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Implantation of a gelatin-sponge as a model for effector recruitment

Tumor growth inhibition by T-lymphocytes recovered from a site of tumor rejection

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Summary. Nylon-wool-eluted lymphocytes, isolated from a site of tumor rejection in Balb/c mice expressing concomitant tumor immunity, were examined for their ability to inhibit the growth of the EMT6 tumor. Tumor growth inhibition was monitored after co-inoculation of lymphocytes and tumor cells into naive mice in a Winn-type adoptive-transfer assay. A pre-implanted gelatin sponge was employed to capture the tumor-infiltrating lymphocytes. Mice harboring primary tumors were implanted 8 days later with gelatin sponges. The pre-implanted sponges were then inoculated with a secondary tumor challenge 2 days after implantation of the sponge (i.e. 10 days after primary tumor challenge). On day 17 (7 days after secondary tumor challenge), the immune sponges were retrieved, digested in collagenase and the T lymphocytes were isolated using a nylon-wool column. Blank sponges (lacking tumor cells), obtained from primary-tumor-bearing or non-tumor-bearing animals, were included for comparison. The data showed that T lymphocytes isolated from immune sponges inhibited tumor growth while T lymphocytes recovered from blank sponges did not. At an effector:target (E:T) ratio of 10:1 the lymphocytes from the immune sponges were able to prevent totally the growth of tumors in all cases (100% inhibition). This ability was reduced (60% inhibition) at an E:T ratio of 1:1. Comparison of the antitumor activites of the immune-sponge-derived cells with those from the spleen of the same animal revealed the superiority of the former. Depletion of immune-sponge-derived cells with anti-Thy1.2, anti-Lyt2.2 or anti-L3T4 and complement resulted in a marked decrease in tumor-inhibitory activity. These results indicate that T lymphocytes, expressing Thy1.2, Lyt2.2 or L3T4 antigens, are involved in conferring protection to Balb/c mice against the EMT6 tumor.

Introduction

Several studies that have examined the effectors mediating antitumor responses have employed immunocytes obtained from spleens or lymph nodes of tumor-bearing or immunized animals [5, 8, 9]. The choice of these organs as sources of sensitized lymphocytes has been based on the realization that during successful tumor rejection, the tumor is no longer discernible and host effectors cannot be recovered from the rejection site. The need to study the cells directly mediating immune responses prompted several investigators to employ implanted sponge matrices to capture host immunocytes infiltrating sites of allograft or tumor rejection [6, 10, 14]. These studies have demonstrated the migration, and retention within sponge matrices (previously coated with allogeneic or tumor cells), of cytotoxic mononuclear cells. Recently Vallera et al. [14] reported the capture of sponge-infiltrating lymphocytes with in vitro tumoricidal activity during the generation of a primary antitumor response to a regressor dose of the tumor induced by the Moloney sarcoma virus.

The study described here employed a gelatin sponge to capture effector lymphocytes mediating the rejection of a secondary tumor during the expression of concomitant tumor immunity to the EMT6 tumor. In an earlier study [3] we demonstrated superior in vitro tumoricidal activity of lymphocytes recovered from a site of tumor rejection over that of counterpart splenic lymphocytes during the manifestation of concomitant tumor immunity. In this report, the in vivo tumor-inhibitory activities of sponge-derived and splenic lymphocytes were compared using a Winn-type adoptive-transfer assay. The data reveal the superiority of immune-sponge-derived cells over immune splenocytes in their tumor-inhibitory activity.

Materials and methods

Mice. Female Balb/c mice, 8–9 weeks old, were used in this study. Mice were purchased from the Jackson Laboratory, Bar Harbor, Me, and housed in the animal facilities at Northern Arizona University. Food and water were supplied ad libitum.

Tumor. The EMT6 tumorigenic cell line was used in this study. The EMT6 cell line is highly antigenic and significantly immunogenic [11, 13]. It is a subline of an early passage of the KHJJ line that was derived from a mammary carcinoma arising in a Balb/c mouse [11]. The tumor was routinely passaged in vivo subcutaneously in syngeneic Balb/c mice for 21 days. The tumors were subsequently disaggregated and cryopreserved at −70° C. Prior to each experiment, the cryopreserved cells were thawed and propagated in vitro for a limited time (two to three serial passages) in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum.

