Europ. J. Cancer Vol. 15, pp. 39–45
Pergamon Press Ltd. 1979. Printed in Great Britain

Immune Mechanisms in Ehrlich Ascites Tumor Growth in Mice

MATKO MARUŠIĆ*

University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences, Biology Division, Oak Ridge National Laboratory,† Oak Ridge, Tennessee 37830, *U.S.A.*

Abstract—*Normal mice immunized with irradiated Ehrlich ascites tumor (EAT) cells rejected EAT challenge given* 2 weeks *later but T-cell-deficient [thymectomized lethally irradiated, and bone-marrow-reconstituted (TIR)] mice succumbed. However, when TIR mice were injected i.v. with thymus, lymph node, or spleen cells from normal syngeneic donors immediately following i.p. injection of irradiated EAT cells, they rejected the subsequent tumor challenge. This induction of immunity in TIR mice was shown to be T-cell dependent. Spleen cells from EAT-bearing mice given immediately after irradiated tumor cells were also able to promote rejection of EAT challenge in TIR mice.*

Spleen cells from EAT-immune mice inhibited EAT growth when admixed with tumor cells prior to i.p. injection into normal recipients, but had no effect on progressive tumor growth when given i.v. immediately after i.p. tumor injection. Immune serum inhibited i.p. EAT growth when given either i.p. or i.v. Whereas inhibition of EAT growth by admixed spleen cells was shown to be T-cell dependent, the activity of the immune serum appeared to be T-cell independent. The data indicate that T lymphocytes are required only in the induction phase of the immune response of mice against EAT, while the efferent phase of the response is accomplished by serum antibodies, perhaps through an interaction with host macrophages.

INTRODUCTION

DESPITE the fact that the immune reaction of mice to Ehrlich ascites tumor (EAT) has been extensively studied, it is still poorly understood. It has been clearly shown that mice can be immunized against EAT [1–6], but very little is known about the mechanisms leading to tumor rejection in immune animals. That anti-EAT immunity could be cell-mediated was suggested by cytological analysis [7] and by cell-transfer studies [3, 8]. However, tumor-bound antibodies which agglutinated but did not kill tumor cells *in vitro* have been detected in EAT-immune mice [9]. Some *in vitro* cytotoxicity against EAT was demonstrated with the serum from mice with advanced EAT growth in a solid form [10], and passive immunization of naive recipients with the serum from EAT-immune donors has been reported [3].

Despite strain nonspecificity of EAT, it is well established that immunity against EAT in mice is not directed against the major histocompatability complex antigens on tumor cells [9, 11]. The precise identity of antigens against which the immune response is directed is not known, but tumor-specific antigens would appear to be major candidates [9]. The contributing role of T and B lymphocytes in immunity against EAT has not been investigated, and we know of no report clarifying the different observations on the role of cellular and humoral immune response against this tumor. Therefore, the present experiments were designed to investigate anti-EAT im-

Accepted 6 June 1978.
*Postdoctoral Investigator supported by Subcontract No. 3322 from the Biology Division of Oak Ridge National Laboratory to the University of Tennessee, and by the Fulbright–Hays Program; on leave of absence from the Department of Physiology, University of Zagreb Faculty of Medicine, Zagreb, Croatia, Yugoslavia. This is where the correspondence should be addressed.
†Operated by the Union Carbide Corporation for the Department of Energy.

munity in more detail, utilizing T-cell deficient [thymectomized, lethally irradiated, bone marrow reconstituted (TIR)] mice [12]. It was expected that, if the induction of the immune response was T-cell dependent, injection of irradiated EAT cells would not induce immunity against the tumor in TIR mice. However, T cells from normal syngeneic donors should permit the development of anti-EAT immunity when given to TIR mice simultaneously with irradiated EAT cells. The present data confirmed these expectations. Induction of anti-EAT immune response was clearly shown to be T-cell dependent. In contrast, studies on the efferent phase of immune reaction against EAT revealed a significant role of serum factors and lack of contribution of T lymphocytes.

MATERIALS AND METHODS

Mice

CBA mice (Jackson Memorial Laboratory, Bar Harbor, ME) of both sexes were used at 16–20 weeks of age. In a given experiment all mice were of a single sex.

