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Immunological Resistance to Growth of Tumours in Syngeneic Multiparous Mice

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Abstract—*The hypothesis that BALB/c mice which have experienced a pregnancy are rendered immune to a 3-methylcholanthrene-induced syngeneic tumour was tested by direct tumour cell challenge of multiparous mice and by carrying out cell transfer assays using purified T cells obtained from the spleens of multiparous mice. Control mice were virgin BALB/c mice of the same age. The results showed that mice which have experienced a single multiparous pregnancy are resistant to the growth of the tumour MC677. In addition, it was shown that purified splenic T cells form multiparous mice inhibited the growth of tumour following implantation of a mixture of tumour cells and splenic T cells to normal recipient mice. Age-matched virgin controls did not show resistance to tumour growth following challenge nor were their lymphoid cells inhibitory to tumour cells. These results demonstrate clearly that pregnancy in mice confers a degree of transplantation resistance against the syngeneic tumour, MC677 and suggest that certain membrane-expressed oncofoetal antigens are operative in resistance against tumours. They also provide support for a possible immunological link between the observed decreased risk of breast cancer and early pregnancy in humans.*

INTRODUCTION

CONSIDERABLE interest has been generated from the study of foetal antigens re-expressed in tumour cells. Oncofoetal antigens are phase-specific antigens which arise in foetal tissue cells during embryonic development and possibly perform important functions in embryogenesis. The genes responsible for their synthesis appear to become repressed in the adult and hence these antigens are absent in normal adult tissue cells [1–3]. Oncofoetal antigens are a valuable group of tumour cell markers but their significance would be greater if it could be shown that they contributed in some degree to the immunological resistance against nascent tumour formation and development. Investigations by Moon [4] and Medawar and Hunt [5] have shown a lower incidence of tumours following pregnancy, or pre-exposure to foetal tissues in rats or mice and McMahon *et al.* [6] have shown a significant decrease in the incidence of breast cancer in women following pregnancy. The present investigations were undertaken to seek direct evidence for an immunological link between the experience of pregnancy and tumour rejection in mice.

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MATERIALS AND METHODS

These have been described previously [7, 8] and only variations in technique are described in detail.

Animals and tumour

Inbred BALB/c mice were used in all experiments. Their origin and maintenance has been described previously [7]. Multiparous mice were $2\frac{1}{2}$–$3\frac{1}{2}$ months of age and had given birth to a single litter of 3–10 young. Tumour MC677 used in the following experiments was induced in a female BALB/c mouse by the subcutaneous (s.c.) injection of 3-methylcholanthrene into the thigh musculature. A part of the first transplant generation tumour was kept biofrozen in liquid nitrogen and the tumour was propagated in the syngeneic strain. The use of this tumour in the present experiments was confined to the first 5 transplant generations. MC677 is a moderately immunogenic tumour and rejection of up to 5×10^5 tumour cells is observed in presensitized mice.

Preparation of tumour cell suspensions

Cell preparations were made from tumour grown s.c. for 10–14 days. The cells were

disaggregated in 0.2% trypsin, cultured *in vitro* for approximately 20 hr, and harvested by trypsinization prior to use in experiments. This procedure removes tumour macrophages and produces a highly viable single-cell suspension of tumour.

Lymphoid cell preparations

Cell suspensions were incubated in Petri dishes for 1 hr in 5% CO_2 in air at 37°C in order to remove adherent cells. The recovered non-adherent cells were treated with 0.8% NH_4Cl in order to remove red cells. The splenic T cell fraction was then separated by elution from a nylon wool column as previously described [8].

Tumour cell challenge and transfer assays

A threshold dose of tumour cells which grew progressively in a very high percentage of implanted mice was first established. It was found that cell doses of 10^4 or larger grew progressively in 10/10 mice tested, whereas only 6/10 mice formed tumours following s.c. inoculation with 10^3 cells. Therefore 10^4 cells were used for both tumour cell challenge as well as for cell transfer assays. In transfer assays tumour cells were mixed with purified splenic T cells at ratios of 1:500 or 1:1000 and the mixture incubated at 37°C for $\frac{1}{2}$ hr before inoculation into recipient mice. Each mouse received 10^4 tumour cells with the appropriate number of lymphoid cells in 0.1 ml of Hepes-buffered Eagles minimum essestial medium s.c. into the flank.

