinomycin D therapy for Wilm's tumor. *Acta Un. int. Cancr. (N.Y.)* **20,** 491 (1964).
7. Y. E. RAHMAN, E. A. CERNY, S. L. TOLLAKSEN, B. J. WRIGHT, S. L. NANCE and J. F. THOMSON, Liposome-encapsulated actinomycin D: Potential in cancer chemotherapy. *Proc. Soc. Exp. Biol. Med.* **146,** 1173 (1974).
8. G. GREGORIADIS, Drug entrapment in liposomes. *FEBS Letters* **36,** 292 (1973).
9. Y. E. RAHMAN, M. W. ROSENTHAL and E. A. CERNY, Intracellular plutonium: Removal by liposome-encapsulated chelating agent. *Science* **180,** 300 (1973).
10. M. W. ROSENTHAL, J. H. MARSHALL and A. LINDENBAUM, Autoradiographic and radiochemical studies of the effect of colloidal state of intravenously injected plutonium on its distribution in bone and marrow, in *Diagnosis and Treatment of Deposited Radionuclides,* (Edited by H. A. KORNBERG and W. D. NORWOOD), Excerpta Medica Foundation, Amsterdam, pp. 73–80 (1968).
11. M. W. ROSENTHAL, E. S. MORETTI and J. J. RUSSELL, Marrow deposition and distribution of monomeric and polymeric ^{239}Pu in the mouse, estimated by use of ^{59}Fe. *Health Phys.* **22,** 743 (1972).
12. H. S. SCHWARTZ, J. E. SODERGREN and R. Y. AMBAYE, Actinomycin D: Drug concentrations and actions in mouse tissues and tumors. *Cancer Res.* **28,** 192 (1968).
13. H. WEISSBACH, B. REDFIELD, T. O'CONNOR and M. A. CHIRIGOS, Studies on the disposition of actinomycin D-3H in virus-infected and tumor-bearing mice. *Cancer Res.* **26,** 1832 (1966).
14. Y. E. RAHMAN and B. J. WRIGHT, Liposomes containing chelating agents. Cellular penetration and a possible mechanism of metal removal. *J. Cell Biol.* **65,** 112 (1975).

Europ. J. Cancer Vol. 11, pp. 891–901. Pergamon Press 1975. Printed in Great Britain

Response of Mouse Skin and the C3HBA Mammary Carcinoma of the C3H Mouse to X-rays and Cyclotron Neutrons: Effect of Mixed Neutron–Photon Fractionation Schemes*

JANET S. R. NELSON, RITA E. CARPENTER and ROBERT G. PARKER

Division of Radiation Oncology, School of Medicine, University of Washington, Seattle, Washington, 98195, *U.S.A.*

Abstract—*The response of mouse foot skin and the C3HBA mammary adenocarcinoma of the C3H mouse to* 250 *kVp X-rays and cyclotron-produced neutrons* (8 *MeV mean energy*) *has been studied. Neutrons or X-rays were given in single fractions* (*fx*); 2 *fx* 24 *hr apart; 2 fx* 96 *hr apart; and* 5 *fx in* 5 *days. Two fx of neutrons* + 3 *fx of X-rays in* 5 *days also were given in the sequence n-n-x-x-x or n-x-x-x-n. When neutron only schemes were compared to X-ray only schemes, RBE's for early skin damage and foot deformity at* 6 *months post-irradiation increased with increasing number of fractions. The RBE for* 2 *fx of neutrons* + 3 *fx of X-rays* (*total dose of neutrons* + *X-rays*) *relative to* 5 *fx of X-rays was* 1·3 *for both mixed fraction sequences. Tumor growth delay following single and fractionated X-rays suggested an hypoxic fraction of cells which undergoes extensive and long lasting reoxygenation, a situation in which fractionated neutrons might not be expected to show an advantage in terms of tumor damage relative to normal tissue injury. Therapeutic gain factors* (*TGF = tumor RBE/skin RBE*) *for single fractions ranged from* 1·6 *to* 1·8 *depending on dose level while TGF's for fractionated neutrons varied from* 0·73 *for* 2 *fx* 24 *hr apart to* 0·96 *for* 5 *fx in* 5 *days. For both the n-n-x-x-x and n-x-x-x-n fractionation schemes a TGF of* 1·1 *suggests that the mixed fractionation schemes may be slightly better than neutron only schemes in this tumor system.*

INTRODUCTION

NEUTRON THERAPY of human cancer has necessarily been preceded by experiments intended to biologically characterize different neutron beams [1–4]. Limited medical access to cyclotron-produced neutron beams has resulted in patients being treated 3 or only 2 times per week, rather than the conventional 5 times per week. This has indicated that the relative importance of the fractionation scheme, as well as radiation quality, in modifying treatment results also needs to be studied and the response of experimental tumors and normal tissue to various neutron only and X-ray only fractionation schemes has been reported [5–8].

This paper describes the response of mouse skin and the C3HBA mammary adenocarcinoma of the C3H mouse to 250 kVp X-rays and 8 MeV (mean energy) neutrons produced by the University of Washington cyclotron, using single and multiple fractions of X-rays or neutrons as well as mixed neutron-photon fractionated radiation. The tumor chosen, a

Accepted 8 August 1975.

*This investigation was supported by Research Grant # CA-12441-04 from the National Cancer Institute, National Institute of Health.

C3H mammary tumor, is one in which neutrons might be expected not to show an advantage because other mammary tumors in this mouse show rapid and extensive reoxygenation, rendering judiciously fractionated X-rays as effective as neutrons [7, 8]. We chose to examine mixed fractionation schemes, utilizing 2 initial neutron fractions followed by 3 fractions of X-rays over a five day period (n-n-x-x-x) or 2 fractions of neutrons on the first and fifth days, with X-rays on the intervening days (n-x-x-x-n). The rationale was that, in a rapidly reoxygenating tumor, one or two initial neutron fractions could efficiently kill hypoxic tumor cells while subsequent fractions of X-rays would be more effective after the initiation of reoxygenation; a mixed scheme with a final neutron fraction (n-x-x-x-n) would also allow more effective killing of any resistent hypoxic foci left at the end of treatment. Such treatment schemes might produce an increase in therapeutic ratio while taking into account limited cyclotron access for patient treatment.

MATERIAL AND METHODS

Radiation sources and dosimetry

X-irradiations were performed using a General Electric Maxitron 250; the settings were 250 kVp, 30 mA, 0·5 mm Cu + 1·0 mm Al added filtration, target to specimen distance (TSD) 50 cm, dose rate 100 R/min. Irradiations were performed with a horizontal beam and an 8 × 8 cm field defined by a $\frac{1}{4}$ in. thick lead shield. Mice were irradiated while taped to a vertical Lucite board placed directly behind the shield. Dosimetry was done with a Victoreen air equivalent exposure meter using a 250 R chamber and a rad/Roentgen conversion factor of 0·95. Measurements were made with the mouse holder in place to include contributions from backscatter. LiF and $LiBO_4$ thermoluminescent dosimenters were also used to verify absorbed dose in several experiments. These dosimeters were taped to the feet of mice, in the case of skin irradiations, or placed under the skin adjacent to the irradiated tumors.

