ELIMINATION OF SYNGENEIC SARCOMAS IN RATS BY A SUBSET OF T LYMPHOCYTES*

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We have recently reported the elimination in vivo of a syngeneic rat Moloney sarcoma (MST)[1] with an established blood supply, by remote intravenous infusion of syngeneic splenic effector cells with specificity for the tumor (1). The splenic effector cells were effective in vivo only after cytotoxicity of the population was augmented in an in vitro mixed lymphocyte tumor cell culture (MLTC). In this paper, we present data on the elimination of an established Moloney sarcoma with a blood supply, ranging from 1 to 7 cm in Diam, by infusion of varying numbers of specific effector cells. The specific effector cells were a subset of T blast cells, $W3/25^+$ and $W3/13^+$ (2), as detected by monoclonal antibodies to rat T antigens, and they apparently functioned as amplifier or helper cells in the tumor-bearing host. A $W3/25^-$ population, a melange of T cells, null cells, macrophages, and B cells, was associated with enhancement of tumor in vivo.

Materials and Methods

Rats. 3-mo-old BN females were used as donors of spleen cells and as test animals bearing an established tumor. They were obtained from the breeding colony of Scripps Clinic and Research Foundation (La Jolla, Calif.)

Tumors. MST-1 is a rat Moloney sarcoma that expresses Moloney sarcoma virus and carries the RT1 surface markers of the BN (3). 5×10^4 MST-1 is lethal in irradiated recipient BN rats within 40 d. BM2 is another BN rat tumor line derived from MST-1. It expresses virus, elicits a high level of cytotoxic effector cells, and is lethal in test-irradiated BN recipients at a dose that exceeds 10^7 cells. Both tumor lines are maintained in vitro as monolayer and suspension cultures. Passage numbers 10–40 of these lines were used both for immunizationof donor rats and as targets for ^{51}Cr release assays in vitro. Immunization with either line protected recipient rats from lethal inocula of the other line. MST-1 was used in all experiments as the challenge tumor in vivo, and BM2 was usually used to immunize donor rats.

Antibodies. Three T cell reagents were used to identify and to isolate rat T lymphocytes: a monoclonal anti-rat T cell antibody (W3/13) and a second monoclonal anti-rat T cell antibody (W3/25), described by Williams et al. (2) and commercially available from Sera Lab, Ltd., Sussex, England. W3/13, by indirect immunofluorescence, i.e., by labeling cells with a fluorescein (Fl)- or rhodamine (Rh)-labeled goat anti-mouse IgG (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) after binding of the primary unlabeled antibody, stained 100% of

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C, complement; CMC, cell-mediated cytotoxicity; FACS, fluorescence-activated cell sorter; Fl, fluorecein isothiocyanate; GAMG, goat anti-mouse IgG antibodies; GARG, goat anti-rat Ig antibodies, polyvalent; MLTC, mixed lymphocyte tumor culture; MST_{Mit}, mitomycin C-treated MST; RART, rabbit anti-rat T lymphocyte antiserum; Rh, tetramethyl rhodamine isothiocyanate; Spc, spleen cells; SIg, surface immunoglobulin.

thymocytes and 40–45% of spleen cells. W3/25 by indirect immunofluorescence, stained 95–100% of thymocytes and 35–40% of spleen cells, about 80–85% of cells stained by W3/13. This latter reagent detected peripheral T lymphocytes; W3/13 detected peripheral and immature bone marrow and thymic T lymphocytes (2). A rabbit anti-rat thymocyte antibody (RART) was prepared by injecting rabbits with rat thymocytes and absorbing the antiserum with rat liver, kidney, and bone marrow until <2% of the bone marrow cells stained by indirect immunofluorescence, and 90–95% of thymocytes were stained (4). Cowan strain *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was coated with this antibody and was used to immunoprecipitate NP40 extracts of rat thymocytes. The antigen-antibody complex was injected to rabbits, and the resultant antiserum stained, by direct fluorescence, 90–95% thymocytes, 2–5% bone marrow cells, and 40–50% spleen cells. RART was used to lyse T-positive lymphocytes and destroyed 95% of thymocytes.

Ia antigens were detected with a mouse antiserum, B10.D2 anti-B10.Br, generously supplied by Dr. David Sachs (5). It was used after fractionation in 50% ammonium sulfate. Surface immunoglobulin (SIg) was identified with $F(ab')_2$ fragments of a polyvalent goat antiserum to rat immunoglobulins (GARG); its preparation and properties have been described (6). $F(ab')_2$ fragments of goat antiserum to mouse IgG (GAMG) (N. L. Cappel Laboratories, Inc.) were used to detect mouse IgG. GAMG was absorbed with rat IgG coupled to Sepharose 4-B to render it specific for mouse IgG; labeled with a fluorophore, it stained 1–2% of rat spleen lymphocytes by direct fluorescence assay.

Fl and Rh conjugates of anti-Ia, GARG, and GAMG were prepared as described elsewhere (7).

Cell Staining. T lymphocytes were labeled by incubating 3×10^6 spleen cells or cells of a 7-d MLTC with 150 μg of GARG in a volume of 0.5 ml of minimal essential medium or RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% fetal bovine serum. The incubation period was 75 min at 37°C, to strip SIg from the cell membrane of rat B cells and to prevent any cross-reactivity of GAMG for rat spleen cells. The cells, after incubation, were washed once in Hanks' balanced salt solution containing 2% bovine serum albumin (BSA) and 0.1% NaN_3, resuspended in 50 μl of this medium, and then incubated for 30 min at 4°C with 25 μl of W3/13 at 1:10 dilution or W3/25 at 1:4 dilution. Cells were washed two times, resuspended in Hanks' balanced salt solution containing BSA and NaN_3, incubated with 20 μg of Fl-GAMG for 30 min at 4°C, and washed three times before final suspension in medium. When T cells were fractionated in a fluorescence-activated cell sorter (FACS), W3/25 stained cells were kept in RPMI-1640 10% fetal bovine serum for the entire procedure.