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Medium. Filter-sterilized α-MEM (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 100 IU/ml penicillin and 100 μg/ml streptomycin, was utilized for long-term culture of EMT6 cells. For the preparation of effector cells from sponge matrices and spleens, α-MEM supplemented with 10% fetal bovine serum and buffered with 20 mM morpholinopropanesulfonic acid was used. For studies on the T cell subset depletion, using monoclonal antibody and complement, RPMI 1640 (Gibco, Grand Island, NY) supplemented with 25 mM HEPES buffer and 0.3% bovine serum albumin was utilized.

Sponge implantation, injection of tumor cells and sponge retrieval. The Balb/c mice were given a primary subcutaneous injection of 1×10^5 viable EMT6 cells in a 0.1-ml volume of sterile saline into the shaven right dorso-lumbar area. Eight days later, animals were administered pentobarbital anesthesia (40 mg/kg body weight) and a $15 \times 15 \times 10$-mm gelatin sponge (Gelfoam, Upjohn, Mich) was implanted through an incision in the dorsal-cervical region away from the primary tumor. Two days after sponge implantation (i.e. 10 days after primary tumor inoculation), 1×10^5 viable EMT6 cells in 0.1 ml saline were injected into the sponge through the skin. Sponges previously injected with tumor cells and obtained from tumor-bearing mice will henceforth be denoted "immune sponges". Blank sponges from tumor-bearing (secondary blank) and non-tumor-bearing (control blank) mice were used as controls. Seven days later (i.e. 17 days after primary tumor inoculation), mice were sacrificed by cervical dislocation and the sponges were retrieved through a skin incision. Sponge matrices retrieved from 10–15 mice were pooled in a beaker containing 5 ml collagenase enzyme mixture, minced with a pair of scissors and digested in the collagenase cocktail as previously described [2].

Determination of cell number and viability. The viability and yield of the dispersed cell suspension was determined by counting the cells in a hemocytometer after staining with propidium iodide and acridine orange as previously described [1].

Enrichment for T lymphocytes from sponges and spleens. The single-cell suspension, obtained after disaggregation of sponges (immune or blank), was adjusted to a concentration of 2×10^7 cells/ml. Three to five milliliters of this suspension were transferred into 150-mm tissue-culture dishes (Falcon, 150 × 25 mm; no. 3025), and then incubated at 37° C for 60 min to allow the adherence of macrophages and residual tumor cells. The non-adherent cells were recovered by carefully rinsing the dishes twice with 10 ml warm α-MEM supplemented with 20 mM MOPS (α-MEM/MOPS). The non-adherent cells were washed once in α-MEM/MOPS (400 *g*, 10 min, 4° C) and the cell pellet was resuspended in 5 ml of the same buffer. Ammonium chloride/potassium chloride lysis buffer (15 ml; 8.29 g NH_4Cl, 1.09 g $KHCO_3$ and 0.0372 g EDTA/l) was added to the cell suspension for the lysis of erythrocytes. After 30 s, the cell suspension was washed once with 50 ml α-MEM/MOPS at 37° C (400 *g*, 10 min, 21° C) and the cell pellet was resuspended in the same buffer to give a concentration of 2×10^5 cells/ml. Fifteen milliliters of the cell suspension were layered over 10 ml Ficoll/Hypaque (Pharmacia, Piscataway, NJ) and centrifuged at 400 *g* for 15 min at 21° C. The mononuclear cell layer was carefully collected using a sterile pasteur pipette, transferred into a 50-ml centrifuge tube and then centrifuged at 400 *g* for 10 min. The resulting cell pellet was adjusted to a volume of 1.5–2 ml in α-MEM/MOPS and then loaded onto a nylon-wool column [7]. After 1 h of incubation at 37° C, the nylon-wool column was eluted with two 10-ml washes of warm α-MEM/MOPS. The eluted T lymphocytes were centrifuged at 400 *g* for 10 min and then adjusted to a cell concentration of 1×10^7/ml. Spleens from eight to ten animals bearing immune sponges were removed aseptically. The disaggregation of splenocytes was accomplished mechanically using the blunt end of a sterile 10-ml plastic syringe plunger and passing them through a 140-μm wire mesh. Single-cell suspensions of spleen cells were similarly treated for the isolation of nylon-wool-non-adherent T lymphocytes. May-Grünwald/Giemsa staining of cytocentrifuge preparations revealed that 76% (range of 62%–89%) nylon-wool-eluted, sponge-derived cells were lymphocytes and 92%–95% nylon-wool-eluted splenocytes were lymphocytes.

