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Enhancing T lymphocytes from tumor-bearing mice suppress host resistance to a syngeneic tumor*

The cell-mediated immune response of animals to a lethal syngeneic tumor was investigated by inoculating C57BL mice with Lewis lung carcinoma (3LL) cells. T lymphocytes, obtained from the enlarged spleens of the tumor-bearing mice were found to be cytotoxic to 3LL target cells *in vitro*. However, we found that such spleen cells enhanced tumor growth *in vivo* when mice were injected with a mixture of spleen cells and tumor cells. Removal of T lymphocytes by treatment of the spleen cells with anti-Θ serum plus complement reduced the enhancement of tumor growth. Hence, the tumor enhancing cells, like the cytotoxic cells, appeared to be T lymphocytes. Removal of T lymphocytes from normal mice by adult thymectomy before tumor inoculation led to a reduction in the number of tumor metastases. Thus, enhancing T lymphocytes appear to exist in normal as well as in tumor-bearing mice. Investigation of this mechanism of tumor enhancement suggested that the enhancing T lymphocytes act as suppressor T cells inhibiting natural immune resistance to tumor growth.

1. Introduction

Tumor-specific antigens have been demonstrated in most tumors investigated [1]. Nevertheless, the occurrence of an immune response against such tumor antigens does not appear to prevent the development of the tumor [1, 2]. One of the ways to explain this paradox is to assume that the host's immune response may enhance as well as inhibit the growth of a tumor [1, 2]. Enhancement has been considered to be mediated by humoral antibodies which are thought to bind to tumor-specific antigens and block an effective immune response. This notion is based on experiments involving allogeneic tumor grafts [4–6]. The evidence for antibody-mediated enhancement of syngeneic tumors is scarce. On the other hand, inhibition of the growth of solid tumors has been considered to result from cell-mediated immunity [3, 7]. However, we recently found that lymphocytes which were sensitized *in vitro* against syngeneic fibroblasts [8, 9] or tumor cells [10] enhanced tumor growth when the lymphocytes were inoculated with the tumor cells into syngeneic mice. The lymphocytes which were sensitized in these experiments were found to consist mainly of T cells [11]. Other investigators have also suggested recently that cell-mediated immunity under certain conditions could promote tumor growth both *in vivo* [12] and *in vitro* [13].

The aim of the present study was to investigate the cell-mediated immune response developing towards a tumor transplanted into a syngeneic host, and to identify the cells which affect the growth of tumor cells *in vivo* and *in vitro*. We found that T lymphocytes from tumor-bearing mice produced cytotoxic effects against the tumor cells *in vitro*, but enhanced the growth of the same tumor cells when injected into syngeneic mice.

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Abbreviations: EM: Dulbecco's modification of Eagle's medium **PBS:** Phosphate buffered saline

2. Materials and methods

2.1. Animals and their irradiation

Inbred C57BL/6 mice were supplied by the Animal Breeding Center of the Weizmann Institute of Science, Rehovot, Israel.

Male mice were used at the age of 6 – 10 weeks. Adult thymectomy of some mice was performed at the age of 6 weeks, according to the technique described by Gross [14]. Control mice were sham thymectomized. A ^{60}Co gamma source (150 A, Atomic Energy of Canada, Ottawa) was used for irradiation of mice. The mice received 450 or 750 r total body irradiation (75 cm distance at 52 r/min).

2.2. Tumor

The Lewis lung carcinoma (3LL), a malignant metastasizing tumor which developed spontaneously in a C57BL/6 mouse [15], was used. It was maintained by transfer of tumor cells subcutaneously to syngeneic male mice. After subcutaneous inoculation of 5 x 10^5 tumor cells local tumors appeared within 1 – 2 weeks and the mice died from lung metastases after 4 to 8 weeks.

Suspensions of tumor cells were prepared by treatment of minced tumor tissue for 30 min with a solution of 0.3 % trypsin. The cells were washed three times with phosphate buffered saline (PBS) and suspended in Dulbecco's modification of Eagle's medium (EM) for inoculation *in vivo*, or in Waymouth's medium supplemented with 5 % calf serum for tissue culture.