SIg was detected with Fl-GARG as described previously (6). Ia was detected by incubating 3×10^6 spleen cells or cells of a 7-d MLTC in 0.1 ml with 25 μg of Fl-anti-Ia for 30 min at 4°C. Cells were washed three times, fixed by addition of 4% paraformaldehyde, final concentration 1%, washed once, and examined. Double labeling was carried out as follows: (*a*) for Ia and SIg, cells were first labeled with Rh-GARG to detect SIg, washed three times in Hanks balanced salt solution-10% rat serum and 0.1% NaN_3, and stained next with Fl-anti-Ia; (*b*) for Ia and T antigens, cells were stained first with unlabeled W3/25 or W3/13 followed by Rh-GAMG to detect T antigens, washed three times in Hanks'-10% mouse serum and 0.1% NaN_3, and next stained with Fl-anti-Ia.

Fluorescence Microscopy and Rapid Flow Microfluorimetry. Cells were examined in a Zeiss epi-fluorescence microscope with Fl- and Rh-specific filters. The number of positively stained cells per 100 lymphocytes was scored for each trial. Doubly labeled cells were quantitated by recording the number of positively stained cells with one fluorophore per 100 lymphocytes and the number of positively stained cells with the other fluorophore per 100 positive cells labeled with the first fluorophore.

Fluorescence intensity and light scatter were determined in an FACS (FACS II; Div. FACS Systems, Beckton, Dickinson & Co., Oxnard, Calif.). The preamplifiers and laser beam were adjusted so that the fluorescence peak was at channel 280, and the scatter peak was at channel 230 when 5 μm fluorescent beads were tested (Coulter Electronics Inc., Hialeah, Fla.). The coefficient of variation was about 10%. Cells in fluorescence channels 100–400 were classified as low density, and 400–1,000 as high density. Percentage of cells under each part of the

fluorescence histogram was determined by the formula: number of cells in specified fluorescence channels/total number of fluorescent cells multiplied by 100. Positive W3/25 cells were isolated by sorting in the FACS at 4–5,000/s. Cells in channels 0–90 were deflected left, and those in channels 110–1,000 were deflected right.

Depletion of B and T Lymphocytes. B cells were eliminated from effector populations by adherence to plastic Petri dishes coated with affinity purified $F(ab')_2$ fragments of goat antibodies to rat immunoglobulins according to method of Mage et al. (8). T cells were removed by lysis with an excess RART and guinea pig serum diluted 1:5 as a source of complement. RART plus complement treatment reduced day 7 immune T cells from 57 to 5%, as detected by W3/13.

Generation of Effector Cells. Rats were immunized by a subcutaneous injection with 10^6 BM2 cells. Spleen cells were removed 7 wk later, 2 wk after the tumors had completely regressed. To augment cytotoxicity, immune spleen cells were cultured in vitro with MST-1 tumor cells treated with 50 $\mu g/10^7$ cells/ml, of mitomycin C (Sigma Chemical Co., St. Louis, Mo.). Pretreatment of tumor cells with trypsin (0.5 g trypsin plus 0.2 g EDTA/liter for 5 min at 37°C in 95% air-5% CO_2; trypsin EDTA (Flow Laboratories, Inc., Rockville, Md.) enhanced their immunogenicity as stimulating elements in the MLTC. The cultures were established in 75-cm^2 tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co.) at a ratio 5:1 spleen (E):tumor cells (T) and incubated for 7 d under the conditions previously described (1). Cytotoxicity of spleen cells only from donor rats in which regression was complete could be augmented in vitro.

^{51}Cr Release Assay. After 7 d of culture the cytotoxic spleen cell activity (CMC) was assessed in a 16-h ^{51}C-release assay as described previously (1). The 7-d cultures of immune spleen cells and mitomycin C-treated MST tumor cells (MST_{Mit}) yielded the maximal CMC, ranging from 80 to 84% specific ^{51}Cr release at E:T ratios of 50:1 to 100:1. In a microassay, 10^4 ^{51}Cr-labeled target cells were incubated at a ratio of 50:1 with effector cells in 0.5-ml microtubes in a volume of 0.3 ml.

Challenge of Established Sarcomas In Vivo. Assay rats were irradiated with 400 rad whole body irradiation, and inoculated subcutaneously in the flank with 5×10^4–2.5×10^5 MST-1, a lethal inoculum within 30–40 d. The dose of tumor varied with different passages of MST-1, and assay rats were tested at 6–8 d when MST-1 was 1–1.5 cm in Diam; at 12 d, when tumors were 2.5 cm in Diam; and at 18 d postinoculum when tumors were 4–7 cm in Diam. Groups of three to five assay rats were infused intravenously with a single or repeated doses of unfractionated cytotoxic effector cells or control effector cells. In some experiments, fractionated populations were used: $W3/25^+$ cells and $W3/25^-$ cells from immune spleen cultures of MLTC; T cell- and B cell-depleted immune effector populations. Tumor size was monitored by measuring with dial calipers every 2–4 d for up to 100 d. Size was recorded as the product of maximal perpendicular measurements and presented in square millimeters as the mean of a group.