Depletion of T cells expressing Thy1.2, Lyt2.2 or L3T4 by monoclonal antibody and complement treatment. Nylon-wool-eluted, sponge-derived cells were adjusted to a concentration of 1×10^7/ml in RPMI 1640 (Gibco, Grand Island, NY) supplemented with L-glutamine, 25 mM HEPES buffer and 0.3% bovine serum albumin. Equal volumes of the monoclonal antibodies Thy1.2 (cell-surface marker for T lymphocytes), Lyt2.2 (marker for cytotoxic/suppressor T cells) or L3T4 (marker for helper/inducer T cells; Accurate Chemicals and Scientific Corporation, Westbury, NY) diluted 1:10, 1:10 and 1:5, respectively, in RPMI 1640, were added separately to test-tubes containing 500 μl effector cells and incubated at 4° C for 60 min. After washing once (400 *g*, 10 min, 37° C) the cell pellet was resuspended in 1 ml 1:10 dilution of Low-Tox rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, NY) and incubated for 60 min at 37° C. The cells were washed twice in RPMI 1640 and resuspended to the original cell volume in normal saline for a Winn-type adoptive-transfer assay. Cells incubated alone with medium as well as cells incubated with complement alone were included as controls.

Winn's in vivo adoptive transfer assay. T lymphocytes from sponges or immune spleen, purified on a nylon-wool column, were mixed with 1×10^5 viable EMT6 tumor cells to give effector:target (E:T) ratios of 10:1 or 1:1. Of the cell mixture containing effector and target cells at the desired E:T ratio, 0.1 ml was injected subcutaneously into the dorso-lumbar region of normal naive Balb/c mice under i.p. pentobarbital anesthesia. At 7, 14, 21, and 28 days post-inoculation, the sizes of the growing tumors were determined by taking measurements in two bisecting planes using a pair of calipers. Animals injected with EMT6 tumor cells only were included for comparison.

Statistical analysis. The mean size of tumors at 28 days post-inoculation was taken as the end point. Statistical significance of results of end-point adoptive-transfer assays was determined by one-way analysis of variance (ANOVA). Multiple comparisons of end-point data sets were done using Bonferroni's adjusted non-paired one-way Student's *t*-test [12]. Values are expressed as means ± SEM. Probability values (P) of < 0.05 were considered to indicate significant differences between data sets.

Results

In vivo tumor-inhibitory activity of T lymphocytes obtained from immune sponges

Effector T lymphocytes obtained from immune sponges on day 7 after secondary challenge were mixed with 1×10^5 viable EMT6 tumor cells at an E:T ratio of 1:1 or 10:1. The mixed cell suspension was injected into normal Balb/c mice. The sizes of growing tumors were measured over a 4-week period. Blank sponge cells from tumor-bearing or non-tumor-bearing mice were used as controls. The data from this experiment show that the T lymphocytes from immune sponges expressed tumor-growth-inhibitory activity at an E:T ratio of 1:1 whereas effector cells from secondary blank or control blank sponges did not show any antitumor activity (Fig. 1a). The growth rates of the control blank-sponge group and the secondary blank-sponge group were comparable to that of the EMT6 tumor control group. In the immune-sponge group the percentage of animals with tumors had diminished from 60% on day 14 to 33% on day 28. In contrast the percentage of animals with tumors in the control groups ranged from 93%–100% on day 14 and remained so through day 28 (Fig. 1c).

