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Influence of Immune Status of Host on Immunogenicity of Tumors Induced with Two Doses of Methylcholanthrene

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Summary. *Previous studies by Prehn demonstrated a direct correlation between the dose of carcinogen used for tumor induction and the immunogenicity of the resulting tumors. The purpose of the present study was to determine the role of the host's immune response and the influence of the carcinogen on immune function in this relationship. For that reason, a comparison was made of the immunogenicities of tumors induced with two doses of carcinogen in immunologically normal mice and in mice immunodepressed by adult thymectomy and irradiation. If the direct relationship between dose and immunogenicity demonstrated in normal mice was due to the degree of immunosuppression produced by the carcinogen, this correlation should not be apparent in mice already immunosuppressed. Although there was some increase in the immunogenicity of tumors induced in the immunosuppressed mice, the same relationship between carcinogen dose and immunogenicity was observed in both groups of mice. These results indicate that the degree of immunogenicity of tumors induced with both high and low doses of carcinogen was influenced by immunoselection, but in addition another, non-immunologic factor was significant in the relationship between carcinogen dose and immunogenicity.*

Introduction

Tumors induced with the carcinogen 3-methylcholanthrene (MCA) are generally immunogenic; the degree of immunogenicity exhibited by a particular MCA-induced tumor, however, appears to be influenced by a number of factors. One such factor is the dose of carcinogen used for tumor induction. A direct correlation between the dose of MCA administered and tumor immunogenicity has been demonstrated [5, 14]. A second factor is the immune response of the host, which has been shown to influence immunogenicity by selection [1, 5, 9].

One characteristic of many carcinogens, which may also be a factor in determining tumor immunogenicity, is their ability to suppress host immune function. MCA has been demonstrated to depress both cellular and humoral responses, and this depression is dose-dependent [16, 17]. This immunosuppression may be an explanation for the correlation between carcinogen dose and immunogenicity. A large dose of carcinogen may depress the host's immune response to the extent that there is no selection against strongly immunogenic tumors. A smaller, less immunosuppressive, dose of carcinogen may not effect immunoselection, in which case weakly immunogenic tumors would develop. If this hypothesis is correct, the degree of immunogenicity of tumors induced in the absence of immunoselection presumably is not related to the dose of the inducing carcinogen. However, if this relationship is based on another mechanism, such as a direct effect of the carcinogen on the transformed cells, this relationship presumably will exist for tumors induced in an immune-free environment.

The purpose of the present study was to determine whether immunoselection and the effect of MCA on immune function was the basis for the correlation between dose and immunogenicity. For that reason, a comparison was made of the immunogenicity of tumors induced with two doses of MCA in normal mice and in mice immunodepressed by adult thymectomy and irradiation. This method of immunodepression was chosen because previous results had demonstrated a lack of immunoselection for tumors induced in the absence of cell-mediated responses [1]. The results of the study suggest that although immunoselection influenced tumor immunogenicity, there was another, non-immunologic, basis for the relationship between carcinogen dose and immunogenicity.

Materials and Methods

Animals. BALB/cByJ (CBy), and C57BL/6J (B6) female mice, obtained from the Animal Resource Facility of the Jackson Laboratory, Bar Harbor, ME were housed 4–5 mice per cage and fed Old Guilford 96W Jax Lab Feed and tap water ad libitum. The mice were 5–6 weeks old at the start of the experiments.

Tumor Induction. CBy mice, thymectomized at 5–6 weeks of age, 2 weeks later received 450R of whole-body irradiation (WBI) from a General Electric Maxitron 250 X-Ray Machine at a rate of 100R/min (250 KVP and 20 MA with a 1 mm Cu and 0.5 mm Al filter). These mice will be referred to as TXR mice. An equal number of CBy mice were sham-thymectomized to serve as controls.

B6 mice, thymectomized at 5–6 weeks of age, received 800R of WBI 2 weeks later, followed by an IV injection of 2×10^6 syngeneic bone marrow cells approximately 4 h after

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Abbreviations used: B6, C57BL/6J; CBy, BALB/cByJ; IP, intraperitoneal; IR, Immunogenicity Ratio; IV, intravenous; LP, latent period; MCA, 3-methylcholanthrene; SC, subcutaneous; TXB, adult thymectomized, lethally irradiated, and injected with 2×10^6 syngeneic bone marrow cells; TXR, thymectomized and sublethally irradiated; WBI, whole-body irradiation

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irradiation. Half of these mice (TXB mice) received no further treatment. The remaining mice received an IP injection of 1×10^7 syngeneic spleen cells 2 h after the bone marrow injection. These mice will be referred to as restored mice.