Results

Effector Cells and Tumor Elimination In Vivo. In the first set of experiments, a tumor inoculum of 2.5×10^5 MST-1 was injected subcutaneously into the right flank of BN rats, which were irradiated with 400 rad total body irradiation. The E:T ratio was varied from 50:1 to 400:1, calculated on the number of tumor cells inoculated on day 0. On day 6, when tumors ranged between 1.0 and 1.5 cm in Diam and had a blood supply (as determined by histologic examination in other experiments) effector cells from 7-d MLTC, with augmented in vitro cytotoxicity, were infused intravenously. The results are shown in Fig. 1.

When 1×10^8 immune spleen cells were infused intravenously (E:T ratio of 400:1), MST-1 was eliminated within 20–30 days after infusion of effector cells. By infusion of 5×10^7 immune spleen cells intravenously (200:1, E:T), MST-1 was eliminated in 40–48 d after infusion of effector cells; by infusion of 1.25×10^7 immune spleen cells intravenously (50:1 E:T) and 2.5×10^7 immune spleen cells intravenously (100:1 E:

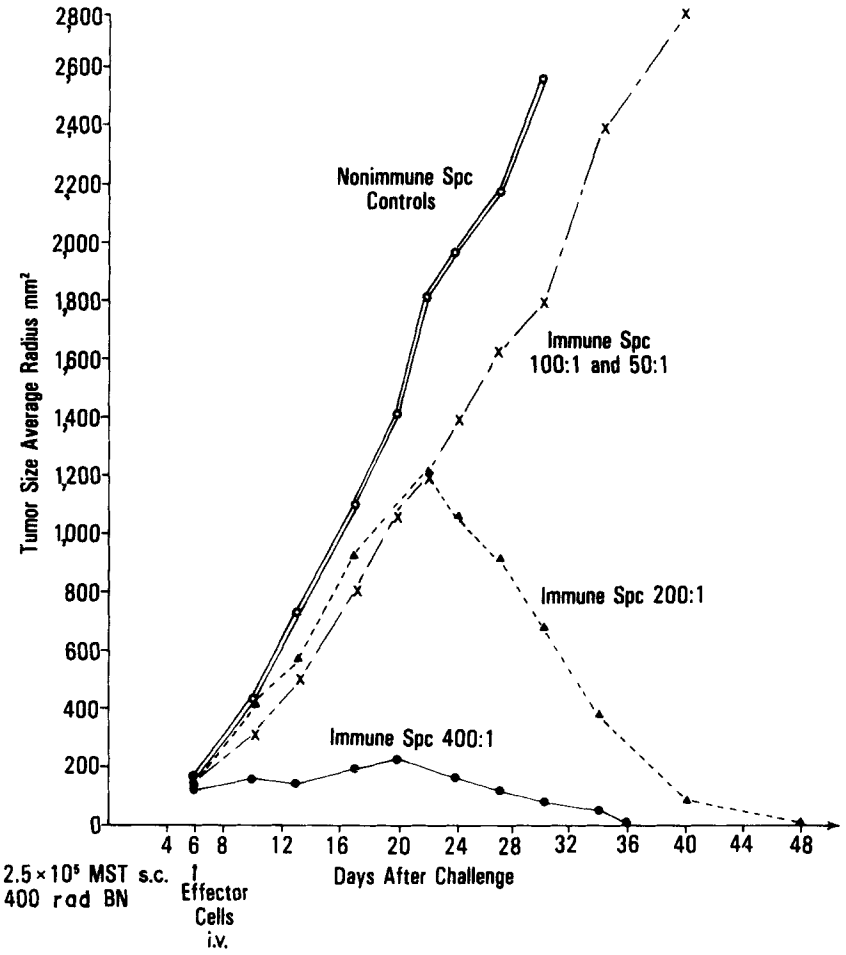

FIG. 1. Effect of the intravenous (i.v.) infusion of varying numbers of immune spleen cells (Spc), with augmented cytotoxicity, in assay rats bearing a day-6-established vascularized subcutaneous (s.c.) MST-1 sarcoma, 1.5 cm in Diam. Controls included infusion of: MST-1 tumor cells alone, MLTC cells from normal nonimmunized donors, and MLTC immune spleen cells incubated with an unrelated tumor (BC5). Data pooled from 4 separate experiments, i.e., 20 assay rats in each group.

T), there may have been some delay in tumor growth, but MST-1 expansion was progressive, and all rats were dead 40 d after tumor inoculation. Untreated controls with tumor only, died within 30 d of tumor inoculation. In rats given cells from normal or progressor donor spleens that were cultured for 7 d in MLTC, growth of tumors was unaffected or enhanced.

Fig. 1 reveals that despite infusion of large number of effector cells, a period of time was required (about 2 wk) before regression was recognizable. Furthermore, the larger the number of effector cells, the greater and sooner the effect on tumor growth. Similar results were also obtained in assay rats that were not irradiated except that tumor elimination was prolonged by several days (data not shown). Irradiated rats were reported in this paper because rate of growth of tumors in irradiated rats was

less variable than in normal rats and because in a small proportion of normal hosts bearing a tumor, infusion of effector cells was inhibitory rather than curative (1).