Statistical evaluation of data

The delay in the appearance or absence of tumours in experimental and control groups of animals was recorded at various time intervals and the proportion of mice in which tumours had developed were compared. Data were tested using Fisher's Exact Test for 2×2 tables. Student's *t*-test was used to compare mean tumour diameters of the test and control groups. Levels of significance are shown in the tables and where appropriate in the text.

RESULTS

In the first experiment, BALB/c mice $2\frac{1}{2}$–$3\frac{1}{2}$ months of age which had produced a single litter were given a challenge dose of 2×10^4 tumour cells s.c. 7–12 days after the birth of the litter. Age-matched virgin mice were given an identical challenge inoculum at the same time and the development of tumours was observed over a period of two months. Tumour challenge with 2×10^4 MC677 cells resulted in tumour formation in all 10 mice in the test group as well as in all 10 controls. There was a significantly decreased mean tumour diameter in the test group as compared to the controls ($P<0.05$). Ten days after tumour challenge, the mean diameters of tumours in the test and control groups were 1.0 ± 0.21 and 2.15 ± 0.46 respectively. A slight delay was observed in the appearance of the tumours, in addition to the smaller mean tumour diameters in the test group at later intervals.

Experiment 2 (Table 1 and Fig. 1) shows that, of the 5 mice challenged with 10^4 tumour cells, 3 rejected the tumour inoculum, in contrast to the control group in which tumour formation occurred in 5/5 mice. The difference between the mean tumour diameters of the test and control groups were highly significant ($P<0.001$) on each of the three intervals recorded. In each instance, the mean diameter in the test group was smaller than that of the controls. Furthermore, a significant delay in tumour appearance was observed in the test group compared to the controls 13 days following tumour challenge ($P<0.05$).

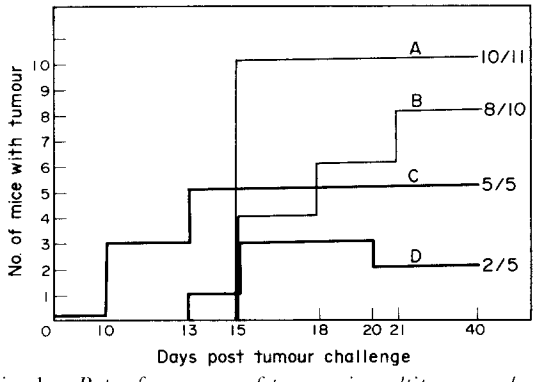

Fig. 1. Rate of occurrence of tumours in multiparous and age-matched virgin control mice following s.c. inoculation of 10^4 *MC677 tumour cells. Exp. 2—C = control group, D = test group.* ($P<0.05$ *at* day 13.) *Exp. 3—A = control group, B = test group.* ($P<0.05$ *at* day 15.)

In experiment 3, a second group of 10 test mice and 11 controls were challenged with 10^4 tumour cells. The development of tumours, was observed and tumour diameters recorded at the intervals indicated (Table 1). Table 1 shows that the time of appearance of tumour 15 days following tumour implantation was dignificantly delayed in the test group compared to controls ($P<0.05$), with only 4/10 mice in the test group showing palp-

Table 1. *Incidence and growth rates of tumours in multiparous mice following tumour challenge*