Neutron irradiations were performed at the University of Washington Cyclotron. The neutron beam was produced by bombarding a 1·5 mm thick beryllium target with 21 MeV deuterons, yielding a neutron beam with mean energy of 8 MeV and an energy spread of 0–25 MeV. Dosimetry was performed with tissue equivalent (TE) ionization chambers (Shonka type A 150 plastic) filled with TE gas [4]. The doses are expressed as total rads of neutrons plus gammas; gamma contamination was 8% in air at the mouse positions. The neutron beam was defined by a Cu Al bronze collimator in the initial skin experiment and animals were irradiated at a TSD of 70 cm and a dose rate of 100–125 rad/min. Subsequent experiments employed a borated water-extended polyester (B/WEP) collimator which defined an 18 × 18 cm field at a TSD of 108 cm; the dose rate was 40–50 rads/min. Mice were irradiated while taped to a vertical Lucite board which was placed 2 cm from the collimator exit.

Skin irradiations

Female BALB/c mice (Jackson Laboratories or Charles River) were anesthetized with Nembutal (sodium pentobarbital, 60 μg/gm body weight) and one rear foot was irradiated with the animals' bodies shielded by the lead shield in the case of X-irradiation and by the bronze or B/WEP collimator in the case of neutron irradiations. Six to nine mice were irradiated at each dose and the feet were immersed in tissue equivalent fluid ($C_5H_{40}O_{18}N$) [9] to provide for secondary charged particle equilibrium in the neutron irradiation. No significant difference in skin response to single doses of neutrons was seen with the two collimators. C3H/HeJ male mice, 6–10 weeks old (Jackson Laboratories), were also used in single dose experiments to verify that the two strains of mice had similar skin responses to X-rays and neutrons and yielded the same neutron RBE. Thereafter, skin response RBE's were determined using BALB/C mice and were compared to tumor response RBE's from C3H mice to calculate Therapeutic Gain Factors (TGF = tumor RBE/skin RBE).

Early skin response following single fractions was scored over the period of 7–35 days postirradiation, using a scale modified from Fowler *et al.* [10]. For experiments using multiple fractions of radiation, the ascending curve for skin response vs. days postirradiation was superimposed over similar curves for single fractions to determine which postirradiation day was equivalent to day 7. In all cases, day 8 or 9 was used as the starting point for skin scoring in multiple fraction experiments, and observations were continued until day 36 or 37. Foot deformity at 6 months post-exposure was scored using the scale of Field *et al.* [11].

Tumor irradiations

C3H/HeJ male mice, 6–10 weeks old (Jackson Laboratories), were housed in the University of Washington Vivarium at least

one week after arrival before C3HBA mammary adenocarcinomas were transplanted by placing small (1–2 mm dia) pieces of non-necrotic tumor under the skin of the upper back. The C3HBA tumor is a transplantable mammary neoplasm which occurred as a spontaneous tumor in a C3H/An mouse in 1946 and has been carried as a transplantable tumor in C3H/HeJ mice by Jackson Laboratories, Bar Harbor, Maine, since 1949. At 12–13 days post-transplant, tumors of average diameter, 2·7 mm to 5·7 mm were chosen for irradiation. Six to eight mice anesthetized with Nembutal (60 μg/g wt.) were irradiated at each dose. The animals were positioned at the corners of the field with their bodies shielded by the lead shield in the case of X-irradiation or the B/WEP collimator in the case of neutron irradiations.

volume was confirmed by comparing calculated volumes to weights and saline displacement volumes of excised tumors. The volume of each tumor on the first day of irradiation was taken as 1·0, and volumes on succeeding days were normalized to this value. The endpoint for tumor radiation response was growth delay, defined as:

> (no. of days for irradiated tumor to reach 5x starting volume) minus (no. of days for control tumor to each 5x starting volume.)

Neutron RBE's were determined by dividing the X-ray dose by the neutron dose which produced equivalent growth delay. RBE's for

Table 1

Radiation type, number of fx	Fractionation scheme M-Tu-W-Th-F	Feet Dose/fx	Feet Total dose	Tumors Dose/fx	Tumors Total dose
1 fx X-rays	x - - - -	1000 - 4500	1000 - 4000	1010 - 4540	1010 - 4540
2 fx X-rays	x x - - -	908 - 2270	1816 - 4540	640 - 1810	1280 - 3620
2 fx X-rays	x - - - x	1025 - 2465	2050 - 4930	1010 - 2220	2020 - 4440
5 fx X-rays	x x x x x	605 - 1260	3025 - 6300	505 - 980	2525 - 4540
1 fx neutrons	n - - - -	700 - 2000	700 - 2000	400 - 2000	400 - 2000
2 fx neutrons	n n - - -	500 - 1000	1000 - 2000	75 - 1000	100 - 2000
2 fx neutrons	n - - - n	500 - 1100	1000 - 2200	400 - 1250	800 - 2500
5 fx neutrons	n n n n n	260 - 500	1300 - 2500	100 - 425	500 - 2125
2 fx neutrons + 3 fx X-rays	n x x x n	50 - 625 (n) 958 or 1261 (x)	2974 - 5033 (n + x)	100 - 400 (n) 908 (x)	2924 - 3524
2 fx neutrons + 3 fx X-rays	n n x x x	100 - 625 (n) 958 or 1261 (x)	2974 - 5033 (n + x)	50 - 400 (n) 908 (x)	2824 - 3524

All doses are in rads.

For each fractionation scheme, mice from the same transplant group were irradiated concurrently with neutrons or X-rays and their responses were used to determine RBE's. However, repeat experiments showed tumor response to a given dose and fractionation scheme was constant enough to allow data from separate experiments employing different transplant groups to be combined without changing RBE values.

Tumors were measured in 3 dimensions 3–5 days/week during and after treatment and tumor volumes were calculated, using the simplified formula:

$$\frac{\text{length} \times \text{width} \times \text{depth}}{2},$$

which closely approximates the volume of an ellipsoid. Measurements were corrected for skin thickness. This method of determining tumor

mixed neutron-photon fractionation schemes were determined by dividing total X-ray dose by total neutron + X-ray dose.

Therapeutic gain factors (TGF), defined as tumor RBE/skin RBE for a specified dose and fractionation scheme, were determined.