TIR mice were used in the experiments 7–9 weeks after irradiation and reconstitution. These mice were thymectomized at 8–10 weeks of age and irradiated and reconstituted 4–6 weeks afterwards.

EAT

The tumor was obtained from Dr. L. H. Smith of the Biology Division, Oak Ridge National Laboratory, who had maintained it in $(C3H \times 101)F_1$ mice. For the present experiments tumor cells were serially passaged i.p. in CBA mice.

Irradiation

Mice were exposed to 800R total-body irradiation with a Philips X-ray machine (0.5 mm Cu HVL with 1.0 Al added filtration, at 250 kV and 15 mA). The target-to-object distance was 60 cm; the exposure rate ranged from 150 to 160 R/min, measured in air. EAT cells were exposed to 10,000 R at a dose of about 1100 R/min.

Preparation and use of anti-Thy 1.2 antiserum

These procedures have been described in detail [13].

Immunization of mice to EAT

Mice were initially injected i.p. with 10^7 irradiated EAT cells and were challenged 2 weeks later by i.p. injection of 10^6 EAT cells. Mice that survived for 2 months were again challenged with 10^6 EAT cells and were used as donors of EAT-immune spleen cells and serum 1–2 months after the second challenge.

Inhibition of tumor growth by admixed lymphoid cells

Equal volumes of spleen and EAT cell suspensions were mixed together and 10^6 EAT cells with the desired number of spleen cells in 1.0 ml were injected i.p. immediately afterwards.

Evaluation of tumor growth

Mice were challenged i.p. with 10^6 EAT cells and mortality scored daily for 2 months. Mice that survived for 2 months were considered to have rejected the tumor. Inasmuch as most CBA mice that survive for 2 months after i.p. injection of 10^6 EAT cells survive a normal life-span, this criterion appeared warranted.

Statistics

Mortality data was compared using the chi-square test with Yates correction.

RESULTS

Requirements for the induction of immunity against EAT in mice

To determine whether the presence of the thymus or thymus-derived T-cells are required for development of anti-EAT immunity in mice, (a) normal mice, (b) adult thymectomized mice, (c) irradiated and bone marrow reconstituted mice and (d) TIR mice who were injected i.p. with 10^7 irradiated EAT cells and challenged i.p. with 10^6 live EAT cells 2 weeks later. Control groups that had not been given irradiated EAT cells were similarly challenged. Table 1 shows that all groups of mice that had not been injected with irradiated EAT cells succumbed after EAT challenge. Of the mice that had been given irradiated tumor cells, normal mice, adult-thymectomized mice and irradiated bone-marrow-reconstituted mice rejected the tumor challenge, but TIR mice succumbed. The result suggested that thymus-dependent T cells are required for the induction of anti-EAT immunity in mice.

At once it follows that injection of T-cells should enable TIR mice to generate anti-EAT immunity. Therefore, TIR mice were injected i.p. with 10^7 irradiated EAT cells, and immediately thereafter i.v. with syngeneic thymus, lymph node or spleen cells. Groups were also included in which TIR mice re-

*Table 1. Thymus-dependence of active immunization of CBA mice against EAT**

Pretreatment of mice	Irradiated EAT cells	Deaths/total No. of recipients
None	−	9/10
	+	9/10
Thymectomized†	−	8/8
	+	0/9
Irradiated and reconstituted	−	7/7
	+	1/10
TIR‡	−	9/10
	+	9/10

*Two weeks after immunization with 10^7 irradiated (10,000 R) EAT cells, mice were challenged with 10^6 EAT cells. Mortality was scored for 2 months.
†Thymectomy was performed at 8–10 weeks of age.
‡At 2–4 weeks after thymectomy, mice were irradiated with a dose of 800 R and reconstituted with 4×10^6 bone marrow cells. They were used in the experiment 2 months later.

ceived spleen cells treated with anti-Thy 1.2 antiserum and complement, spleen cells treated with normal serum and complement and, finally, spleen cells from TIR mice. As seen in Table 2, thymus, lymph node and spleen cells facilitated development of anti-EAT immunity in TIR mice, the effect clearly being cell dose-dependent. While the effectiveness of lymph node cells was similar to that of spleen cells, thymus cells appeared to be less effective. In contrast to TIR mice given spleen cells pretreated with normal serum and complement, TIR mice given spleen cells pretreated with anti-Thy 1.2 antiserum and complement failed to reject EAT challenge ($P < 0.05$). This and the inefficiency of spleen cells from TIR mice clearly demonstrated that it is the T lymphocyte which enables resistance to be generated in the TIR recipients. The requirement for live cells is illustrated by the failure of irradiated spleen cells to induce any anti-EAT resistance.