		Days post tumour challenge†						
Exp. 2	Group	10	13	15	18	20	22	40
Incidence of tumours*	Multiparous	0/5	1/5	3/5	3/5	3/5	2/5	2/5
	Controls	3/5	5/5	5/5	5/5	5/5	5/5	5/5
		NS	$P<0.05$	NS	NS	NS	NS	NS
Mean tumour diameter (mm) ± S.E.M.	Multiparous	ND	ND	0.6±0.25	ND	0.6±0.25	0.5±0.36	ND
	Controls	ND	ND	1.3±0.44	ND	2.25±0.83	5.28±1.68	ND
		—	—	$P<0.001$	—	$P<0.001$	$P<0.001$	—
		Days post tumour challenge†						
Exp.3				15	18	21	40	
Incidence of tumours*	Multiparous			4/10	6/10	8/10	8/10	
	Controls			10/11	10/11	10/11	10/11	
				$P<0.05$	NS	NS	NS	
Mean tumour dimeter (mm) ± S.E.M.	Multiparous			1.15±0.62	1.15±0.72	2.45±0.88	ND	
	Controls			2.64±0.61	3.09±0.76	4.3±1.19	ND	
				NS	NS	NS	—	

*Number of mice with tumours/total number in group.
†Tumour cell challenge with 10^4 MC677 cells.
ND not done.
NS not significant.

able tumours as compared to 10/11 in controls. However, at later times, this slow growth rate in the test group, as evidenced by mean tumour diameter, was not significantly different from that of the controls.

Experiments 4 and 5 (Table 2 and Fig. 2) show the results of transfer assays in which the inhibitory activity on tumour development of lymphoid cells taken from mice that have given birth to a single litter was studied. Purified splenic T cells were mixed with tumour cells at ratios of 500:1 and 1000:1 and the cell mixtures inoculated into normal male mice. Each recipient was injected with 10^4 tumour cells and the appropriate number of lymphoid cells in 0.1 ml of medium s.c. into the flank. Control splenic T cells were obtained from normal age-matched virgin mice. Table 2 shows that tumours developed in all 10 test and 10 control mice using a splenic T cell: tumour cell ratio of 500:1. A significantly decreased mean tumour diameter was observed in the test group after 15 days ($P<0.05$). Table 2 and Fig. 2 show the results of a second transfer experiment, in which a splenic T cell: tumour cell ratio of 1000:1 was used. The incidence of tumours in the two groups showed a highly significant difference at 7 days ($P<0.001$), when only 1/14 of the test group, compared to 13/14 of controls, had developed palpable tumour. At day 10, 14/14 controls had developed tumour but there was no change in test mice. Although between days 13 and 20 tumours became palpable in 5 further mice in the test group, the incidence of tumours as compared to controls was significantly different ($P<0.01$). No further tumours developed over the next 20 days and it was concluded that, in the 8/14 mice which had not formed palpable tumours over the 40 days period observed, rejection of the tumour had occurred. A comparison of the mean tumour diameters also showed a highly significant difference between the test and control groups with a consistently decreased mean tumour diameter on day 7 ($P<0.001$), day 10 ($P<0.001$), day 13 ($P<0.001$) and day 30 ($P<0.0001$).

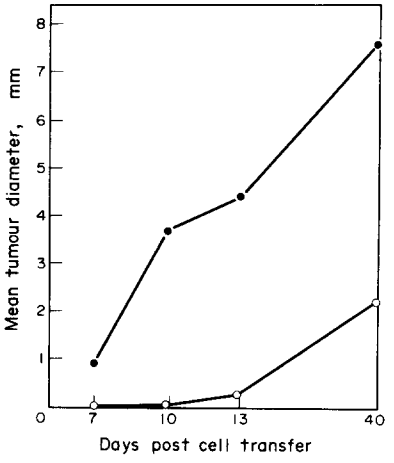

Fig. 2. Rate of growth of tumours following transfer of 10^4 *MC677 cells together with* 10^7 *splenic T cells into normal mice. Splenic T cell donors were multiparous (○——○) or age-matched virgin mice (●——●). Each point is the mean tumour diameter (mm) in each group (14 mice per group).* $P<0.001$ *at* days 7, 10 *and* 13. $P<0.0001$ *at* day 30.