RESULTS

The fractionation schemes, total doses, and dose per fraction used in skin and tumor response experiments are outlined in Table 1. One fraction of neutrons, two fractions 24 or 96 hr apart, and 5 fractions in 5 days were compared to X-rays given in identical fractionation schemes. In the mixed fractionation schemes, 2 fractions of neutrons plus 3 fractions of X-rays were given in 5 days, in the sequence n-n-x-x-x or n-x-x-x-n, and the responses were compared to those obtained with 5 fractions of X-rays in 5 days. In both the all X-ray and mixed neutron-

photon options in these experiments, the X-ray dose per fraction was held constant while the neutron dose per fraction was varied. Within the context of such an experiment, an RBE could be determined by seeing which dose of neutrons + X-rays was equivalent to the constant total X-ray dose in that experiment (for example, see Fig. 2 and 7).

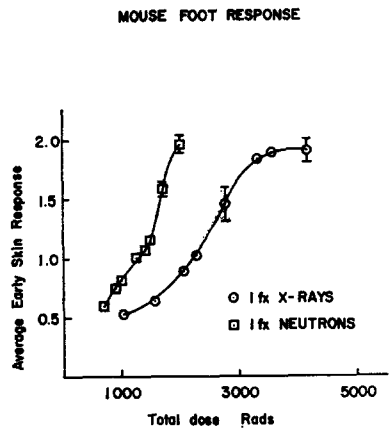


Fig. 1. Average early skin response vs dose in rads for single fractions of X-rays or neutrons. Each point is the mean ± standard error of the average skin response over the period 7–35 days postirradiation for the mice in each dosage group.

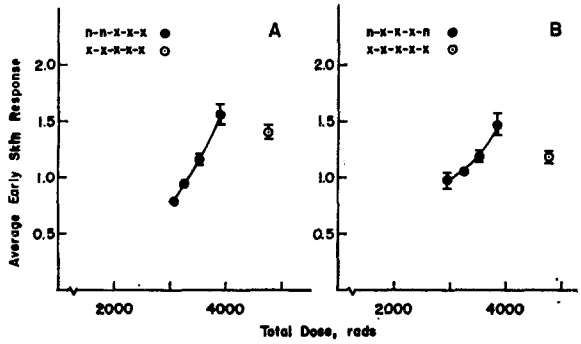


Fig. 2. Average early skin response vs total dose for 5 fx of X-rays in 5 days (5 × 958 rads/fx) and for 2 fx of neutrons (variable dose/fx) + 3 fx of X-rays (958 rads/fx). Each point is the mean ± standard error for early skin response (7–35 days postirradiation) for all mice in a dosage group.

A. 5 fx X-rays in 5 days vs n-n-x-x-x
Neutron doses/fx were 100, 200, 325 and 425 rads.

B. 5 fx X-rays in 5 days vs n-x-x-x-n
Neutron doses/fx were 50, 200, 325 and 475 rads.

Skin response

Early skin response to single doses of neutrons or X-rays is shown in Fig. 1, and response to mixed fractionation schemes is shown in Fig. 2(a and b). Each value is the mean ± standard error of the average skin response over the period 7–35 days postirradiation for all mice in one dosage group. Foot deformity at 6 months after single doses of X-rays or neutrons is also shown (Fig. 3). Neutron RBE's for early and late damage were read directly from smooth curves fitted to the points on these plots and similar ones for all other fractionation schemes, and are summarized in Tables 2–4.

Tumor response

Of the mice irradiated for tumor growth delay, 4% died from anesthetic overdose, 4% died within the first 10 days after irradiation, due mostly to radiation effect on the intestine, and 4% died later than 10 days but too soon for their tumors to be included in the growth delay analysis.

Tumor growth following graded single doses of X-rays or neutrons is shown in Fig. 4(a and b). Tumor response to radiation was assessed as tumor growth delay, determined from volume change against time for control and irradiated

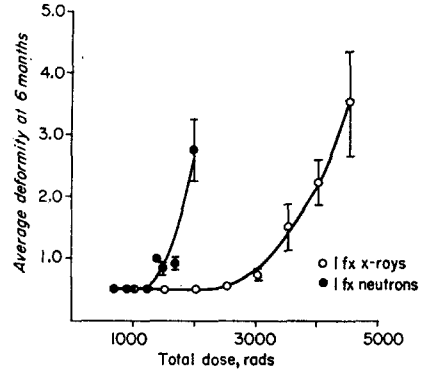


Fig. 3. Average foot deformity at 6 months post treatment vs dose for single fractions of neutrons or X-rays. Each point is the mean ± standard error for all animals in one dosage group. Points without error flags have standard error smaller than the size of the point.

tumors. Unirradiated control tumors had a doubling time of 4·0 ± 0·2 days starting at 12–13 days post-transplant (mean ± standard error for 52 tumors from 8 experiments). In these experiments 5/434 irradiated mice died or were terminated at 69–114 days post-treatment with no measurable tumor. Other mice in the same dosage group who lived longer all had recurrent tumors and some mice dying without a measurable tumor were found upon autopsy to have a small tumor at the primary site. Since recurrences were found to occur up to 150 days after treatment, these mice had not survived long enough to be considered cured and were eliminated from the analysis.

Growth delay in reaching 5x starting volume as a function of total dose of neutrons vs total dose X-rays is shown in Figs. 5 and 6. Growth delay as a function of total dose of X-rays vs total dose of neutrons + X-rays in the mixed

fractionation scheme is shown in Fig. 7 (a and b). Each value is the mean ± standard error of delay in days for all tumors in a particular dosage group. The neutron and mixed neutron-photon RBE's for tumor growth delay are summarized in Tables 5 and 6.

Estimation of errors

In the absence of an established statistical test for variation in RBE's for skin damage and tumor growth delay read from the graphs, the following method was used. Envelopes of the standard bars were drawn on each curve of early skin damage, foot deformity, or tumor growth delay vs dose. RBE's relative to a specified X-ray dose were read directly from the curves. The maximum and minimum X-ray and neutron doses likely to produce the same level of damage as the specified X-ray dose were determined from the envelopes of the standard error bars. Dividing the maximum X-ray dose by the minimum neutron dose, and vice-versa, gives a range of RBE's, which are included in parenthesis below the RBE values listed in Tables 2–6. For sample sizes used here this approximates a 70% confidence limit [12]. The RBE range was expressed as per cent of the RBE value read directly from the curves, and these per cent ranges were used to calculate the ranges of TGF values in Table 6.