Discs of 5% or 0.05% MCA in paraffin [1] were transplanted SC dorsally into TXR and normal CBy, and TXB and restored B6 mice. These mice were checked weekly for the appearance of tumors at the site of pellet implantation. The latent period (LP) for each tumor was calculated as the interval between administration of the MCA disc and development of a tumor measuring at least 7 mm in diameter.

Tumor Immunogenicity. Tumors were transferred from primary hosts into syngeneic mice to produce the first transplant generation, which was used for in vivo immunogenicity tests, as described elsewhere [6]. For each tumor, 8–12 syngeneic mice were inoculated with first-transplant-generation tumor by trocar; 8–12 mice received a sham-inoculation. The resulting tumors were excised 10 days later, when the control mice received sham-excisions. Ten days later, the mice received 450R WBI. Such irradiation has been demonstrated to inhibit the primary, but not the secondary immune response [2, 8], and for this reason has been used to make the immunogenicity test more sensitive [4]. In the case of the CBy tumors, X-irradiation was administered. For the B6 tumors gamma irradiation from a Cesium Mark 1 irradiator (J. L. Shepard and Assoc., Glendale, CA, USA) was administered at a rate of 230R/min. One day after irradiation, the mice were challenged with an SC ventral injection of $1-5 \times 10^5$ tumor cells (total cell count) suspended in 0.2 ml Eagles Minimum Essential Medium (Microbiological Associates, Walkersville, MD, USA). The suspension was prepared by enzymatic disaggregation of minced tumor fragments with 2.5 mg Pronase and 50 µg DNase (Calbiochem, Los Angeles, CA, USA) per ml in HBSS. Tumor cell suspensions prepared in this manner generally have greater than 90% viability.

Tumor growth was measured weekly, and the first reading in which the mean tumor diameter in the control mice was 5 mm or greater was the reading used for analysis. Only tests in which growth occurred in at least 67% of the control group were used for analysis. The immunogenicity ratio (IR) for each tumor was calculated by the formula:

$$\mathrm{IR} = \frac{\text{mean tumor diameter (control mice)}}{\text{mean tumor diameter (immunized mice)}}.$$

All mice, including those without tumors, were included in the calculation of the IR, which therefore reflects both tumor incidence and size in both groups of mice. An IR = 1 means that tumor growth was the same in the immunized and control mice; ratios greater than 1 indicate inhibition in the immunized mice; ratios smaller than 1 suggest stimulation. Because large IRs usually represent growth in only one or two immunized mice and the value can be easily skewed, the IRs were arbitrarily truncated at 10.

Skin Grafting. To determine the immunocompetence of the various groups of mice, split-thickness grafting was performed [10]. Donor and recipient mice differed at one or more minor histocompatibility (non-H-2) loci. The skin was removed from the donor mouse, the underlying fat scraped away with a dull scapel blade, and pieces of skin 13 mm in diameter were punched out with a number 9 cork borer.

A split-thickness graft bed (with underlying fat intact) was prepared in recipient mice on the right side just behind the forelimb. The graft was placed on the bed, and covered with a small piece of adaptic non-adhesive dressing (Johnson & Johnson, New Brunswick, NJ, USA) impregnated with Vaseline petroleum jelly. A bandage of Clear Dermicel Tape (Johnson & Johnson), wrapped around the midriff of the mouse, covered the graft and dressing. Bandages were kept in place for 6–8 days. Thereafter, grafts were examined daily for the first 3 weeks and then twice weekly. The first reading in which no trace of the graft was visibly detectible was considered the time for 100% rejection. Technical failures due to grafts slipping off the graft bed were excluded from the results.

Statistical Methods. Tumor incidences were compared by Chi-square. The Mann-Whitney U-test was used to compare tumor immunogenicities and skin graft rejection times [15].

Results

Tumor Induction and Immunogenicity

As expected, tumors arose more rapidly and with greater frequency in mice receiving 5% MCA than in mice receiving 0.05% MCA. In addition, immunodepression was found to have no significant effect on tumor induction with either dose of MCA (Table 1).