DISCUSSION

These experiments show that the experience of a single multiparous pregnancy in BALB/c mice leaves them with a degree of immunity which can significantly affect the development of tumours following tumour cell challenge. The anti-tumour immunity against a challenge inoculum was relatively small, as judged by the criterion of resistance to tumour-specific transplantation antigens of chemically- and virally-induced tumours, but sufficient to inhibit temporarily a threshold dose of tumour cells following tumour challenge, and possibly also to kill and destroy a proportion of the injected cells. Although 5/15 mice in the test group, compared to 1/16 of controls, failed to develop tumours following challenge, this was not found to be significant. However, taken together with the delay in the appearance of tumours in the experimental groups compared to the controls in both experiments, and also the highly significant difference in mean tumour diameters between the two groups in experiment 2, it is reasonable to conclude that the increased tumour rejection observed in the test groups was due to anti-tumour activity in these animals.

The demonstration of resistance to tumour in multiparous mice was strongly substantiated by the clear results obtained in cell transfer experiments. A cell mixture containing 10^4 tumour cells plus 5×10^6 splenic T cells (500 times as many splenic cells as tumour cells) when transferred to normal recipients showed a significant retardation of tumour growth. However, using 10^7 splenic T cells (1000 times as many splenic cells as tumour cells) highly significant suppression of tumour formation occurred in the test group compared to controls. Only 3/14 test mice developed tumours after 13 days as compared to 13/14 controls after 7 day and the final incidence of tumours in the

Table 2. Incidence and growth rates of tumours in normal mice after transfer of splenic T cells from multiparous mice together with tumour cells

Exp. 4 Ratio of spleen to tumour cells		Spleen cell donor mice	Days post cell transfer* 15	19	23
500:1	Mean tumour diameter (mm) ± S.E.M.	Multiparous	3.07±0.08	4.75±0.98	8.75±1.05
		Controls	5.5±0.81	7.3±1.22	12.15±2.02
			$P<0.05$	$P<0.1$	NS

Exp.5 Ratio of spleen to tumour cells		Spleen cell donor mice	Days post cell transfer* 7	10	13	16	20	30	40
1000:1	Mean tumour diameter (mm) ± S.E.M.	Multiparous	0.07+0.07	0.07±0.07	0.29±0.16	ND	ND	2.21	ND
		Controls	0.93±0.07	3.71±0.41	4.41±0.32	ND	ND	7.78	ND
			$P<0.001$	$P<0.001$	$P<0.001$	—	—	$P<0.0001$	—
	Incidence of tumours†	Multiparous	1/14	1/14	3/14	6/14	6/14	6/14	6/14
		Controls	13/14	14/14	14/14	14/14	14/14	14/14	14/14
			$P=0.001$	$P=0.001$	$P=0.001$	$P=0.0019$	$P=0.0019$	$P=0.0019$	$P=0.0019$

*Cell transfers wer carried out using multiparous or age-matched virgin BALB/c splenic T cells plus 10^4 MC677 tumour cells.
†Number of mice with tumour/total number in group.
ND not done.
NS not significant.

test and control groups was 6/14 and 14/14, a highly significant suppression of tumour.

Previous work has shown that lymphoid cells from multiparous mice, as compared to age-matched virgin mice, were inhibitory to tumour cells *in vitro*, and not to adult normal cells [1, 2, 9]. On the other hand, the demonstration *in vivo* of resistance developing in animals that have been presensitized to foetal tissues has been variously reported. Thus, resistance to tumour development could not be demonstrated in some reports [10–12] whereas successful demonstration of transplantation resistance to tumours induced by presensitization with foetal tissue in mice, guinea pigs and rats has been documented [13–16], although in some experiments, sub-threshold doses of tumour cells and large numbers of animals were required [14]. Amongst possible explanations for the difficulties and failures to demonstrate resistance which have been suggested [17], sensitivity of the techniques employed is an important consideration, and in addition, the selection of the dose of tumour cells used both for challenge and transfer, is a critical factor, as shown in the present series of experiments.