Table 2. Neutron RBE's for early skin damage (7–35 days postirradiation) and foot deformity at 6 mos

			Skin response		Deformity	
Fractionation scheme	Total X-ray dose, rads	X-ray dose/fx, rads	Equivalent* neutron dose/fx	RBE**	Equivalent neutron dose/fx	RBE**
1 fx	2500	2500	1390 (1290 - 1450)	1.8 (1.6 - 1.9)*	1260	2.0 (———)
	4000	4000	1940 (1880 - 2000)	2.1 (1.8 - 2.6)	1900 (1750 - 1980)	2.1 (1.9 - 2.4)
2 fx - 24 hrs. apart	2500	1250	575 (545 - 580)	2.2 (2.2 - 2.3)	605 (500 - 650)	2.1 (1.9 - 2.5)
	4000	2000	915 (865 - 935)	2.2 (2.2 - 2.3)	925 (850 - 960)	2.2 (1.8 - 2.4)
2 fx - 96 hrs. apart	2500	1250	515 (515 - 540)	2.4 (2.3 - 2.4)	525	2.4 (2.2 - 2.7)
	4000	2000	890 (850 - 910)	2.2 (2.1 - 2.4)	965 (955 - 1005)	2.1 (1.8 - 2.3)

* The numbers in parentheses under each neutron dose/fx are the range of doses producing an equivalent effect as determined from envelopes drawn to standard error bars on plots of skin response or deformity vs. dose.

** RBE ranges are approximately 70% confidence limits. See text for explanation

Table 3. Neutron and mixed neutron-photon RBE's for early skin damage

Fractionation scheme	X-ray dose/fx	Equivalent* neutron dose/fx	RBE** (X dose/fx / N dose/fx)	Total X-ray dose	Equivalent total dose: test radiation	RBE** (total X-ray dose / total test dose)
5 fx X-rays vs. 5 fx neutrons in 5 days	800	276 (258 - 290)	2.9 (2.6 - 3.2)	4000	1380	2.9 (2.6 - 3.2)
	950	346 (340 - 382)	2.7 (2.3 - 2.9)	4750	1760	2.7 (2.3 - 2.9)
2 fx neutrons + 3 fx X-rays in 5 days (n-n-x-x-x) vs. 5 fx X-rays in 5 days***	958	430 (390 - 455)	2.2	4790	3734	1.3 ---
	1261	555 (510 - 600)	2.3	6305	4893	1.3 ---
2 fx neutrons + 3 fx X-rays in 5 days (n-x-x-x-n) vs. 5 fx X-rays in 5 days***	958	320 (285 - 345)	3.0	4790	3514	1.3 ---
	1261	500 (475 - 540)	2.5	6305	4783	1.3 ---

* The numbers in parentheses under each neutron dose/fx are the range of doses producing equivalent effect as determined from envelopes drawn to standard error bars on plots of skin response vs. dose.

** RBE ranges are approximately 70% confidence limits. See text for explanation.

***The mixed fractionation schemes employ a constant X-ray dose/fx (958 or 1261 rads/fx) while the neutron dose/fx, on those days when neutrons are given, is varied. The early skin response (7–35 days post irradiation) produced by these treatments is compared to damage produced by 5 x 958 or 5 x 1261 rads of X-rays given in 5 days.

Table 4. Neutron and mixed neutron-photon RBE's for late foot deformity

Fractionation scheme	X-ray dose/fx	Equivalent* neutron dose/fx	RBE** $\left(\frac{\text{X dose/fx}}{\text{N dose/fx}}\right)$	Total X-ray dose	Equivalent total dose: test radiation	RBE** $\left(\frac{\text{total X-ray dose}}{\text{total dose test radiation}}\right)$
5 fx X-rays vs. 5 fx neutrons in 5 days	800	300 (270 - 346)	2.7 (2.2 - 3.4)	4000	1500	2.7 (2.2 - 3.4)
	950	340 (322 - 390)	2.7 (2.2 - 3.1)	4750	1740	2.7 (2.2 - 3.1)
	1250	490 (450 - 530)	2.6 (2.3 - 2.8)	6250	2450	2.6 (2.3 - 2.8)
5 fx X-rays in 5 days vs. n-n-x-x-x***	958	370 (340 - 405)	2.6	4790	3614	1.3 ---
	1261	520 (455 - 580)	2.4	6305	4823	1.3 ---
5 fx X-rays in 5 days vs. n-x-x-x-n***	958	330 (220 - 400)	2.9	4790	3534	1.4 ---
	1261	550 (510 - 605)	2.3	6305	4883	1.3 ---

* The numbers in parentheses under each neutron dose/fx are the range of doses producing equivalent effect as determined from envelopes to standard error bars on plots of foot deformity.

** RBE ranges are approximately 70% confidence limits. See text for explanation.

*** The mixed fractionation schemes employ a constant X-ray dose/fx, (958 or 1261 rads/fx) while the neutron dose/fx, on those days when neutrons are given, is varied. The late foot deformity produced by these treatments is compared to damage produced by 5 x 958 or 5 x 1261 rads of X-rays given in 5 days.