2.3. Spleen cells

The spleens of C57BL mice injected subcutaneously with 3LL cells were found to progressively enlarge up to 2 – 3 times normal weight during the first 3 weeks of tumor growth. From 12 to 28 % of the nucleated cells in these enlarged spleens were usually found to be large blast-like lymphocytes. Only 1 – 3 % of such cells were found in normal spleens. After the first 3 weeks of tumor growth the spleens were observed to shrink in size as tumor metastases appeared in the lungs of the mice.

In these studies we used spleen cells taken from mice 10 days after they were inoculated subcutaneously with 10^6 3LL tumor cells. At this time the local tumors were 0.5 to 1.5 cm in diameter and the spleens were all near their peak enlargement. Spleens were removed aseptically and suspensions of spleen cells were prepared by using a fine wire mesh. The cells were washed three times with PBS and suspended in EM for inoculation into mice or in EM + 15 % fetal calf serum for cytotoxic assay *in vitro*.

2.4. Assays of tumor development

2.4.1. Local tumor development

Spleen cells from normal or tumor-bearing mice mice were mixed with suspensions of tumor cells and the cell mixtures were inoculated subcutaneously into the right hind footpads of syngeneic C57BL mice using a No. 27 needle. In some experiments (Fig. 4) spleen cells were injected 5 days before tumor inoculation. Each experimental group contained 9 – 11 mice. The animals were examined daily for tumors. The development of a visible tumor, as compared to the uninjected left footpads, was considered significant.

2.4.2. Evaluation of number of metastases

In some experiments we evaluated tumor growth by counting the number of spontaneous lung metastases. Groups of 12 to 16 treated or control mice were injected subcutaneously with 5 x 10^5 tumor cells. Three weeks later the animals were killed and their lungs were observed under a low power dissecting microscope for the presence of subpleural metastatic nodules. The number of subpleural tumors has been found to provide an accurate estimate of the total number of metastases present in the lungs [16].

2.5. *In vitro* assay of cytotoxicity

The microtest developed by Takasugi and Klein [17] was used with minor modifications [10]. In brief, 2 x 10^3 3LL tumor cells in a volume of 10 µl of culture medium (Waymouth's medium + 5 % calf serum) were dispensed into the wells of microplates (Falcon Plastic Products, Los Angeles, Calif.). Twenty hours later the medium was removed by suction and replaced by immune or control spleen cells suspended in EM supplemented with 15 % fetal calf serum. After an additional 24 h of incubation the plates were flooded with Waymouth's medium and gently shaken in order to remove the nonadherent tumor cells and the lymphoid cells. This procedure was repeated until there remained only attached 3LL target cells. The number of surviving tumor cells was recorded in the wells using a phase contrast microscope. Only spindle shaped 3LL cells were counted and the identity of the wells was revealed only after the results were tabulated. The percent of cytotoxicity was calculated as follows:

$$\frac{\text{Number of cells in control group} - \text{number of cells in test group}}{\text{Number of cells in control group}} \times 100$$

Significance of the differences between experimental and control groups was evaluated by Student's t-test.

2.6. Removal of Θ-positive spleen cells

Anti-Θ serum was prepared according to the method of Reif et al. [18]. AKR/J mice were immunized by CBA/Lac thymus cells. The serum killed 100 % of C57BL thymus cells at a dilution of 1:128. Spleen cells from normal or from tumor-bearing mice, at a concentration of 50 x 10^6 cells/ml were incubated for 30 min at 37 °C with normal AKR/J serum or with anti-Θ serum diluted 1:2 in EM + 1 % fetal calf serum The cells were centrifuged, resuspended and incubated at 37 °C for 30 min with guinea pig complement (Difco, Detroit, Mich.) diluted 1:9 with Kolmer medium. The cells were then washed 3 times with PBS and counted for viability by the exclusion of 0.1 % trypan blue. The anti-Θ serum killed 15 – 22 % more spleen cells than did normal AKR serum.

3. Results

3.1. *In vitro* cytotoxicity caused by spleen cells of tumor-bearing mice

The activity of spleen cells from tumor-bearing mice was tested against 3LL cells *in vitro* (Table 1). We found that such spleen cells had a strong cytotoxic effect on the tumor target cells.