Efferent mechanisms of the immune response to EAT

To assess the efferent phase of anti-EAT immune response, spleen cells from EAT-immune mice were tested for inhibition of EAT growth when admixed with tumor cells before injection to normal and TIR recipients. Table 3 shows that spleen cells from EAT-immune mice inhibited tumor growth when admixed with EAT cells and injected i.p. to

*Table 2. Development of anti-EAT immunity in TIR mice injected with normal syngeneic thymus, lymph node or spleen cells immediately after i.p. injection with irradiated EAT cells: requirement for T-cells**

Transferred lymphoid cells			Deaths/total No. of recipients	P†
Origin	Number ($\times 10^{-6}$)	Treatment		
No lymphoid cells			24/27	
Thymus	10	None	10/10	NS
	30	None	6/10	NS
	60	None	4/10	0.01
Lymph nodes	1	None	6/10	NS
	10	None	2/10	0.001
	30	None	1/10	0.001
Spleen	1	None	5/10	0.05
	10	None	3/10	0.001
	30	None	1/10	0.001
	30	Irradiated	10/10	NS
	20	Normal serum plus complement	2/10	0.001
	20	Anti-Thy 1.2 antiserum plus complement	8/10	NS
	30	From TIR donors	10/10	NS

*Two months after irradiation and reconstitution TIR mice were given i.p. 10^7 irradiated (10,000 R) EAT cells, then injected immediately i.v. with lymphoid cells. Two weeks later mice were challenged i.p. with 10^6 EAT cells and mortality was scored for 2 months.
†Comparison with mortality of TIR mice given no lymphoid cells (top line) using chi-square test with Yates correction.

*Table 3. Effect of spleen cells from EAT-immune mice on the intraperitoneal growth of 10^6 EAT cells in normal and T-cell deficient recipients**

Spleen cells			Deaths/total No. recipients	
Number ($\times 10^{-6}$)	Route of injection	Treatment	Normal	TIR
No spleen cells			10/10	8/8
50	i.p.	None	0/9	0/10
25	i.p.	None	0/6	0/10
12	i.p.	None	0/6	ND
6	i.p.	None	1/6	
3	i.p.	None	6/6	ND
20	i.p.	None	0/10	ND
20	i.p.	Normal serum plus complement	1/10	ND
20	i.p.	Anti-Thy 1.2 antiserum plus complement	8/10	ND
40	i.v.	None	7/8	10/10
20	i.v.	None	10/10	10/10

*TIR mice were used 2 months after irradiation and reconstitution. Spleen cells injected i.p. were admixed with EAT cells before injection. Spleen cells injected i.v. were given immediately after tumor injection. Spleen cells from normal donors were always used as control. In no case they influenced progressive EAT growth in the recipient mice. ND = not done.

normal recipients at ratios as low as 6:1. EAT growth was also inhibited when the mixture of spleen and EAT cells was injected to TIR mice. To test if a T-cell component in the spleen cells is required for the inhibition of EAT growth, immune spleen cells were treated with anti-Thy 1.2 antiserum and complement or normal serum and complement before mixing with the EAT cells. It can be seen in Table 3 with immune spleen cells that were pretreated with anti-Thy 1.2 antiserum and complement failed to inhibit EAT growth ($P < 0.01$ for comparison with the effect of cells treated with normal serum and complement). It should be noted that for each experimental group a control was set up in which normal rather than immune spleen cells were used. Normal spleen cells had no influence on the progressive EAT growth in all recipients (data not shown). In contrast to the preceding results, as many as 40×10^6 spleen cells from EAT-immune mice were totally ineffective in inhibiting tumor cell growth when injected i.v. immediately after i.p. injection of EAT in either normal or TIR recipients.