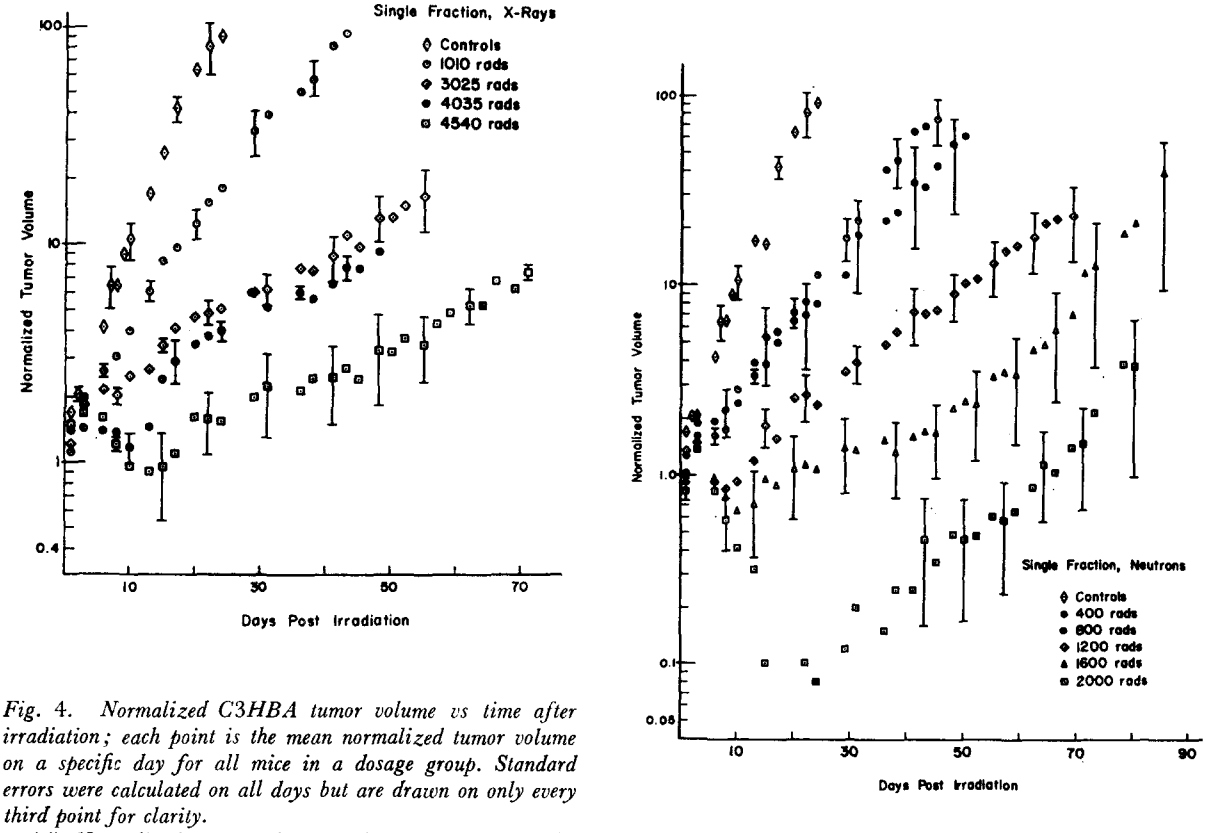


Fig. 4. Normalized C3HBA tumor volume vs time after irradiation; each point is the mean normalized tumor volume on a specific day for all mice in a dosage group. Standard errors were calculated on all days but are drawn on only every third point for clarity.

(A) Normalized tumor volume vs days postirradiation for single doses of X-rays.

(B) Normalized tumor volume vs single doses of neutrons.

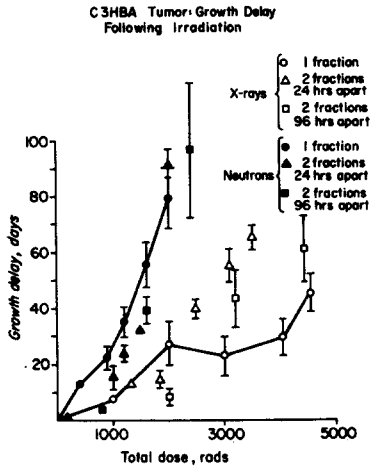


Fig. 5. Growth delay in days, for the C3HBA tumor to reach 5x the volume at the beginning of irradiation vs dose of neutrons or X-rays, for single fractions, 2 fx 24 hr apart, and 2 fractions 96 hours apart. Each point is the mean growth delay in days ± standard error for all mice in one dosage group.

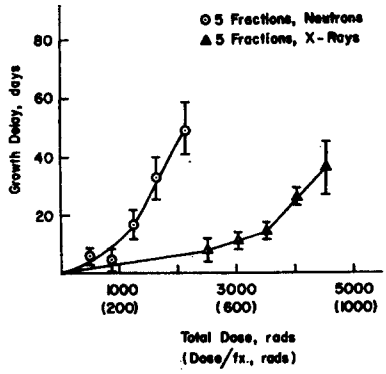


Fig. 6. C3HBA tumour growth delay in days, to reach 5x starting volume vs dose for 5 fx X-rays or 5 fx neutrons in 5 days. Each point is the mean growth delay ± standard error for all tumors in a single dosage group.

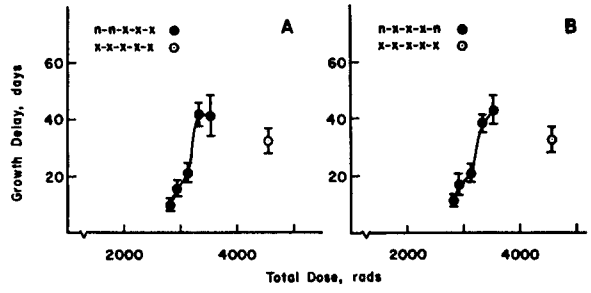


Fig. 7. C3HBA tumor growth delay, in days, to reach 5x starting volume vs total dose for 5 fx of X-rays in 5 days (5 × 908 rads) and for 2 fx of neutrons (variable dose/fx) + 3 fx of X-rays (908 rads/fx).

(A) 5 fx X-rays in 5 days vs n-n-x-x-x
Neutron doses/fx were 50, 100, 200, 300 and 400 rads.

(B) 5 fx X-rays in 5 days vs n-x-x-x-n
Neutron doses/fx were 50, 100, 200, 300 and 400 rads.

DISCUSSION

The neutron RBE's for early mouse skin response and foot deformity at 6 months after irradiation with the University of Washington neutron beam (8 MeV mean energy) increase with increasing number of fractions and decreasing dose/fx, as reported previously [7, 11, 13]. The skin RBE's calculated for total dose of 2 fx of neutrons + 3 fx of X-rays, given in the mixed fractionation scheme and compared to 5 fx of X-rays in 5 days, are the same for both mixed schemes, n-n-x-x-x and n-x-x-x-n: 1·3 (Table 3). The deformity RBE for total dose of neutrons + X-rays in the mixed scheme, relative to 5 fx of X-rays in 5 days, is 1·3 for the n-n-x-x-x scheme and 1·3 or 1·4 for the n-x-x-x-n scheme.

The response of the C3HBA tumor to single doses of X-rays (Fig. 5) shows that growth delay increased quite rapidly with increasing dose up to 2000 rads and then shows less rapid change between 2000 and 4500 rads. The mean values for delay at each dose define a plateau in response between 2000 and 4000 rads, with increasing response as a function of dose above 4000 rads. This is similar to the response of the RIB-5 tumor of the rat to single doses of X-rays [11] and this refractoriness between 2000 and 4000 rads indicates that this C3H mammary tumor contains an hypoxic fraction of cells which render it resistant to further growth delay over an intermediate dose range. A plateau region in the growth delay vs dose curve is not seen for single fractions of neutrons; this is expected even if hypoxic cells are present because they provide relatively less protection against high LET radiation [14].

Between 2000 and 4500 rads, two fractions of X-rays given 24 or 96 hr apart are more effective than the same dose given in a single fraction (Fig. 5). Neutrons given in 2 fractions 24 or 96 hr apart are slightly less effective in producing tumor growth delay than the same total dose given in one fraction (Fig. 5). The net effect is a reduced neutron RBE for 2 fx 24 or 96 hr apart, relative to a single fraction (Table 5).