At an E:T ratio of 10:1, effector cells from the immune-sponge group were highly effective in totally inhibit-

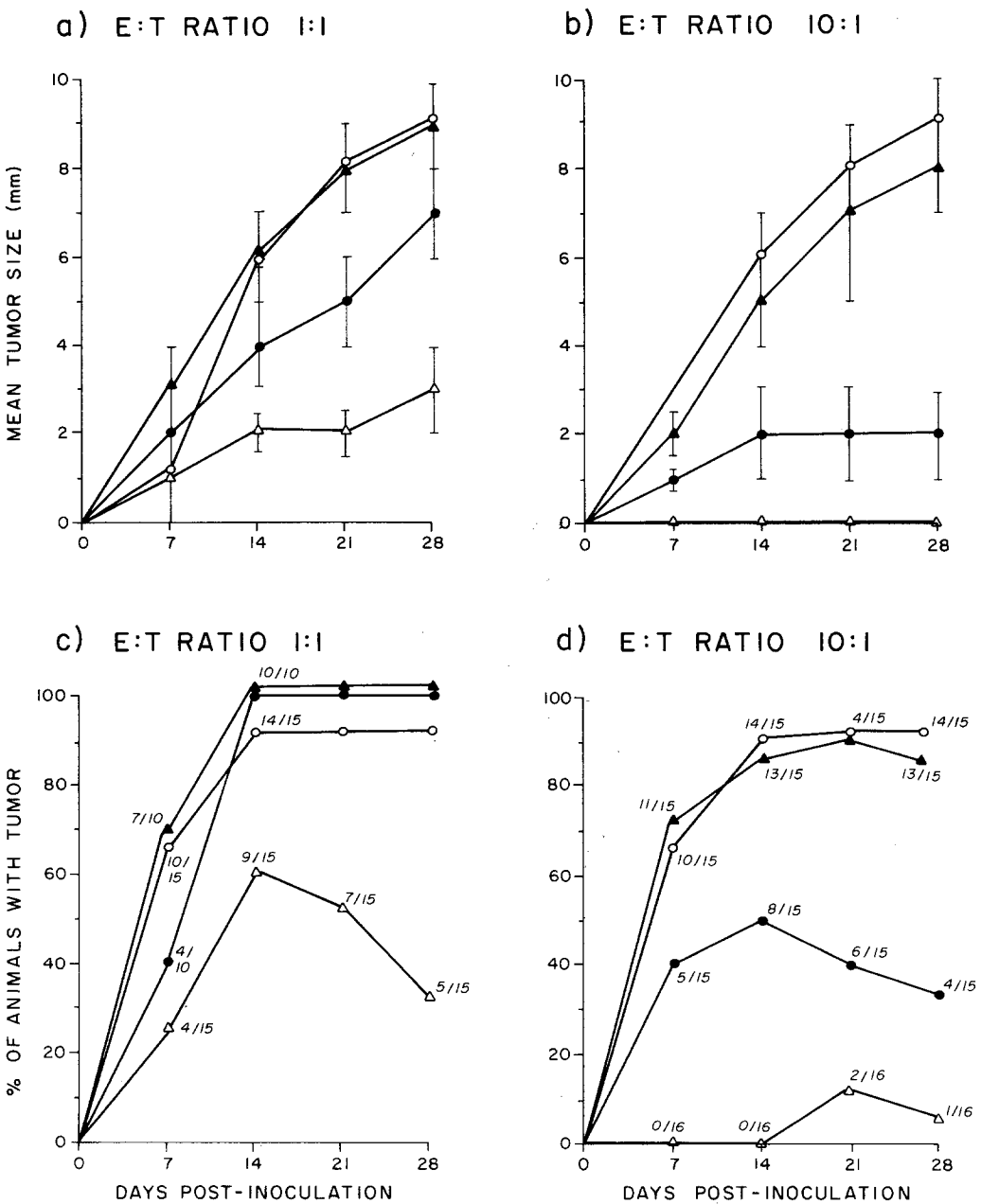

Fig. 1. In vivo cytotoxicity of sponge-derived T lymphocytes. Effector cells from immune sponges (△), blank-sponge effectors from tumor-bearing (secondary blank, ●); and non-tumor-bearing mice (control blank, ▲); were mixed with 1×10^5 viable EMT6 tumor cells at an effector:target ratio of 1:1 (**a, c**) or 10:1 (**b, d**) and inoculated into normal Balb/c mice. The sizes of growing tumors were serially measured with a pair of calipers in two bisecting planes at weekly intervals for 4–5 weeks. Each data point represents mean values from 10–15 mice; *bars*, ±SE. All the experiments included a group of mice injected with only EMT6 cells (○)

ing the growth of the EMT6 tumor in Balb/c mice (Fig. 1b). The data also show that T lymphocytes isolated from secondary blank sponges expressed a certain degree of antitumor activity when compared with those from the control blank sponges. However, the mean tumor size of the secondary blank group (2.1 ± 1.0 mm) was higher than that of the immune-sponge group (0.16 ± 0.1 mm; $P < 0.05$) on day 28. In the control blank-sponge group, there was a progressive increase in the mean tumor size, which was comparable to the EMT6 tumor control group. Also, in the immune-sponge group, two tumors that appeared in two mice (mean tumor size 0.22 ± 0.2 mm) on day 21 (Fig. 1d) had regressed by days 28 (Fig. 1d) and 35 (data not shown) respectively. In contrast, the residual tumors in the secondary blank-sponge group (four mice on day 28) had not regressed by day 35 (data not shown).

Comparison of tumor-inhibitory activity of immune-sponge cells versus immune spleen cells

Nylon-wool-eluted T lymphocytes, isolated from immune sponges from mice expressing concomitant tumor immunity, were compared with their counterpart splenic lymphocytes for determining their tumor-inhibitory activity in a Winn-type adoptive-transfer assay. The data (Fig. 2a) show that at an E:T ratio of 1:1 the effector cells derived from both the immune sponge and the immune spleen were moderately effective in inhibiting tumor growth. The mean tumor sizes in the immunesponge and