To further characterize the efferent mechanisms of the immune response to EAT, serum from EAT-immune mice was mixed with 10^6 EAT cells and injected i.p. or EAT-immune serum was injected i.v. immediately following i.p. injection of 10^6 EAT cells (Table 4). Whereas the serum from normal mice had no effect on tumor growth, as little as 0.1 ml of the immune serum inhibited EAT growth in normal mice when given either i.v. or i.p. It was also found that 0.1 ml of serum from EAT-immune mice given i.v. inhibited tumor growth in both TIR recipients and in recipients that were thymectomized 11 months earlier. Inasmuch as both the TIR mice and mice thymectomized 11 months previously are T-cell deficient [14], it appears that the serum from EAT-immune mice does not interact through T lymphocytes in the inhibition of tumor growth.

In an effort to see if the immune serum "armed" recipients' macrophages, splenectomized or silica-treated recipients were given 0.1 ml of serum from EAT-immune mice i.v. immediately following i.p. tumor injection. Splenectomy was performed 2 weeks before the test. Silica particles (Silica DQ 12, 5 μm, was supplied by Dr. K. Robock, Wirtshaftverband Azbestzement e.e. 404 Neuss, West Germany), which are cytotoxic for macrophages [15], were given i.v. in a dose of 5 mg per animal 6 hr before serum injection. Immune serum afforded protection equally well to either splenectomized or normal recipients. In contrast, only minimal protection (in comparison to normal recipients, $P < 0.1$) was afforded with immune serum in silica-treated recipients suggesting, but not proving, peritoneal macrophage involvement.

*Table 4. Effect of the serum from EAT-immune mice on the intraperitoneal growth of 10^6 EAT cells**

Serum			Deaths/total No. of recipients given		
Amount (ml)	Route of injection	Recipients	Immune serum	Normal serum	P
0.3	i.v.	Normal	0/10	7/7	0.001
0.1	i.v.	Normal	1/10	7/7	0.001
0.025	i.v.	Normal	10/10	10/10	NS
0.1	i.p.	Normal	0/10	9/9	0.001
0.025	i.p.	Normal	10/10	7/7	NS
0.1	i.v.	TIR	3/20	7/8	0.001
0.1	i.v.	Thymectomized 11 months earlier	0/10	6/6	0.001
0.1	i.v.	Splenectomized	0/14	8/8	0.001
0.1	i.v.	Silica treated	6/10	8/8	NS

*TIR mice were used 2 months after irradiation and reconstitution, splenectomized mice were used 2 weeks after splenectomy. Silica particles were given i.v. in a dose of 5 mg/recipient, 6 hr before serum injection. Mortality of immune serum-injected and normal serum-injected recipients were compared using chi-square test with Yates correction. NS = not significant.

Anti-EAT reactivity of spleen cells from EAT-bearing mice

Spleen cells from EAT-bearing mice were given i.v. to TIR mice immediately following an i.p. injection of irradiated EAT cells to determine if the spleen cells from EAT-bearing mice can generate anti-EAT resistance in TIR recipients as did the spleen cells from normal mice (see Table 2). Spleen cells from EAT-bearing mice were simultaneously tested for inhibition of EAT growth when admixed with tumor cells prior to injection to normal recipients. The two tests were thus compared in order to see whether they provide the same or different information on the the anti-tumor activity of spleen cells from tumor-bearing animals. Spleen cells to be tested were harvested from tumorous mice 6, 9, 12 and 15 days after i.p. injection of 10^6 EAT cells. Spleen cells from normal (non-immunized) donors and from donors immune to EAT were used as controls. As previously described, TIR mice are injected i.p. with 10^7 irradiated EAT cells and immediately thereafter i.v. with 2×10^7 spleen cells. They are challenged i.p. with 10^6 EAT cells 2 weeks later. Ratio of spleen to tumor cells in the admixture-inhibition assay was 20:1 (10^6 EAT cells per recipient). Table 5 shows that spleen cells from EAT-immune mice inhibited tumor growth in both tests. However, the activity of spleen cells from normal (non-immune) mice and from tumor-bearing mice differed strikingly in two tests. Neither spleen cells from normal donors nor those from EAT-bearing donors had any effect on the progressive EAT growth in the admixture-inhibition assay, but both enabled TIR mice to reject EAT challenge.