Field *et al.* suggested [5] that this elimination of a plateau region in the growth delay response curve with fractionated X-irradiation is due to reoxygenation. Fowler *et al.* [7] and Suit and Schiavone [15] showed that, following large single doses of X-rays rapid reoxygenation occurred in primary and early generation explants of C3H mouse mammary tumors, and Howes [16] showed that the minimum *proportion* of hypoxic cells occurred at 96 hr after

Table 5. Neutron RBE's for tumor growth delay

Fractionation scheme	Total X-ray dose, rads	X-ray dose/fx rads	Equivalent neutron* dose/fx	RBE**	TGF = tumor RBE ** / skin RBE
1 fx	2000	2000	980 (910 - 1130)	2.0 (1.2 - 4.6)	——
	2500	2500	1000 (860 - 1180)	2.5 (1.4 - 4.8)	2.5/1.6 = 1.6(0.90-3.1)
	4000	4000	1050 (950 - 1200)	3.8 (2.5 - 4.5)	3.8/2.1 = 1.8(1.2-2.1)
2 fx - 24 hrs. apart	2000	1000	600 (550 - 630)	1.7 (1.6 - 1.9)	——
	2500	1250	790 (760 - 835)	1.6 (1.5 - 1.7)	1.6/2.2 = 0.73(0.65-0.9)
	3500	1750	940 (860 - 940)	1.9 (1.7 - 2.1)	——
2 fx - 96 hrs. apart	2000	1000	445 (430 - 450)	2.2 (2.0 - 2.5)	——
	2500	1250	600 (585 - 650)	2.1 (1.8 - 2.4)	2.1/2.4 = 0.88(0.7-1.0)
	4400	2200	1100 (1010 - 1125)	2.2 (1.7 - 2.8)	——

* The numbers in parentheses under each neutron dose/fx are the range of doses producing equivalent effect as determined from envelopes drawn to standard error bars on plots of tumor growth delay vs. dose.

** RBE ranges are approximately 70% confidence limits, and are used to calculate ranges of TGF values. See text for explanation.

Table 6. Neutron and mixed neutron-photon RBE's for tumor growth delay

Fractionation scheme	X-ray dose/fx	Equivalent* neutron dose/fx	RBE** (X dose/fx / N dose/fx)	Total X-ray dose	Total dose of test Radiation	RBE** (Total X-ray dose / Total dose of test radiation)	TGF** = tumor RBE / skin RBE
5 fx in 5 days	706	240 (210 - 272)	2.9 (2.2 - 3.6)	3530	1200	2.9 (2.2 - 3.6)	——
	807	295 (266 - 312)	2.7 (2.4 - 3.2)	4035	1475	2.7 (2.4 - 3.2)	2.7/2.9 = 0.93(0.8-1.1)
	908	350 (306 - 356)	2.6 (2.2 - 3.5)	4540	1750	2.6 (2.2 - 3.5)	2.6/2.7 = 0.96(0.81-1.3)
2 fx neutrons + 3 fx X-rays in 5 days (n-n-x-x-x) vs. 5 fx X-rays in 5 days ***	908	250 (235 - 270)	3.6	4540	3224	1.4	1.4/1.3 = 1.1
2 fx neutrons + 3 fx X-rays in 5 days (n-x-x-x-n) vs. 5 fx X-rays in 5 days ***	908	250 (215 - 275)	3.6	4540	3224	1.4	1.4/1.3 = 1.1

* The numbers in parentheses under each neutron dose/fx are the range of doses producing equivalent effect as determined from envelopes drawn to standard error bars on plots of tumor growth delay vs. dose.

** RBE ranges are approximately 70% confidence limits and are used to calculate limits for TGF's. See text for explanation.

*** The mixed fractionation schemes employ a constant X-ray dose/fx (908 rads) while the neutron dose/fx, on those days when neutrons are given, is varied. The tumor growth delay produced by these treatments is compared to delay produced by 5 x 908 rads of X-rays given in 5 days.

exposure, although, more importantly, the minimum *number* occurred at 48 to 72 hr. Like primary and early explant C3H mammary tumors, the C3HBA mammary adenocarcinoma probably undergoes rapid reoxygenation, rendering 2 fx of X-rays at 24 or 96 hr intervals more effective than the same dose delivered as a single fraction. To accomplish this reoxygenation must outweigh the effect of repair between fractions in the case of the 24 hr interval and additionaly the effect of repopulation during the 96 hr interval.

When 5 fx of neutrons in 5 days are compared to 5 fx of X-rays in 5 days, RBE's are increased relative to two fractions, presumably because the accumulated effect of less repair between fractions of neutrons tends to raise the RBE, although differences in the pattern of reoxygenation during the 5 fx X-ray treatment relative to the 2 fx treatments cannot be ruled out (Table 6).

The RBE for neutrons plus X-rays in the mixed fractionation schemes (2 fx of neutrons + 3 fx of X-rays in 5 days in the sequence n-n-x-x-x or n-x-x-x-n) relative to 5 fx X-rays in 5 days, is 1·4 for both schemes (Table 6). The RBE's here are lower than those calculated when neutron only schemes are compared to

X-ray only schemes and reflect the relatively large contribution of the 3 X-ray fractions to the total dose.

TGF's calculated by dividing tumor RBE by skin RBE for a particular dose and fractionation scheme are close to 1·0 for all treatment regimens except single fractions of neutrons (Table 5, 6). The TGF for both mixed fractionation schemes is 1·1, which is marginally better, although the confidence limits for TGF values for some neutron only schemes overlap this value (Table 6). The TGF values of less than 1·0 can be explained in terms of the rapid and extensive reoxygenation in C3H mammary tumors such that fractioned X-irradiation can be highly effective. Fowler and others [7, 8, 17] have studied the response of first and second generation explants of C3H mouse mammary tumors to neutrons and X-rays and have shown that the appropriate fractionation of X-rays, to take advantage of the known reoxygenation pattern of those tumors, makes photon treatment as effective as neutrons when treatment effectiveness is expressed in terms of tumor growth delay or cure probability relative to skin damage. Neutrons may give more reliable results, being less dependent on fractionation scheme [18], but not necessarily better results. These experiments described here are consistent with these findings. The TGF's for all schemes other than single fx are close to 1·0. Also, the sequence of neutron fractions in the mixed irradiations does not alter the RBE. The RBE for skin damage (1·3) is the same whether neutrons are given on the first and second days or the first and fifth days of a 5 day treatment schedule; the tumor growth delay RBE of 1·4 is the same for both sequences.

The hypothesis to be tested was that an advantage might be seen with neutrons in a rapidly reoxygenating tumor if neutrons were given as the first one or two fractions, when hypoxic cells were present in the largest numbers, followed by X-ray fractions to kill increasingly better reoxygenated cells. The hypothesis is not borne out; the TGF's of 1·1 for both mixed fractionation schemes are not significantly different from TGF's for 5 fx of neutrons in 5 days. At any rate, the mixed schemes are no worse than and may be slightly better than 5 fx of neutrons in 5 days.