Previous studies [1, 5, 9, 12] have demonstrated an inverse correlation between LP and immunogenicity. For that reason, tumors with similar LP were chosen from the various groups to be tested for their immunogenicities. A total of 39 CBy and 56 B6 individually induced tumors were tested for their immunogenicities. The latencies of tumors tested ranged from 90 to 145 days (median = 125) for the CBy tumors, and from 98 to 189 days (median = 146) for the B6 tumors. The IR for the tumors tested have been plotted on scatter diagrams (Figs. 1 and 2) and the mean IR for each group appears in Table 2. In general, tumors induced with 5% MCA were more immunogenic than those induced with 0.05% MCA in both control and immunodepressed mice. This difference reached statistical significance (Mann-Whitney U-test) only in the case of tumors induced in TXR CBy mice and in restored B6 mice. However, in all four groups the mean IR was larger, and the percentage of tumors with IR of 5 or greater was higher for tumors induced with 5% MCA than with 0.05% MCA (Table 2). In addition, there was a slight increase in the immunogenicities of tumors induced with both doses of MCA in the immunodepressed, as against the respective control, mice. In no case, however, was the increase significant.

Skin Grafting

Skin grafting was performed to measure the immunocompetence of TXR and TXB mice and mice bearing discs of 5% and 0.05% MCA. TXR and control (sham-thymectomized and irradiated) CBy mice received discs of either paraffin or either 5% or 0.05% MCA in paraffin and grafts of DBA/2J skin 41 days after irradiation. Graft rejection was significantly delayed in TXR compared with control mice, but the presence of 5% or 0.05% MCA discs had no significant effect on graft rejection (Table 3). TXB and restored B6 mice were grafted with 129/J skin 47 days after irradiation. Graft rejection was significantly later in the TXB than in the restored mice

Table 1. Percent tumor incidence 200 days after administration of discs of 5% or 0.05% MCA

Mice	Concentration of MCA		P value[b] 5% vs 0.05%
	5%	0.05%	
BALB/cByJ			
Normal	100% (54/54)[a]	35% (24/68)	< 0.001
TXR	98% (48/49)	36% (21/58)	< 0.001
P value, normal vs TXR	NS[c]	NS	
C57BL/6J			
Restored	93% (28/30)	33% (54/162)	< 0.001
TXB	93% (28/30)	32% (42/132)	< 0.001
P value, restored vs TXB	NS	NS	

[a] Number of mice with tumors/total number of mice
[b] P value determined by Chi square
[c] Not significant

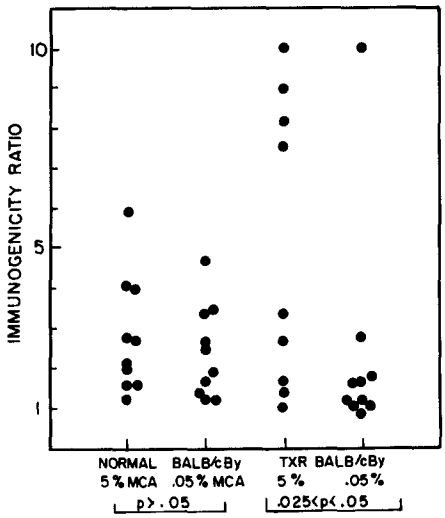

Fig. 1. Immunogenicity ratios [IR = Mean tumor diameter (control)/Mean tumor diameter (immune)] of tumors induced with 5% and 0.05% MCA in normal and TXR BALB/cByJ mice. P values determined from Mann-Whitney U-test

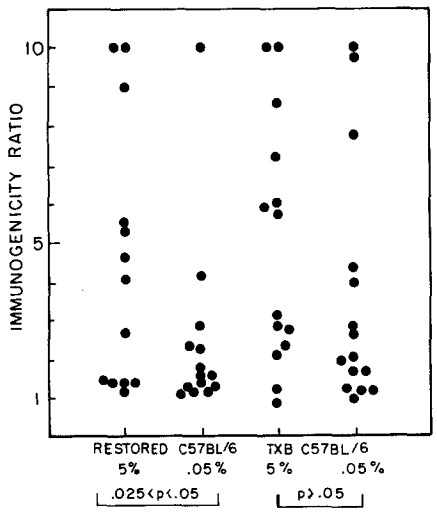

Fig. 2. Immunogenicity ratios [IR = Mean tumor diameter (control)/Mean tumor diameter (immune)] of tumors induced with 5% and 0.05% MCA in TXB and restored C57BL/6J Mice. P values determined by Mann Whitney U-test

Table 2. Mean IR and percent tumors with IR > 5 for tumors induced with 5% and 0.05% MCA