In a second set of experiments, subcutaneous inocula of MST-1 were allowed to expand in size for 12 and 18 d after inoculation, when they were over 2.5 and 5.0 cm in Diam, respectively. These data are shown in Fig. 2. The larger the tumor load at the time of infusion of effector cells, the greater the number of effector cells that was

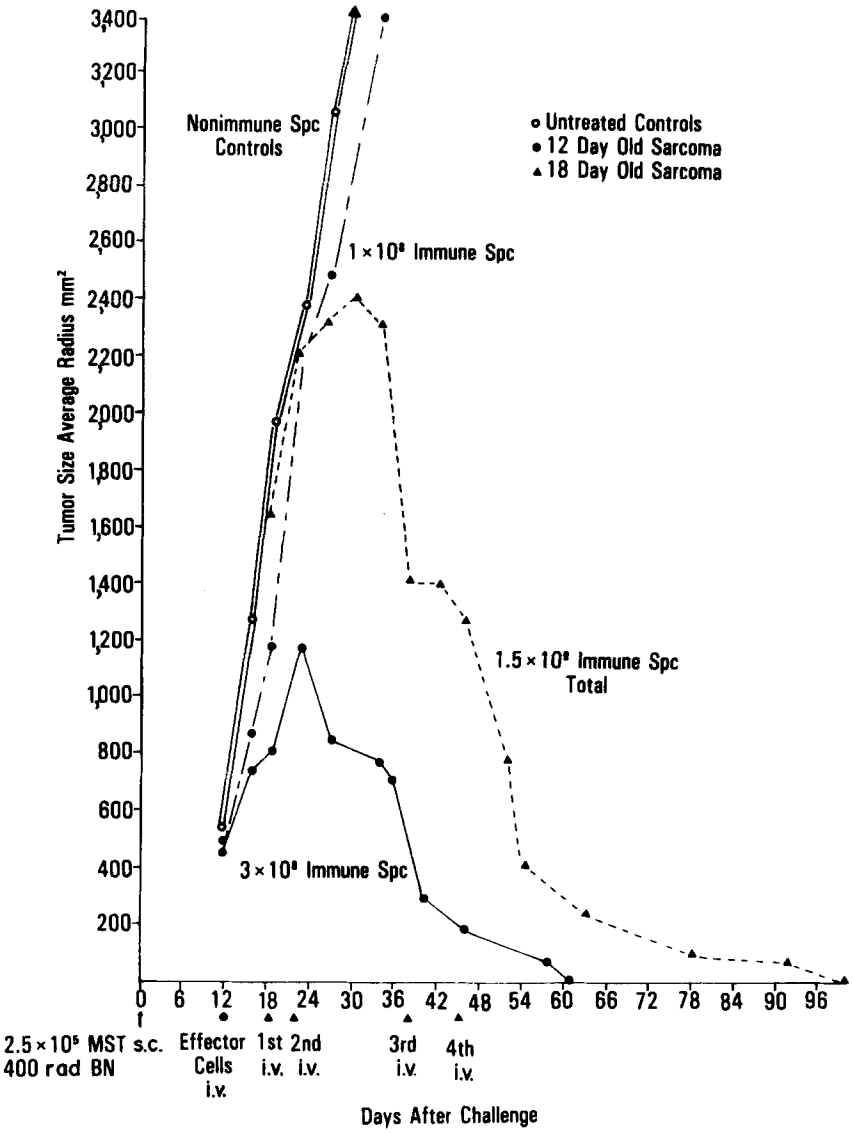

FIG. 2. Elimination of malignant sarcomas, 2.5–7 cm in Diam, by infusion of immune spleen cells with augmented cytotoxicity. Assay rats bearing a 12-d-old sarcoma (●) were treated with a single infusion of effector cells and assay rats bearing an 18-d-old malignancy were treated with repeated infusions (▲) of effector cells. Data pooled from three separate experiments. Each point of the experimental groups represents the mean of 6 assay rats, and each point of the control curves is mean of 15 assay rats. O, untreated controls.

needed to eliminate the tumor. As before, there was continued growth of tumor for 10–14 d after infusion of effector elements. Elimination of a 12-d-old sarcoma required a minimal dose of 3×10^8 effector cells, and complete elimination was achieved 50–60 d after injection. However, in 18-d-old sarcomas, the total number of effector cells needed to induce detectable regression was 7.5×10^8, given in two intravascular infusions. Two additional infusions of effector cells were administered even when it was apparent that MST-1 was diminishing, in order to ensure that the tumor would not resume growth. Fig. 3 is a photograph showing the size of the tumor in one assay rat at its peak size on day 27 after inoculation of MST-1, and also its complete elimination by day 98. There was no recurrence in any of the rats cured of MST sarcomas, some of them having been followed for more than 18 mo. Furthermore, the treated rats retained the capability to reject a lethal dose of MST-1 when challenged 1 yr later (data not shown).