The C3HBA tumor's response to 2 fx of X-rays vs single doses strongly suggests that reoxygenation occurs in this tumor as in primary C3H mammary tumors and early generation explants. These latter tumors reoxygenate after large single doses of X-rays such that the proportion of hypoxic cells 24 to 96 hrs after irradiation is considerably less than in the unirradiated tumor [16]. Such extensive reoxygenation is not seen in other experimental tumors including the KHT mouse sarcoma [18, 19], a mouse osteosarcoma [20] and the RIB-5 tumor of the rat [21], where at best the proportion of hypoxic cells in irradiated tumors returns to levels close to those in untreated controls. Thus C3H mammary tumors are probably the system least likely to demonstrate an advantage for neutron therapy. The theoretical basis for using one or two initial fractions of neutrons followed by fractionated photons may be valid in other tumors.

The practice of expressing neutron RBE in terms of dose/fx rather than total dose has been widely used and has yielded useful insight into the variation of RBE with increasing number of fractions and decreasing dose per fraction in cell systems which can repair sublethal damage [13]. In the mixed fractionation schemes, a variable neutron dose/fx was coupled with a fixed X-ray dose/fx, the latter being given on 3 of the 5 days. The response to the mixed radiation was compared to 5 fx of X-rays using the same X-ray dose per fraction as in the mixed scheme. Neutron dose/fx relative to X-ray dose/fx can be used to calculate an RBE here if one wishes to focus on neutron dose/fx equally effective as a fixed X-ray dose in the context of these experiments. In neutron only vs X-ray only schemes, RBE's are the same, whether one uses dose/fx or total dose in the calculations while in the mixed schemes, an elevated RBE is obtained if one uses dose/fx rather than total dose (Tables 3, 4, 6). The dose/fx approach to calculating RBE's ignores the 3 fx of X-rays common to the mixed scheme and to 5 fx of X-rays in 5 days. The definition of RBE is: (dose of X-rays necessary to produce a specific effect)/(dose of test radiation to produce the same effect). No effect was examined which resulted from only the neutron component of the mixed irradiation. All endpoints were the result of the total treatment, and consequently RBE's calculated from (total X-ray dose)/(total dose of (neutrons + X-rays)) should be used to calculate TGF's.

Clinical correlation

The conventional pattern of irradiation with external beams, 5 consecutive daily increments per week, developed empirically. Although there are factors of custom and convenience, this so-called "Coutard method" is based on clinically useful therapeutic ratios resulting from preservation of the integrity of normal tissues concurrent with tumor cell killing. This

single pattern of treatment is unlikely to be the best possible for all, or even many, clinical situations. However, at this time, there is no scientific basis for tailoring the pattern of treatment to maximize the therapeutic ratio for each specific clinical problem.

Whenever a new radiotherapeutic modality is to be tested, the pattern of application is likely to be conventional, based on custom, or expedient, based on a variety of extraneous factors.

Restricted patterns of application may be based on the limited availability of the neutron generators rather than on any biological evidence. Therefore, it is essential that a wide spectrum of treatment patterns be studied prior to any commitment for definitive clinical trials.

Mixed neutron-photon fractionation schemes would facilitate the selected treatment of a larger number of patients with each expensive neutron generator, and are attractive on this basis alone. However, a favorable therapeutic ratio must be shown. In this relatively unfavorable tumor system, this mode of treatment is at least as effective as any fractionated neutron only scheme and deserves further consideration in other tumor systems.

Acknowledgements—We gratefully acknowledge the support of Dr. Juri Eenmaa, Dr. Keith Weaver, and Dr. David Williams of the Division of Medical Radiation Physics for performing the neutron dosimetry and providing other services at the University of Washington Cyclotron.

REFERENCES

1. J. F. Fowler and R. L. Morgan, Pre-therapeutic experiments with the fast neutron beam from the Medical Research Council cyclotron. VIII. General review. *Brit. J. Radiol.* **36,** 115 (1963).
2. J. L. Montour, J. D. Wilson, C. C. Rogers, R. B. Theus and F. H. Attix, Biological characterization of a high energy neutron beam for radiation therapy. *Cancer (Philad.)* **34,** 54 (1974).
3. P. Todd, C. B. Schroy, F. H. Attix and R. B. Theus, Spatial distribution of human cell survival in a neutron beam designed for therapy. *Cancer (Philad.)* **34,** 33 (1974).
4. P. Wootton, K. Alvar, H. Bichsel, J. Eenmaa, J. S. R. Nelson, R. G. Parker, K. A. Weaver, D. L. Williams and W. G. Wyckoff, Fast neutron beam radiotherapy at the University of Washington. *J. Canad. Assoc. Radiol.* **26,** 44 (1975.)
5. S. B. Field, T. Jones, and R. H. Thomlinson, The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. II. Fractionation, recovery and reoxygenation. *Brit. J. Radiol.* **41,** 597 (1968).
6. J. Denekamp, E. W. Emery and S. B. Field, Response of mouse epidermal cells to single and divided doses of fast neutrons. *Brit. J. Radiol.* **45,** 80 (1971).
7. J. F. Fowler, J. Denekamp, A. L. Page, A. C. Begg, S. B. Field and K. Butler, Fractionation with X-rays and neutrons in mice: response of skin and C3H mammary tumors. *Brit. J. Radiol.* **45,** 237 (1972).
8. J. F. Fowler, S. B. Field and J. Denekamp, The effect of fractionated doses of neutrons on C3H mouse mammary tumours. *Europ. J. Cancer* **10,** 281 (1974).
9. N. A. Frigerio, R. F. Coley and M. J. Sampson, Depth dose determinations. I. Tissue equivalent liquids for standard man and muscle. *Physics Med. Biol.* **17,** 792 (1972).
10. J. F. Fowler, R. J. Berry, R. E. Ellis, K. Kragt and P. J. Lindop, The effect of divided doses of 15 MeV electrons on the skin response of mice. *Int. J. Radiat. Biol.* **9,** 241 (1965).
11. S. B. Field, T. Jones and R. H. Thomlinson, The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. I. Single doses. *Brit. J. Radiol.* **40,** 834 (1967).
12. A. Goldstein, *Biostatistics: An Introductory Text,* Macmillan, New York (1964).
13. S. B. Field and S. Hornsey, RBE values for cyclotron neutrons for effects on normal tissues and tumors as a function of dose and dose fractionation. *Europ. J. Cancer* **7,** 161 (1971).
14. G. W. Barendsen, Cellular responses determining the effectiveness of fast neutrons relative to X-rays for effects on experimental tumours. *Europ. J. Cancer* **7,** 181 (1971).