The effector cells in the spleens might be immune T lymphocytes [19], B lymphocytes [20] or macrophages [21]. To explore this question we either treated the spleen cells with anti-Θ serum plus complement to kill T lymphocytes (Table 1) or removed the macrophages by adherence to a plastic surface for 1 h (Table 2) before application to the target cells. We found that elimination of T cells from the spleens of tumor-bearing mice abrogated their cytotoxic activity (Table 1). On the other hand, removal of adherent macro-

Table 1. Effect of removing Θ-positive lymphocytes on cytotoxicity mediated by spleen cells from tumor-bearing mice[a)]

Exp. no.	Treatment with anti-Θ serum + C	Mean no. of target cells remaining after incubation with spleen cells ± S. E. Normal	Immune	Cytotoxicity (%)	P value
1	None	113.0 ± 8.9	68.1 ± 8.5	31.7	<0.005
	Yes	99.4 ± 13.9	101.8 ± 14.0	−2.4	N.S.[b)]
2	None	14.3 ± 2.3	5.0 ± 2.0	65.0	<0.01
	Yes	12.2 ± 1.6	13.7 ± 3.6	−12.3	N.S.

a) Spleen cells from normal or 3LL tumor-bearing mice were treated with normal serum or with anti-Θ serum and complement, and assayed for cytotoxicity against 3LL tumor cells *in vitro*.
b) Not significant.

phages only slightly reduced cytotoxicity in one experiment and had no effect in the other (Table 2). These results indicate that immune T cells were the effector cells which mediated cytotoxicity *in vitro*.

Table 2. Effect of removing macrophages on cytotoxicity mediated by spleen cells from tumor-bearing mice[a)]

Exp. no.	Absorption	Mean no. of target cells remaining after incubation with spleen cells ± S.E. Normal	Immune	Cytotoxicity (%)	P value
1	None	115.2 ± 24.8	48.5 ± 10.7	57.9	<0.05
	Yes	148.0 ± 3.2	98.8 ± 6.8	33.2	<0.001
2	None	95.3 ± 12.9	46.2 ± 5.8	51.5	<0.005
	Yes	126.7 ± 27.2	57.4 ± 10.0	54.7	<0.05

a) Spleen cells from normal or 3LL tumor-bearing mice were unabsorbed or depleted of macrophages by absorption on plastic petri dishes for 1 h at 37 °C. The remaining spleen cells were assayed for cytotoxicity against 3LL tumor cells *in vitro*.

3.2. The influence of immune spleen cells on tumor development

To study the influence of these spleen cells on tumor development *in vivo*, we injected the footpads of C57BL mice with tumor cells mixed together with different numbers of spleen cells taken either from normal or from tumor-bearing syngeneic mice (Fig. 1). We found that the rate and incidence of tumor development was greater in mice which received immune spleen cells as compared to mice which received normal spleen cells. This occurred at ratios of tumor cells to spleen cells of 1:10 and 1:100. Hence, the spleens of tumor-bearing mice contained cells which could enhance the growth of tumor transplants *in vivo*.

3.3. The role of T cells in the enhancement of tumor growth

We found that T lymphocytes from the spleens of tumor-bearing mice were cytotoxic for 3LL tumor cells *in vitro* (Table 1). What type of lymphoid cells caused the enhancement of tumor growth which was observed in the above *in vivo* experiments? To study the role of T lymphocytes in