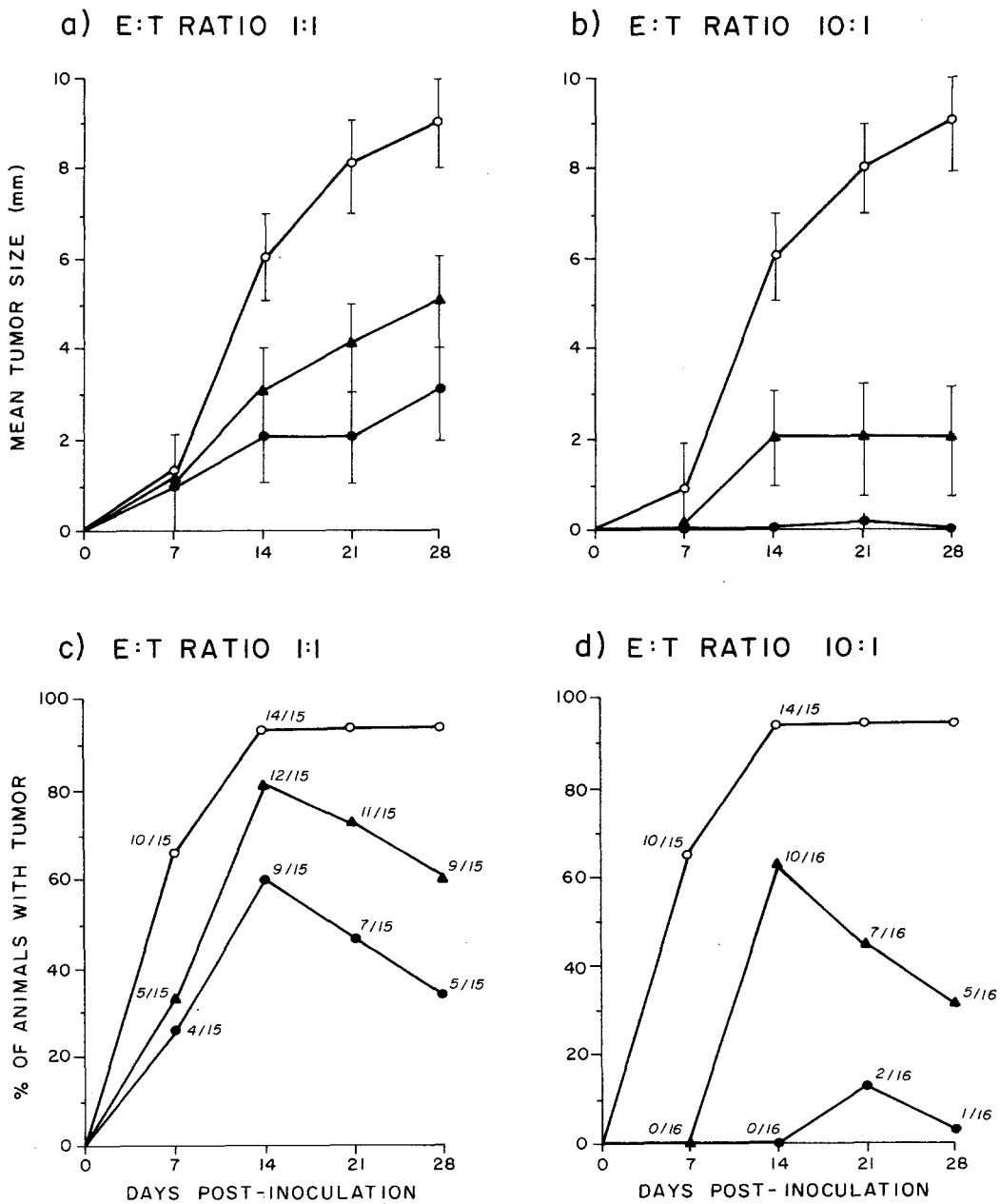

Fig. 2. Comparison of antitumor activity of immune-sponge-derived versus immune spleen-derived T lymphocytes. Tumor-inhibitory activity of immune-sponge-derived T lymphocytes (●) was compared with that of immune spleen cells (▲) obtained from the same mouse in an adoptive-transfer assay as described in materials and methods. Each group consisted of 15 animals. The effector:target ratio was 1:1 (**a, c**) or 10:1 (**b, d**). All the experiments included a group of animals injected with only EMT6 cells (○), *bars*, ±SE

the immune-spleen groups (3.13 ± 1.2 mm and 4.95 ± 1.1 mm, respectively) were lower than that of the EMT6 tumor control group (9.04 ± 1.0 mm; $P < 0.05$) on day 28. The fraction of animals harboring tumors was reduced from 60% on day 14 to 33% on day 28 in the immune-sponge group whereas in the immune-spleen group this percentage, which was 80 on day 14, diminished to only 60 on day 28 (Fig. 2c). There was no reduction in the percentage of animals bearing tumors in the EMT6 tumor control group.

At an E:T ratio of 10:1, the effector cells from the immune-sponge group were superior to those of the immune-spleen group in providing tumor protection as shown in Fig. 2b. On day 28, the mean tumor size in the immune-spleen group was 2.2 mm, compared to 0.16 mm in the immune-sponge group. The residual tumors (one in the immune-sponge group and five in the immune-spleen group on day 28) were followed for another 2 weeks. It was observed that the residual tumor in the sponge group regressed and completely disappeared. In contrast, the residual tumors in the immune-spleen group did not disappear even after a 14-day follow-up (data not shown). In the immune-sponge group, 12% of the animals harbored tumors on day 21; this value was reduced to 6% on day 28. In contrast, in the immune-spleen group this percentage diminished from 43 on day 21 to 31 on day 28 (Fig. 2d).