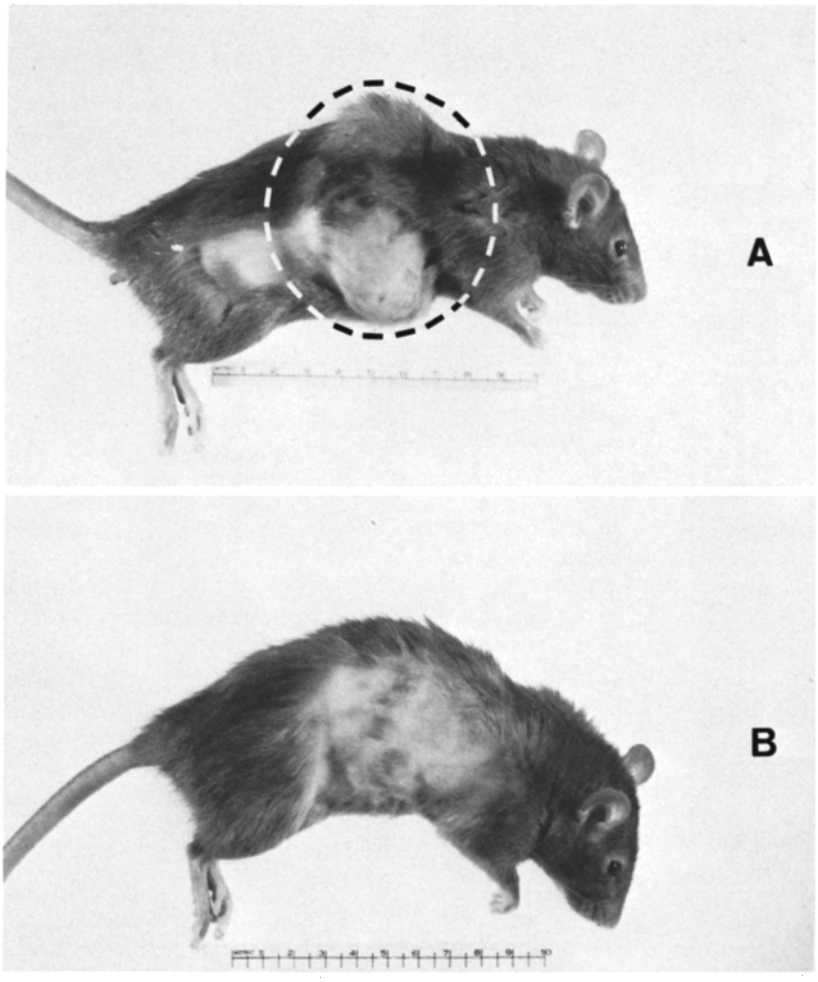

FIG. 3. Photograph showing: (A) one assay rat bearing a lethal subcutaneous MST-1 sarcoma, 6 cm in Diam, on day 27 after tumor inoculation. The test rat was treated with repeated intravenous infusions of effector spleen cells (total = 1.5×10^9), beginning on day 18 after inoculation of tumor. (B) Elimination of the tumor 98 d after tumor inoculation and 80 d after infusion of effector cells.

Nature of Effector Cells. The composition of spleen cells after removal from normal BN rats and BN rats with regressed or progressive tumors (day 0 of culture) is presented in Table I. There were no significant differences in the percentages of T-positive, Ig^+, and Ia^+ cells. The non-T, Ig^- cells included macrophages (5–10%) and null cells and other unidentified elements, and this mixed population of cells was also not significantly different in relative numbers among the three groups of spleens. It should be noted that spleens of rats with progressive tumors were about two times larger in weight (2–3 g) than were spleens of normal control rats or spleens from rats in which tumors had regressed, and yielded twice as many cells per spleen.

After 7 d of culture of a mixture of spleen cells and mitomycin-treated MST-1, there was a significantly altered composition of spleen cells from regressor rats (Table I). T cells were selectively increased (50–57%), Ig^+ cells were decreased; and Ia^+ cells were increased. The total number of cells in 7-d cultures of spleens from regressor animals averaged 50% of the number cultured on day 0; in 7-d cultures of spleens from normal rats or rats with progressive tumors, cells were reduced to 15% of the day 0 number. The relative percentages shown in Table I, indicate that there was a fivefold increase of $W3/25^+$ cells in day 7 cultures of regressor spleens compared with day 7 cultures of controls. For in vivo infusion, experimental and control cultures were pooled. When an equal number of cells was administered to tumor-bearing hosts, the absolute number of $W3/25^+$ T cells in pools of regressor spleen cultures delivered to test rats, was significantly greater than the number of $W3/25^+$ cells delivered in pools of cultures derived from controls or uncultured regressor spleens. Examination of spleens from normal rats and rats with progressive tumors also revealed that after 7 d of culture in MLTC, there was a relative decrease of T cells, Ig^+, and Ia^+ cells. These decreases were not statistically significantly different from the percentages of cell types found in day 0 cultures.

There was also a significant increase in the number of Ia^+ cells in the 7-d cultures of regressor spleens. Approximately 55% of $W3/25^+$ T cells became Ia^+ (Table I). In the three groups of spleens at day 0, Ia^+ T cells ranged from 1 to 3%. Our data

TABLE I

*Surface Markers on Uncultured and Day 7 MLTC Cells**

	T ($W3/13^+$)	T ($W3/25^+$)	Ig^+	Ia^+	T ($W3/13^+$) Ia^+	T ($W3/25^+$) Ia^+	T^- Ig^-‡
				%			
Uncultured spleen cells, day 0							
Normal spleens	45 ± 1 (4)§	38 ± 1 (4)	48 ± 2 (4)	27 ± 1 (12)	3 ± 1 (7)	2 ± 1 (7)	7
Progressor spleens	46 ± 2 (4)	34 ± 2 (4)	46 ± 1 (4)	28 ± 1 (2)	4 ± 1 (2)	4 ± 1 (2)	8
Regressor spleens	46 ± 1 (4)	38 ± 1 (4)	45 ± 1 (4)	29 ± 1 (2)	5 ± 1 (2)	4 ± 1 (2)	9
Day 7 MLTC cells							
Normal spleens	39 ± 3 (3)	35 ± 3 (3)	31 ± 1 (2)	18 (1)	1 (1)	2 (1)	30
Progressor spleens	37 ± 6 (3)	35 ± 3 (3)	39 ± 2 (2)	21 ± 2 (2)	5 ± 1 (2)	4 ± 2 (2)	24
Regressor spleens	57 ± 3 (6)	50 ± 3 (6)	24 ± 6 (4)	58 ± 4 (5)	54 ± 7 (4)	55 ± 7 (4)	19
P‖	$P = 0.02$	$P < 0.02$	$P < 0.02$	$P < 0.01$	$P < 0.01$	$P < 0.01$	

* Data shown are the mean percentage of stained cells ± SEM. 3×10^6 uncultured and day 7 MLTC spleen cells were stained with F1-GARG, anti-W3/13, or anti-W3/25 followed by F1-GAMG. 1×10^5 viable cells were analyzed for fluorescence intensity in a FACS. For the determination of Ia-positive cells and Ia-positive T cells, 3×10^6 cells were stained with anti-W3/13 or anti-W3/25 and Rh-GAMG followed by F1-anti-Ia. The percentage of Ia-positive lymphoid cells and Ia-positive T cells was determined by fluorescence microscopy.