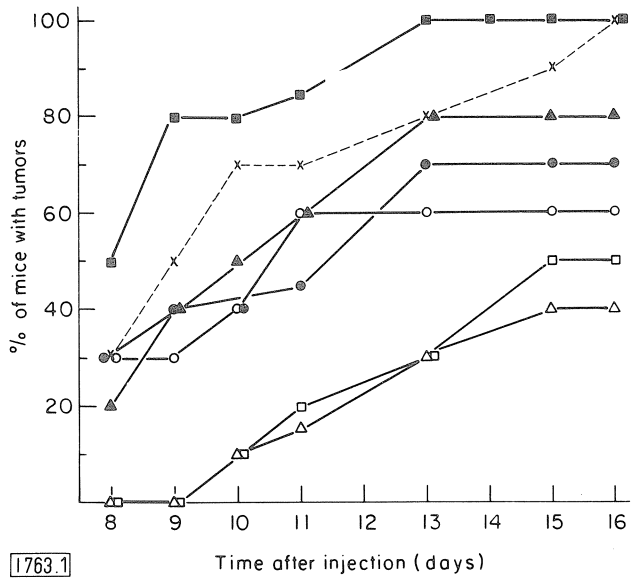

Figure 1. Effect on tumor development of normal and immune spleen cells; 10^5 3LL tumor cells were mixed with 10^5 (circles); 10^6 (triangles); or 10^7 (squares) spleen cells from tumor-bearing (black figures) or from normal control (empty figures) animals. The cells were injected into the right footpads of C57BL mice. An additional group was injected with 10^5 tumor cells alone (dotted line).

tumor enhancement, we treated normal and immune spleen cells with anti-Θ serum plus complement before inoculation into syngeneic recipients which had been irradiated with 450 r one day earlier (Fig. 2). We found that injection of spleen cells from tumor-bearing animals led to increased tumor growth as compared to uninjected controls or to those injected with normal spleen cells. This relative enhancement of tumor growth was inhibited by treating spleen cells of tumor-bearing mice with anti-Θ serum plus complement. In addition, the relative inhibition of tumor growth,

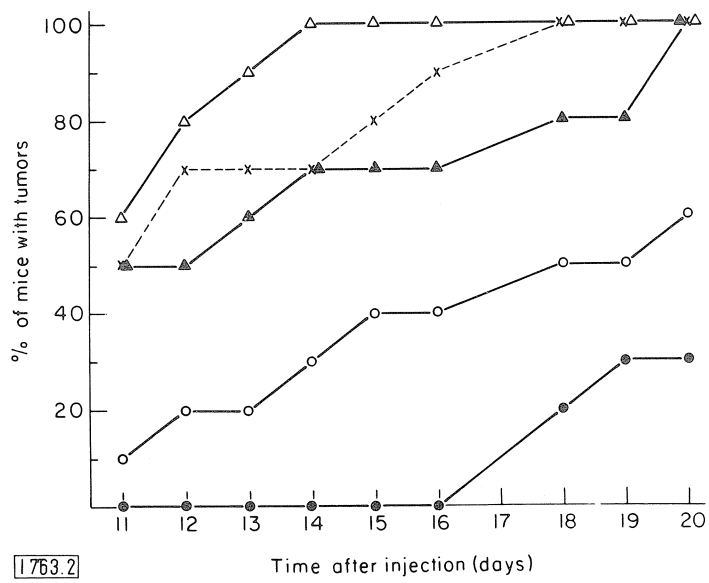

Figure 2. Effect on tumor development of treating normal or immune spleen cells with anti-Θ serum; 5 x 10^4 3LL tumor cells were mixed with 5 x 10^6 spleen cells from tumor-bearing (triangles) or from normal control (circles) mice. The spleen cells were treated with anti-Θ serum (black figures) or with normal serum (empty figures) before mixing. The cells were injected into the right footpads of C57BL mice which had been irradiated with 450 r 1 day earlier. An additional group was injected with 5 x 10^4 tumor cells alone (dotted line).

mediated by normal spleen cells, was augmented by treating the cells with anti-Θ serum plus complement. Hence, removal of Θ-positive lymphocytes from spleens of normal or tumor-bearing mice led to decreased tumor growth. Two additional consecutive experiments produced essentially the same results.

3.4. Effect of thymectomy on the development of tumor metastases

The results of the experiments shown in Fig. 2 suggest that T lymphocytes from normal as well as from tumor-bearing mice may accelerate tumor growth *in vivo*. If this were the case, removal of T lymphocytes from normal mice could be expected to lead to a decrease in tumor growth. Adult thymectomy of mice has been shown to produce partial removal of T lymphocytes [22]. We therefore challenged thymectomized or intact control mice with 0.5 x 10^6 3LL tumor cells at different intervals after thymectomy. The number of lung metastases were counted 3 weeks later. This method provided a sensitive and quantitative assay for tumor development. As seen in Table 3, adult thymectomy resulted in a significant reduction of tumor metastases. This suggests that normal mice possess a subpopulation of T lymphocytes which appears to enhance metastasis after tumor challenge.