Table 5. Anti-EAT-activity of spleen cells from normal, EAT-immune† and EAT-bearing mice tested in the Winn inhibition assay‡ and in TIR mice§*

Spleen cells donors	Deaths/total No. recipients	
	Inhibition assay	Assay in TIR mice
None	6/6	15/17
Normal	6/6	6/24
EAT-immune	0/8	0/14
EAT-bearing		
6 days	7/8	4/17
9 days	8/8	1/17
12 days	8/8	5/16
15 days	8/8	0/17

*Both spleen cell donor and recipient mice were injected i.p. woth 10^6 EAT cells.

†Initially injected with 10^7 irradiated (10,000 R) EAT cells. Rejected two subsequent i.p. challenges with 10^6 EAT cells.

‡Spleen and tumor cells were admixed *in vitro* in a 20:1 ratio and immediately injected i.p.

§Two months after irradiation and reconstitution, TIR mice were injected i.p. with 10^7 irradiated EAT cells (10,000 R), then immediately injected i.v. with tested spleen cells. Two weeks later they were challenged i.p. with 10^6 live EAT cells. Mortality was scored for 2 months.

DISCUSSION

The present study demonstrates a T-cell dependency of the induction phase of the immune response of mice against EAT. The obligatory role of the thymus for the maturation of the fully functional T cells necessary to mount an immune response against EAT has been shown (Table 1) and the sensitivity of the afferent limb of anti-EAT immune response to anti-Thy 1.2 antiserum and complement treatment demonstrated (Table 2). Accordingly, cells from the thymus, lymph nodes and spleen (i.e., from the lymphoid organs that contain T lymphocytes) facilitated anti-EAT immunity in TIR mice when given immediately after irradiated tumor cells (Table 2). The lower activity of thymus cells (in comparison to lymph node and spleen cells) might be explained by the immunological immaturity of the majority of these cells [14]. The essential role of the antibody in the efferent phase of EAT rejection in both admixture-inhibition and passive immunization experiments (Table 4), indicates that the required T-cell function was limited to helper activity for antibody synthesis. Furthermore, the lack of requirement for T cells in the efferent phase of tumor rejection is documented by the ability of the serum from the immune donors to inhibit EAT growth in T-cell-deprived recipients (Table 4). The latter is in accord with the data obtained by others [16].

The observed T-cell dependency of the inhibition of EAT growth by admixed spleen cells from EAT-immune mice (Table 3) is not at variance with the finding that T-cells do not contribute in the efferent phase of anti-EAT immune response. It is reasonable to assume that the inhibition of EAT growth by admixed EAT-immune spleen cells involved both the afferent and efferent limb of the immune reaction for tumor destruction. Therefore, the observed T-cell dependency would only reflect a requirement for spleen-derived T-cell helper function in antibody production. The failure of immune spleen cells given i.v. to inhibit i.p. EAT growth (Table 3) most probably was due to their inability to produce immediately sufficient amounts of circulating anti-EAT antibodies to contain tumor growth. Furthermore, spleen cells given i.p. were in intimate contact with EAT cells before injection into the recipients, and the failure of spleen cells given i.v. to ensue rejection might also be due to the lack of adequate antigen stimulus immediately after injections.

Although the data presented in Table 4 suggest that T cells are not involved in the effector phase of the immune response against EAT, the cell type involved in the destruction of the tumor, if any, remains to be definitively identified. The weaker anti-EAT effect of the immune serum in silica-treated recipients than in non-treated recipients suggested macrophage involvement, but additional data is needed before a conclusion can be reached. The finding that the serum was as effective in splenectomized as in normal recipients (Table 4) indicates that the cytotoxic action of the serum can be accomplished in the absence of the heterogeneous spleen cells population, but does not exclude a contribution of the lymphoid cells from other lymphoid organs.

Despite a remarkable cell proliferation in the spleens of CBA mice injected i.p. with EAT cells [17], spleen cells from EAT-bearing mice were unable to inhibit EAT growth when admixed with tumor cells before injection to normal recipients (Table 5). On the other hand, they were as effective as normal and immune spleen cells in the induction of anti-EAT resistance in TIR mice (Table 5). These findings suggest that spleen cells from EAT-bearing mice were neither immune nor suppressed in their ability to respond to EAT antigens. Taken out of the tumorous mouse environment and exposed for 2 weeks to tumor antigens in TIR recipients they were able to develop a good antitumor immunity. This is in contrast to the reports suggesting T-cell depletion [18] and impaired immune response [19] in EAT-bearing mice.

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