‡ Determined by subtracting from 100% the total number of T cells ($W3/13^+$) and B cells (Ig^+).

§ Number of trials in parentheses.

‖ P value was calculated by Student's t test and compares uncultured (day 0) regressor spleen cells and day 7 MLTC regressor spleen cells.

apparently underestimated the total number of Ia^+ cells present on days 0 and 7. Fluorescence microscopy of day 0 spleen cells showed that about 30% are Ig^+, and 90% of the Ig^+ cells are Ia^+. FACS analysis showed that 48% of cells are Ig^+, hence, Ia-positive cells could be expected to be about 40%.

Microscopic examination of 7-d cultures disclosed that 75% of the cells were blastlike cells (Fig. 4): there were very few tumor cells and macrophages. With appropriate staining, of the blast cells 65% were T cells; there were also about 10% Ig^+ cells. In 7-d cultures of progressor and normal spleens, there were relatively large numbers of tumor cells, few blast cells, and some large vacuolated elements, which may have been macrophages (Fig. 4). A light scatter histogram disclosing the change in cell size of 7-d MLTC cultures of regressor spleens confirmed a significant shift from small to large cells. Over 70% of day 7 cultures were larger than average spleen cells of day 0 cultures (data not shown).

The observations recorded in Table I and Fig. 4 implicated T cells as the main effector cell population. T cells were, therefore, reacted with W3/13 and W3/25 anti-T reagents and examined in a FACS for staining intensity, i.e., for density of T antigens on T cell surfaces. Cultures, prepared immediately after removal of spleens from donor rats and from cultures of 7-d MLTC, were labeled. Results are shown in Fig. 5 and Table II. In Table II cells were analyzed for their fluorescence intensity: low intensity channels 100–400, and high intensity, channels 400–1,000. The significant change in the day 7 effector cell population was an increase of poorly stained T cells and a significant decrease of brightly stained cells when Fl-W3/13 was used. This decrease in fluorescence intensity occurred on cells that had increased in size as determined by light scatter. The opposite changes occurred when Fl-W3/25 was used (Fig. 5).

Elimination of Tumor In Vivo with Fractionated T Cells. Day 7 cultures of MLTC derived from regressor spleens were sorted in a FACS into $W3/25^+$ and $W3/25^-$ populations. W3/25 by indirect immunofluorescence identified 87% of total day 7 immune T cells. Of the $W3/25^+$ populations, 95% were positively stained after examination of sorted cells, and 55% were Ia^+. Rats were infused intravenously with 5×10^6 $W3/25^+$ cells, with equal numbers of $W3/25^-$ cells, or with $10–15 \times 10^6$ immune spleen cells of 7-d MLTC, stained with W3/25 but not sorted. The latter unfractionated population contains approximately $5–7.5 \times 10^6$ $W3/25^+$ T cells and $5–7.5 \times 10^6$ $W3/25^-$ cells. The results of one such experiment are shown in Fig. 6. With infusion of $W3/25^+$ T subset, an inoculum of 10^5 MST given subcutaneously 6 d before was completely eliminated in 6–8 d. $W3/25^-$ cells were ineffective against the established tumor; indeed, MST was significantly larger than tumors in control rats. $10–15 \times 10^6$ unfractionated day 7 immune spleen cells inhibited, but did not eliminate, the tumor. This type of experiment has been performed four times with similar results. However, when the sorted $W3/25^+$ T lymphocytes could not be infused in sufficient numbers into rats bearing a 0.7- to 1-cm sarcoma, growth of the tumor was inhibited, but MST was not eliminated; the challenged hosts survived 75–95 d. In assay rats treated with $W3/25^-$ cells, tumors were larger than in controls, and challenged hosts survived 28–30 d. Mean survival time in untreated controls was 38 ± 4 d SE.

A number of other fractionated populations of effector cells were assayed as described above. $10–50 \times 10^6$ immune spleen cells deprived of T cells by lysis with