Table 3. Effect of adult thymectomy on the number of lung metastases[a)]

Time between thymectomy and tumor challenge (Weeks)	Mean no. of lung metastases per mouse ± S.E. Control	Thymectomized	P value
2	23.0 ± 3.9	9.6 ± 2.3	<0.01
5	42.7 ± 9.9	20.5 ± 4.6	<0.05
24	18.9 ± 3.3	6.8 ± 1.6	<0.005

a) Normal 6-week-old mice were thymectomized and inoculated subcutaneously with 0.5 x 10^6 3LL tumor cells after various time intervals. The number of lung metastases was scored 3 weeks after tumor challenge.

3.5. The mechanism of enhancement mediated by T lymphocytes

It is conceivable that T lymphocytes from tumor-bearing mice might enhance the growth of tumors by either of two mechanisms. (a) Such lymphocytes could by themselves directly stimulate the growth of tumor cells or (b) they could suppress mechanisms of the recipient mice that might otherwise inhibit tumor growth.

To distinguish between these possibilities we irradiated groups of recipient mice with either 450 r or 750 r and assayed the effects of immune spleen cells on tumor growth (Fig. 3). We found that spleen cells from tumor-bearing mice markedly enhanced the growth of tumors in recipients irradiated with 450 r. However, recipients irradiated with 750 r demonstrated an equally rapid growth of tumor cells, regardless of whether or not they were injected with lymphocytes from normal or tumor-bearing mice.

These results indicate that the enhancement produced by lymphocytes of tumor-bearing mice was relative to the state of the recipient mice. Hence, the enhancing T lymphocytes appeared to facilitate tumor growth by suppressing an anti-tumor mechanism in the recipient mice.

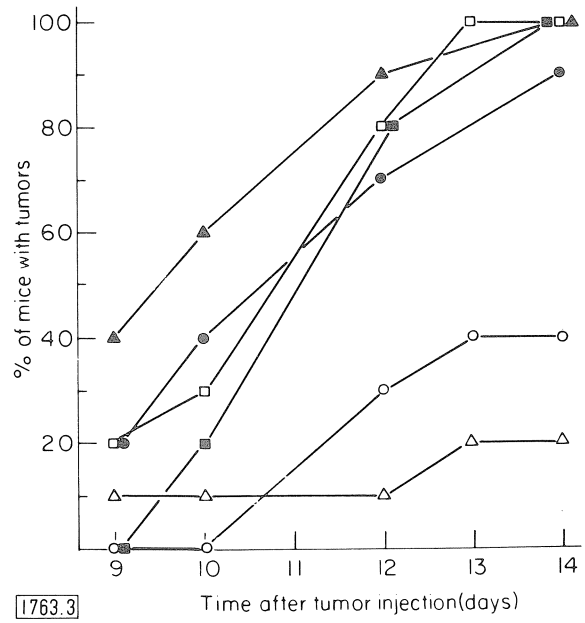

Figure 3. Effect on tumor development of normal and immune spleen cells in irradiated recipients; 5 x 10^4 3LL tumor cells were mixed with 2.5 x 10^6 spleen cells from tumor-bearing (squares) or from normal control (triangles) mice, or were left without spleen cells (circles). The cells were injected into the right footpads of C57BL mice which had been irradiated 1 day earlier with 450 r (empty figures) or with 750 r (black figures).

Fig. 4 shows the results of an experiment in which unirradiated recipient mice were injected with spleen cells from normal or tumor-bearing mice 5 days before challenge with 3LL tumor cells in the same footpads. It can be seen that spleen cells from tumor-bearing mice enhanced the growth of tumor cells even when there was no contact between the lymphocytes and the tumor cells *in vitro*. These results support the conclusion that enhancing T lymphocytes act on the recipient mice rather than directly on the tumor cells.