Effects of depletion of T cell subsets on the tumor-inhibitory activity of immune-sponge-derived cells

Immune-sponge-derived cells bearing the Thy1.2 marker were depleted using anti-Thy1.2 monoclonal antibody and complement. The data (Fig. 3a) show that the tumor-inhibitory activity of immune-sponge-derived effector cells was markedly reduced by the depletion of Thy1.2-expressing T lymphocytes. In the Thy1.2-depleted group, 80% of the animals harbored tumors on day 28, while only 10% of the animals in the untreated group bore tumors (Fig. 3b). However, there was a significant difference between the mean tumor size of the Thy1.2-depleted group (5.7 ± 0.9 mm) and that of the EMT6 tumor control group (9.2 ± 1.0 mm) on day 28 (Fig. 3a; $P < 0.05$).

In order to identify the T-cell subsets mediating antitumor activity, immune-sponge-derived lymphocyte suspensions were treated with anti-Lyt2.2 or anti-L3T4 monoclonal antibodies and complement and then tested for tumor-inhibitory activity. As shown in Fig. 3a, the antitumor activity of the immune-sponge-derived effector cells was greatly reduced by treatment with anti-Lyt2.2 or anti-L3T4 antibodies and complement. However, the mean tumor sizes of the groups treated with Lyt2.2 (4.9 ± 1.3 mm) and L3T4 antibody (5.8 ± 1.3 mm) were similar and were not significantly different from the Thy1.2-antibody-treated group on day 28 (5.7 ± 0.7 mm). The mean tumor size of the groups treated with monoclonal antibody was higher than that of the untreated immune-sponge cell group (1.0 ± 1.0 mm) (Fig. 3a; $P < 0.05$). Also, in the groups depleted of L3T4 and Lyt2.2, the percentage of animals harboring tumors on day 28 were 80% and 67% respectively. In contrast only 10% of the animals receiving untreated immune-sponge-derived T lymphocytes harbored tumors at this time (Fig. 3b). There was no significant difference between the mean tumor size of the animals receiving immune-sponge cells treated with complement alone (0.7 ± 0.7 mm) and that of animals receiving the immune-sponge cells not treated with complement (1.0 ± 1.0 mm).

Discussion

By employing a gelatin-sponge model of tumor implantation this study has made it possible to capture, recover and ascertain the function of host effector cells infiltrating a secondary tumor undergoing rejection. T lymphocytes were isolated from the site of secondary tumor challenge in animals expressing concomitant tumor immunity to the