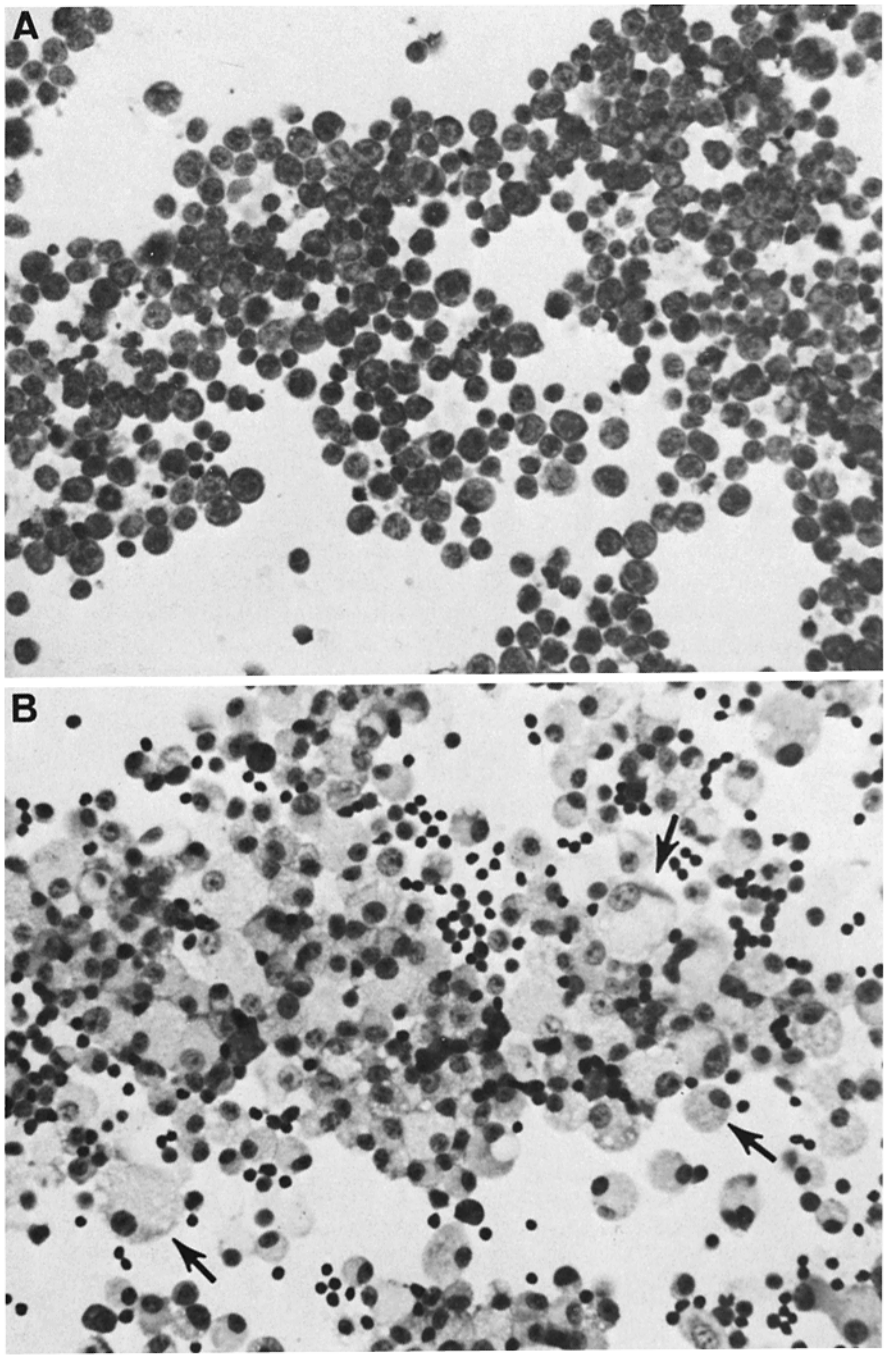

FIG. 4. (A) Photomicrograph of effector cells in 7-d MLTC containing immune spleen cells from donor with regressed tumor and MST-1_{Mit}. Blast lymphocytes are numerous and tumor cells are rarely encountered. Giemsa stain; cytocentrifuged preparation; × 250. (B) Photomicrograph of cells in 7-d MLTC containing immune spleen cells from donor with progressing tumor and MST-1_{Mit}. A mixture of tumor cells (arrows) and small lymphocytes are seen. Giemsa stain; cytocentrifuged preparation. × 250.

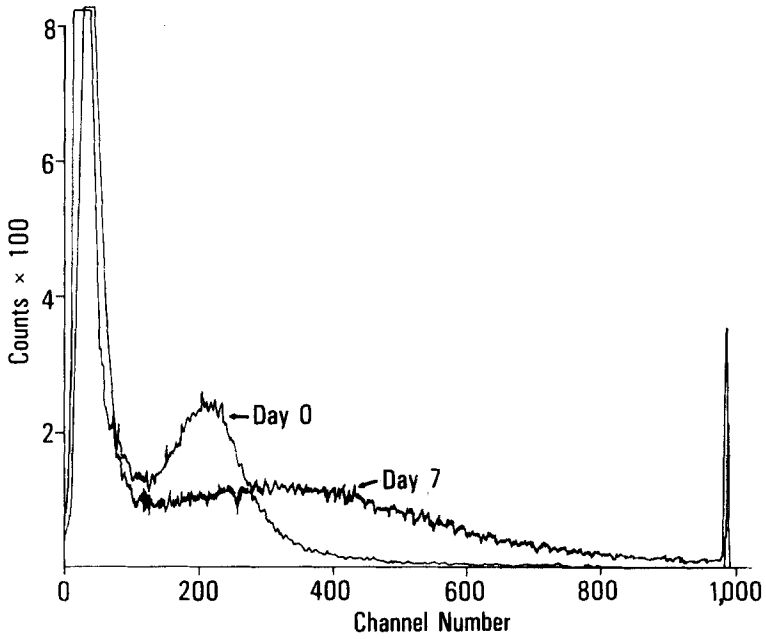

FIG. 5. Fluorescence histograms of day 0 and day 7 MLTC. 10^5 immune spleen cells, from a donor with a regressed tumor were reacted with W3/25 and Fl-GAMG. Cell number is plotted on the vertical axis, fluorescence intensity on the horizontal. There is a marked increase of W3/25 antigen after 7 d in culture.