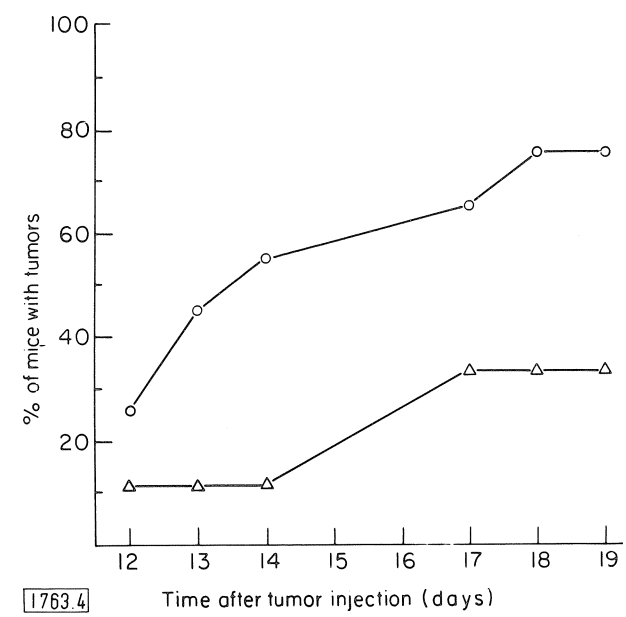

Figure 4. Effect on tumor development of normal and immune spleen cells injected before tumor challenge; 7 x 10^6 spleen cells from tumor-bearing mice (circles) or from normal control mice (triangles) were injected into the right footpads of C57BL mice. Four days later the mice were injected with 0.8 x 10^5 3LL tumor cells in the same footpads.

4. Discussion

A malignant syngeneic tumor was used to study the cell-mediated immune response of the host animal. Cells obtained from the enlarged spleens of mice with progressively growing tumors were found to be cytotoxic towards tumor target cells *in vitro* (Table 1). The cytotoxic reaction was mediated by T lymphocytes, since a pretreatment of the immune lymphocytes with anti-Θ serum plus complement abrogated their cytotoxic activity. Removal of macrophages did not seem to affect the cytotoxic reaction (Table 2).

The activity of the immune spleen cells was tested *in vivo* by inoculating syngeneic recipients with a mixture of tumor cells and spleen cells. We found that spleen cells from tumor-bearing mice enhanced tumor growth *in vivo* as compared to normal spleen cells (Fig. 1). Tumor enhancement was reduced if the spleen cells were treated with anti-Θ serum plus complement before inoculation. Hence, the tumor-enhancing cells, like the cytotoxic cells, appeared to be T lymphocytes.

The results of two kinds of experiments suggest that enhancing T lymphocytes exist in normal as well as in tumor-bearing mice. We found that eliminating T lymphocytes from normal spleens caused a relative inhibition of tumor growth when the remaining spleen cells were used in passive transfer experiments (Fig. 2). In addition, thymectomy of normal adult mice appeared to inhibit the development of metastases after challenge with 3LL tumor cells (Table 3). Thymectomy in adults leads to only a partial depletion of T lymphocytes [22]. The finding that protection against the development of metastases occurred as soon as 14 days after thymectomy suggests that enhancing T lymphocytes have a relatively short life span. The markedly increased enhancement produced by T lymphocytes from tumor-bearing mice indicates that the number and/or activity of enhancing T lymphocytes is increased in these mice.

We succeeded in gaining some insight into the mechanism of action of enhancing T lymphocytes by investigating the role of the recipient mouse. We found that enhancement was relative to the immune state of the host (Figs. 3 and 4). Mice irradiated by 750 r supported a maximal rate of tumor growth that was not affected by the presence or absence of donor lymphocytes. Hence, irradiation by 750 r appeared to abolish a mechanism present in mice which exerts an inhibiting effect on tumor growth. Enhancing lymphocytes seemed, therefore, to produce a similar effect by allowing the maximal rate of tumor growth (Fig. 3). Thus, the presence of enhancing T lymphocytes acted to reduce the natural resistance of the recipient mice to tumor growth. Indeed, enhancing T lymphocytes were found to be effective even when injected 5 days before the tumor cells (Fig. 4). Therefore, it can be concluded that enhancement by T lymphocytes in the experiments described here results from suppression of host resistance and is probably independent of a direct effect on tumor cells. Thus, the enhancing lymphocytes act as suppressor T cells, inhibiting host resistance to tumor growth.