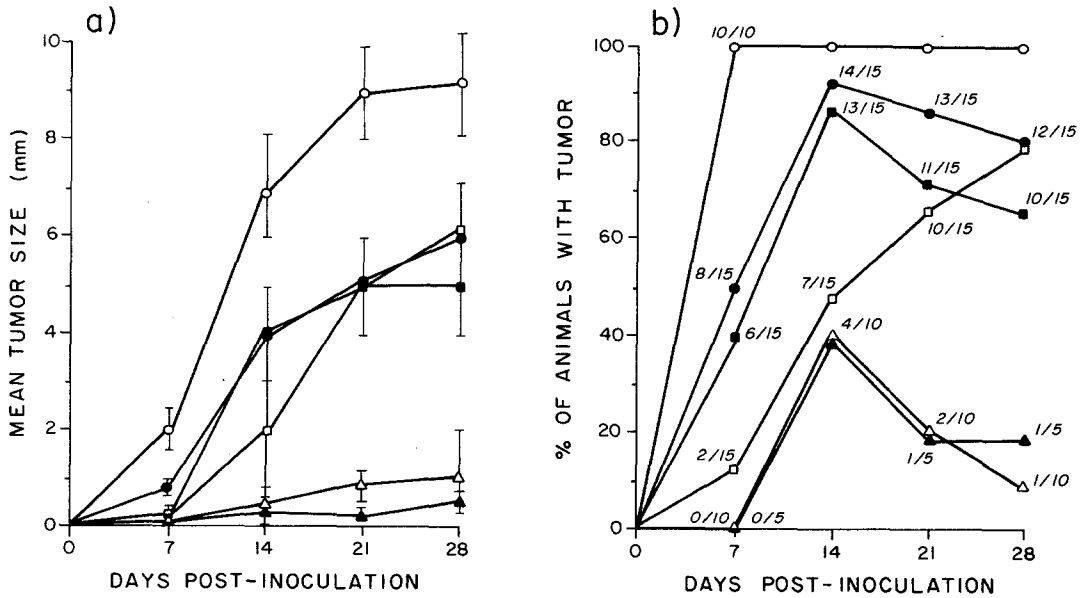

Fig. 3. Tumor-inhibitory activity of immune-sponge-derived cells depleted of Thy1.2, Lyt2.2 and L3T4 T lymphocyte subsets. Immune-sponge-derived effector cells were untreated (△), or treated with complement alone (▲), or Thy1.2 and complement (●), or Lyt2.2 and complement (■), or L3T4 and complement (□). The antitumor activity of sponge-derived immune cells was examined at an effector:target ratio of 10:1 in an adoptive-transfer assay. Points represent mean cytotoxicity of two separate experiments, each using 5–10 mice. Both experiments included a group of mice injected only with EMT6 cells (○); *bars*, ±SE

EMT6 tumor and were examined for their in vivo tumor-inhibitory activity. This activity was compared with that of counterpart splenic lymphocytes from the same animal or blank-sponge-derived lymphocytes from tumor-bearing or non-tumor-bearing animals. The data revealed the superior ability of cells recovered from immune sponges to partially or totally inhibit the growth of the EMT6 tumor in an adoptive-transfer assay at E:T ratios of 1:1 and 10:1, respectively. The significant antitumor activity of immune-sponge-derived cells can be explained on the basis of recruitment and selective retention of specifically sensitized and activated T lymphocytes at the tumor site in response to chemoattractants derived from tumor or lymphocytes. Thus, while the spleen may contain cytotoxic T cells with comparable efficacy, these cells only constitute a small fraction of the splenocyte population used. The alternative suggestion is that sensitized but precytotoxic T cells originate in the spleen during the growth of the primary tumor and only after migration to the site of the secondary tumor and interaction with tumor antigens do they mature into cytotoxic cells [4]. The property of the sponge matrix to provide anchorage for the injected tumor cells and to serve as a "trap" for the recruited immune cells ensures continued antigenic stimulation.

The ability of spleen-derived or blank-sponge-derived lymphocytes from tumor-bearing animals to confer a degree of tumor protection, even though they demonstrated no tumoricidal activity in an in vitro assay [3], would suggest the presence of sensitized precytotoxic T cells in the spleens and blank sponges of these animals that become cytotoxic after contact with tumor antigens, as occurs in the Winn assay. The failure of cells derived from blank sponges from non-tumor-bearing animals to provide tumor protection reinforces the notion that these non-cytotoxic cells are non-specifically recruited in response to an inflammatory response.

In the experiments described, Thy1, Lyt2 and L3T4 monoclonal antibodies plus complement were used to eliminate T cells and their functional subsets from the immune-sponge-deried cell population. The elimination of cells bearing the Thy1 epitope from the immune-sponge population did not totally eliminate the in vivo tumor-inhibitory activity. This observation raises the possibility of the participation of cells other than T cells, such as natural killer cells, in tumor inhibition. The $Lyt2^+$ and $L3T4^+$ T cell subsets were comparable in their ability to confer tumor resistance. While the participation of the $Lyt2^+$ subsets may be attributed to direct tumor cytotoxicity, the involvement of the L3T4 (helper T cell) subset in tumor protection in the adoptive-transfer assay may be to the extent that they secrete interleukin-2 to facilitate the proliferation and activation of the $Lyt2^+$ cytotoxic cells at the tumor site.

The superior antitumor activity of tumor-associated T lymphocytes, recovered from a site of tumor rejection, over counterpart splenic lymphocytes suggests a need to re-evaluate the choice of splenic lymphocytes as effectors in studies aimed at curbing tumor growth. It would appear that information of greater relevance would be gained by examining the antitumor activity of tumor-associated immunocytes directly mediating tumor rejection in vivo. The gelatin-sponge model of concomitant immunity, described here, would make this line of investigation possible.

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