The importance of these findings relates not only to resistance to appearance of nascent tumours developed as a result of pregnancy but also to the potential role of oncofoetal antigens in immunotherapeutic measures. Nascent tumours may be considered to develop from foci consisting of very few tumour cells and the presence of pre-existing immunity in mice having experienced pregnancy could a role in inhibiting or retarding growth of tumour cells, which carry oncofoetal antigens, until host resistance to tumour-specific transplantation antigens has developed and the tumour focus overcome. Whether other factors, in particular the hormonal environment, as suggested by other studies [4, 5] play a part in host resistance remains an unresolved question. However, the present findings go some way towards substantiating the role of an immunological mechanism in resistance to tumour decelopment in multiparous mice which is mediated by T cells. Since foetal antigens appear to be re-expressed in all tumours in which they have been investigated this may have implications for human tumours. The decreased risk of breast cancer occurring in women who have undergone a full-term pregnancy in early life [6] may have, at least in part, an immunological explanation.

REFERENCES

1. R. J. Brawn, Possible association of embryonal antigen(s) with several primary 3-methylcholanthrene-induced murine sarcomas. *Int. J. Cancer* **6,** 245 (1970).
2. I. Hellström and K. E. Hellström, Cytotoxic effect of lymphocytes from pregnant mice on cultured tumour cells. I. Specificity nature of effector cells, blocking by serum. *Int. J. Cancer* **15,** 1 (1975).
3. G. Steele, H. O. Sjögren and M. R. Price, Tumour associated and embryonic antigens in soluble fractions of a chemically induced rat colon carcinomas. *Int. J. Cancer* **16,** 33 (1975).
4. R. C. Moon, Relationship between previous reproductive history and chemically induced mammary cancer in rats. *Int. J. Cancer* **4,** 312 (1969).
5. P. B. Medawar and R. Hunt, Vulnerability of methylcholanthrene-induced tumours to immunity aroused by syngeneic fetal cells. *Nature (Lond.)* **271,** 164 (1978).
6. B. MacMahon, P. Cole and J. Brown, Etiology of breast cancer. A review. *J. nat. Cancer Inst.* **50,** 21 (1973).
7. K. D. Chandradasa, The stimulation and specific suppressions of concomitant immunity in two syngeneic tumour-host systems. *Int. J. Cancer* **11,** 648 (1973).
8. K. D. Chandradasa and R. M. R. Barnes, The correlation of the ongoing proliferative responses to tumour in lymphoid organs with the anti-tumour activity of the lymphoid cells. *Europ. J. Cancer* **14,** 279 (1978).
9. I. Hellström and K. E. Hellström, Cytotoxic effect of lymphocytes from pregnant mice on cultured tumour cells. II. Blocking and unblocking of cytotoxicity. *Int. J. Cancer* **15,** 30 (1975).
10. G. A. H. Buttle and A. Frayne, Effect of previous injection of homologous embryonic tissues on the growth of certain transplantable murine tumours. *Nature (Lond.)* **215,** 1495 (1967).

11. C. C. TING, D. RODRIGUES and R. B. HERBERMAN, Expression of foetal antigens and tumour specific antigens in SV40-transformed cells. II. Tumour transplantation studies. *Int. J. Cancer* **12,** 519 (1973).
12. R. W. BALDWIN, D. GLAVES and B. M. VOSE, Immunogenicity of embryonic antigens associated with chemically induced rat tumours. *Int. J. Cancer.* **13,** 135 (1974).
13. J. H. COGGIN, K. R. AMBROSE and N. G. ANDERSON, Foetal antigens capable of inducing transplantation immunity against SV40 Hamster tumour cells. *J. Immunol.* **105,** 524 (1970).
14. B. P. LE MEVEL and S. A. WELLS, JR., Fetal antigens cross-reactive with tumour specific transplantation antigens. *Nature* (*Lond.*) **244,** 183 (1973).
15. J. P. GRANT and S. A. WELLS, JR., Tumour resistance in rats immunized to fetal tissues. *J. surg. Res.* **16,** 533 (1974).
16. J. P. GRANT, S. LADISCH and S. A. WELLS, JR., Immunologic similarities between fetal cell antigens and tumour cell antigens in Guinea pigs. *Cancer Res* **33,** 376 (1974).
17. R. W. BALDWIN, M. J. EMBLETON, R. M. PRICE and B. M. VOSE, Embryonic antigen expression in experimental rat tumours, *Transplant. Rev.* **20,** 75 (1974).