What host resistance mechanisms are susceptible to such enhancing or suppressing T lymphocytes? Although we have no direct proof, it is very likely that resistance to these tumor cells is mediated by the immune system [23]. Hence, enhancing T lymphocytes might produce their effects by suppressing an immune response damaging to tumor cells. Recently, it has been shown that T lymphocytes from tolerant animals can actively inhibit immune responses. Tolerant T lymphocytes were found to transfer nonreactivity to other animals in antibody production by B lymphocytes [24], or in delayed hypersensitivity mediated by other T lymphocytes [25]. It was also shown that immune spleen cells could directly suppress the ability of immunized lymphocytes to mediate a cytotoxic effect [26]. Hence, it is tempting to speculate that enhancing T lymphocytes from tumor-bearing mice could function as suppressor T cells controlling the function of T effector lymphocytes. It is also conceivable that enhancement was mediated by humoral antibodies produced by B lymphocytes [4–6]. Such antibodies directed against thymus-dependent antigens present on the tumor cells could have been stimulated by the donor T lymphocytes. Thus, the controlling T cells could function by way of enhancing antibodies.

Recent evidence indicates that T lymphocytes can enhance the growth of tumor cells also by a direct interaction between the cells. We found that T lymphocytes autosensitized *in vitro* against syngeneic fibroblasts mediated specific enhancement of fibrosarcoma cells when injected into recipients irradiated with 750 r [27]. These findings suggest that T lymphocytes may produce tumor enhancement by two different mechanisms: (a) by controlling or suppressing the host response as observed in tumor-bearing mice, or (b) by direct stimulation of tumor cell proliferation as observed following autosensitization *in vitro*. Different systems of sensitization appear to favor the expression of one or the other mechanism. An open question is whether separate subpopulations of T lymphocytes are responsible for these effects.

The relationship between cytotoxic and enhancing T lymphocytes is an open question. It is conceivable that the same cells are the agents of both phenomena. Contact between a tumor cell and a particular lymphocyte might lead to the death of the tumor cell *in vitro*, but stimulate its proliferation *in vivo*. A more attractive alternative is that enhancement and cytotoxicity are mediated by separate subpopulations of T lymphocytes.

In previous studies we found that spleen cells sensitized *in vitro* against syngeneic antigens also were cytotoxic against 3LL tumor cells *in vitro* but enhanced growth of the tumor *in vivo* [10]. The results presented here extend these earlier observations.

The existence of enhancing T lymphocytes might appear to be contradicted by the results of experiments showing that adult thymectomy promoted rather than inhibited development of tumors [28]. In addition, immune T lymphocytes have been found to protect against the growth of tumor cells [29–31]. However, these experiments involved antigenic stimulation by tumor cells bearing strong tumor-specific antigens, alloantigens [29, 30], or virus-associated tumor antigens [31]. The syngeneic 3LL tumor used in our experiments was only very weakly antigenic [29]. It is possible that the immune response to strong cell-bound antigens obscures or does not involve an increase in the activity of enhancing T lymphocytes. Enhancement by T lymphocytes may be relatively more prominent in the immune response to relatively weak tumor antigens [32]. Indeed, it has been reported that weak immune responses stimulated tumor growth and that this immunostimulation could be transferred by lymphoid cells [12]. The results presented here suggest the possibility that enhancing T lymphocytes mediated this immunostimulation.

It has been shown in a number of studies that the latent period of tumor development is characterized by effective

immunity of the host against its tumor [33]. The overt clinical appearance of the tumor was associated with disappearance of this immune state. It is possible that suppression of immune resistance to the tumor is mediated by enhancing (suppressor) T lymphocytes as well as by tolerogenic tumor antigens [34]. Further study of the development and function of these cells may help us understand some of the host-tumor relationships.

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