15. H. D. SUIT and J. V. SCHIAVONE, Effect of a single dose of radiation on proportion of hypoxic cells in a C3H mouse mammary carcinoma. *Radiology* **90,** 325 (1968).
16. A. E. HOWES, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
17. J. F. FOWLER, J. DENEKAMP and S. B. FIELD, RBE values for regrowth of C3H mouse mammary carcinomas after single doses of cyclotron neutrons or X-rays. *Europ. J. Cancer* **9,** 853 (1973).
18. L. M. VAN PUTTEN and R. F. KALLMAN, Oxygenation status of a transplantable tumor during fractionated radiotherapy. *J. nat. Cancer Inst.* **40,** 441 (1968).
19. R. F. KALLMAN, L. J. JARDINE and C. W. JOHNSON, Effects of different schedules of dose fractionation on the oxygenation status of a transplantable mouse sarcoma. *J. nat. Cancer Inst.* **44,** 369 (1970).
20. L. M. VAN PUTTEN, Tumour reoxygenation during fractionated radiotherapy: Studies with a transplantable mouse osteosarcoma. *Europ. J. Cancer* **4,** 173 (1968).
21. R. H. THOMLINSON, Changes in oxygenation in tumours in relation to irradiation. *Front. Radiat. Ther. Oncol.* **3,** 109 (1968).

Europ. J. Cancer Vol. 11, pp. 903–904. Pergamon Press 1975. Printed in Great Britain

Recent Journal Contents (1975)

International Journal of Cancer — *December, 1975*

I. Horváth, P. Arányi A. Náray, I. Földes and Á. Gyuris: Tyrosine aminotransferase induction in normal and tumor-bearing chickens.

G. T. Stevenson and D. J. R. Laurence (Editors): Special report: Immune response to solid tumours in man.

A. E. Powell, A. M. Sloss, R. N. Smith, J. T. Makley and C. A. Hubay: Specific responsiveness of leukocytes to soluble extracts of human tumors.

R. van Nie and A. A. Verstraeten: Studies of genetic transmission of mammary tumour virus by C3Hf mice.

L. Weiss, J. R. Subjeck and G. Poste: Some electrical properties of the peripheries of murine 3T3 cells with respect to viral transformation and reversion.

R. J. Dattwyler and T. B. Tomasi: Inhibition of cytotoxic sensitization of T cells by alpha-fetoprotein.

K. Nelson, S. B. Pollack and K. E. Hellström: *In vitro* synthesis of tumor-specific factors with blocking and antibody-dependent cellullar cytotoxicity (ADC) activities.

A. Senik, F. Pozzo Hebrero and J. P. Levy: *In vitro* interactions between lymphoid and tumor cells in the MSV system. Study of the secondary specific antitumor response.

A. Senik, S. Gisselbrecht and J. P. Levy: Antigenic specificities of the cell-mediated antitumor reactions in the MSV system. Study by the secondary chromium release test.

B. Mukherji, D. Vassos, A. Flowers, S. C. Binder and L. Nathanson: Selective and non-selective lymphocytotoxicity in human melanoma: Observation on the effect of long term culture and fetal bovine serum on target cell sensitivity to lymphocytes.

S. Cornain, J. E. de Vries, J. Collard, C. Vennegoor and P. Rümke: Antibodies and antigen expression in human melanoma detected by the immune adherence test.

M. H. Moar and K. W. Jones: Detection of virus-specific DNA and RNA base-sequences in individual cells transformed or infected by Adenovirus type 2.

M. Dalens, L. Zech and G. Klein: Origin of lymphoid lines established from mixed cultures of cord blood lymphocytes and explants from infectious mononucleosis, Burkitt lymphoma and healthy donors.

M. Dorizzi, G. Ortiz-Muniz, D. M. Lopez, M. M. Sigel and R. S. Epstein: Increase in the proportion of cells with the C'_3 receptor in Balb/c mice bearing mammary tumors.

G. W. Wood and G. Y. Gillespie: Studies on the role of macrophages in regulation of growth and metastasis of murine chemically induced fibrosarcomas.

H. Schmitz, D. Kampa and W. Heidenreich: Sensitive method to detect non-complement-fixing antibodies to Epstein–Barr virus nuclear antigen.

A. W. Boddie, Jr., M. M. Urist, D. O. Chee, E. C. Holmes and D. L. Morton: Inhibition of leukocyte migration in agarose by KCl extracts of a human melanoma cell line grown in serum free media.

British Journal of Cancer *December 1975*

J. G. Hall, R. G. Scollay, M. S. C. Biebeck and G. H. Theilen: Studies on FeSV-induced sarcoma in sheep with particular reference to the regional lymphatic system.

M. C. Bibby and G. M. Smith: Increase in Type A virus particles induced in BALB/c mouse epidermis during chemical carcinogenesis.

M. N. Goldrosen and P. B. Dent: Significance of arming, potentiating and blocking factors as correlates of tumour–host interaction in the hamster SV40.

R. D. Barnes and K. Brown: The lack of association of θ status and murine leukaemia virus content in the AKR.

E. Langvad, H. Hyden, H. Wolf and N. Kroeigaard: Extra-corporeal immuno-absorption of circulating specific serum factors in cancer patients.

A. M. Attallah and J. C. Houk: Cytotoxic effects of splenic ultrafiltrates upon leukaemic lymphocytes.

C. C. Bird, A. W. Waddell, A. M. G. Robertson, A. R. Currie, C. M. Steel and J. Evans: Cytoplasmic receptor levels and glucocortiloid response in human lymphoblastoid cell lines.

J. H. Westwood and P. Thomas: Studies on the structure and immunological activity of carcinoembryonic antigen—the role of bisulphide bonds.

T. G. Lavigne, A. Barry, C. d'Auteuil and J. M. Delage: Increase in the gastrointestinal absorption and in tissue storage of cyclosphosphamide in L-1210 leukaemic mice at an advanced stage of the disease.

R. D. Rubens, R. K. Knight and J. L. Hayward: Chemotherapy of advanced breast cancer: a controlled, randomised trial of cyclophosphamide versus a four-drug combination.

Short Communications

M. Munzarova and A. Trnka: Immunoelectrophoretic abnormality in sera from patients with different malignant diseases.

A. H. Feildsteel, P. J. Dawson, C. Kurahara and R. E. Brooks: Unusually high incidence of spontaneous mammary carcinomas in a colony of BALB/c mice.

Letters to the Editor

A. Jacobs, M. Wagstaff and M. Worwood: Serum ferritin, alpha$_2$ H globulin and malignant disease.

W. I. Aderle, O. Serike and B. O. Osunkoya: Pleural effusion in Burkitt's lymphoma.

Book Reviews

R. C. Garner: Chemical carcinogenesis.

H. M. Anthony: Biology of Cancer.

R. Yule: Mass screening for carcinoma of the uterine cervic.

A. H. W. Nias: ESRB Pre-Edinburgh Meeting.