Mice	Concentration of MCA			
	5%		0.05%	
	Mean IR	% IR > 5	Mean IR	% IR > 5
BALB/cByJ				
Normal	2.8	10% (1/10)[a]	2.4	0% (0/10)
TXR	5.0	44% (4/9)	2.3	10% (1/10)
C57BL/6J				
Restored	4.5	38% (5/13)	2.5	7% (1/14)
TXB	4.9	50% (7/14)	3.6	20% (3/15)

[a] Number of tumors with IR > 5/total number of tumors tested

Table 3. Time for 100% rejection of skin grafts

Expt. 1: BALB/cByJ mice grafted with DBA/2J skin 41 days after irradiation

Disc present					
5% MCA		0.05% MCA		Paraffin	
TXR[a]	Control[b]	TXR	Control	TXR	Control
15[c]	15	12	13	15	12
19	15	19	15	21	12
19	15	21	17	23	13
29	15	21	17	29	15
29	17	29	18	29	17
29	17	29	21	39	17
39	29	29		40	18
		29			19
		29			19
		39			
$P = 0.02$[d]		$P = 0.02$		$P < 0.01$	

Expt. 2: C57BL/6J mice grafted with 129/J skin 47 days after irradiation

TXB[e]	Restored[f]
24	13
24	15
29	17
29	17
34	17
45	17
52	17
52	18
63	19
66	20
72	21
	22
	26
$P < 0.001$	

[a] TXR mice were thymectomized and received 450R WBI
[b] Control mice were sham-thymectomized and received 450R WBI
[c] Rejection time in days
[d] P value determined by Mann-Whitney U-test
[e] TXB mice were thymectomized, received 800R WBI, 2×10^6 BM cells IV
[f] Restored mice were thymectomized, received 800R WBI, 2×10^6 BM cells IV, 1×10^7 spleen cells IP

(Table 3). Similar results (data not shown) were obtained in mice grafted 21 and 81 days after irradiation.

Discussion

Previous results reported by Prehn [14] and Johnson [5] demonstrated a direct correlation between the dose of MCA used for tumor induction and the immunogenicity of the tumors produced. This relationship was confirmed in the present study for tumors induced in normal and immunologically restored mice. The results of these studies, however, are in disagreement with those of Stutman, who found no such relationship [18]. Differences in the strains of mice used, the method of carcinogen administration, and, most importantly, the doses of MCA used may account tor this discrepancy. All the doses of MCA used by Stutman were relatively large, and the tumors were, for the most part, strongly immunogenic.

The purpose of the present study was to investigate the role of immunoselection in the relationship between carcinogen dose and immunogenicity. Immunoselection was originally described by Old et al., as an explanation for the inverse correlation between tumor latency and immunogenicity [9]. If selection by the immune response influences tumor induction, immunosuppression by carcinogen, especially large doses of carcinogen, may alter or eliminate this selection. This could be one explanation for the direct correlation between carcinogen dose and immunogenicity. Tumors induced with small doses of carcinogen would be less immunogenic than those induced with larger doses that had suppressed selection. If this hypothesis is correct, tumors induced in the absence of immune influence should not display any relationship between carcinogen dose and immunogenicity. The results of one study by Mondal et al. [7], on in vitro transformation of prostate cells by various doses of MCA, support this hypothesis. No apparent relationship was detected between the dose of MCA used for transformation and the immunogenicity of the resulting tumors. However, the majority of the tumors tested were strongly immunogenic; perhaps smaller doses of carcinogen would have produced less immunogenic tumors.

To examine the role of immunoselection in the relationship between carcinogen dose and immunogenicity in the present study, tumors were induced with two doses of MCA in control mice and mice immunodepressed by adult thymectomy and irradiation. Although they do not rule out immunoselection as a factor, the results do indicate that another, non-immunologic, mechanism was at least partially responsible for this relationship. Tumors induced in immunodepressed mice tended to be more immunogenic than those induced with the same dose of MCA in control mice, suggesting that immunoselection of tumors induced with both doses of MCA was diminished in the immunodepressed mice. However, the direct correlation between carcinogen dose and tumor immunogenicity was demonstrated in the immunodepressed mice as well as the controls. The fact that skin graft rejection was significantly delayed in the TXB and TXR mice indicates that T-cell-dependent responses were substantially inhibited in these mice and were not essential for the relationship between carcinogen dose and immunogenicity to occur. Similarly, other work has suggested that the weak immunogenicity of many spontaneous tumors is not due to host immunoselection [3, 13]. The results of this study indicate that immunoselection was a factor in determining tumor immunogenicity, but that in addition, another, non-immunologic mechanism was essential for the direct correlation between carcinogen dose and immunogenicity.

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