TABLE II

*Fluorescence Intensity of Stained Spleen Cells**

Fluorescence channel	Normal, day 0	Regressor, day 0	Regressor, day 7	P‡
		%		
T ($W3/13^+$)				
100–400	69 ± 4 (4)§	67 ± 4 (4)	85 ± 4 (4)	<0.02
400–1,000	31 ± 5 (4)	32 ± 2 (4)	15 ± 2 (4)	<0.001
T ($W3/25^+$)				
100–400	92 ± 2 (4)	94 ± 2 (4)	72 ± 4 (7)	<0.01
400–1,000	7 ± 1 (4)	5 ± 1 (4)	28 ± 4 (7)	<0.01

* Data shown are the percentages of SEM of fluorescent cells present in the specified channels. 3×10^6 uncultured and day 7 MLTC spleen cells were incubated with anti-W3/13 or anti-W3/25 and stained with Fl-GAMG. 1×10^5 viable cells were analyzed for fluorescence intensity in a FACS. The percentage of cells under each portion of the curve was determined as follows: cells in specified fluorescence channels/total fluorescent cells × 100.

‡ P value was calculated by Student's t test and compares uncultured (day 0) regressor spleen cells and day 7 MLTC regressor spleen cells.

§ Number of trials in parentheses.

RART and complement (C) were ineffective against MST (Fig. 6). At an E:T ratio in vivo of 1,000:1 (1×10^8 immune spleen cells), the tumor was inhibited but not eliminated (5–7% T^+ cells remained; data not shown). 50×10^6 immune spleen cells, after reduction of SIg^+ cells to 3% of the total, completely eliminated MST-1 in about 40 d, a result similar to that obtained with unfractionated day 7 effector cell

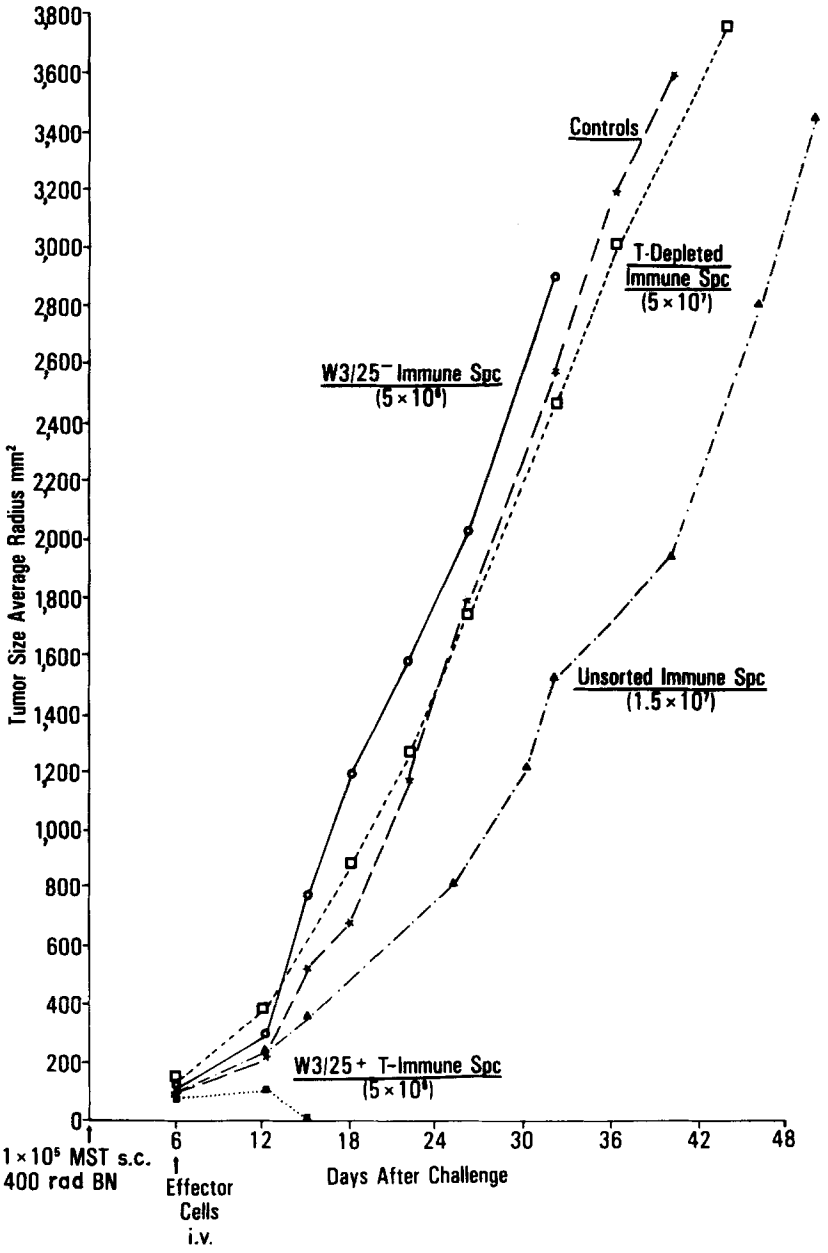

FIG. 6. Effect of W3/25$^+$ and W3/25$^-$ immune spleen cells on tumor growth in vivo. Each curve represents one trial. Similar results were obtained in four different experiments (total = four assay rats) with W3/25$^+$ and W3/25$^-$ cell fractions, but these data are not pooled because different numbers of sorted cells and different doses of tumor challenge were used in each experiment. The growth curves of tumors treated with unfractionated cells, of controls, and of tumors treated with T-depleted immune spleen cells represent the mean of three different experiments with three assay rats per group (nine observations per point).

populations. A ($W3/13^+$, $W3/25^-$) T cell population could not be obtained in sufficient numbers to infuse into tumor-bearing rats.

In Vitro Cytotoxicity of Fractionated T Effector Cells. Immune spleen cells of 7-d cultures in MLTC were sorted in a FACS into $W3/25^+$ and $W3/25^-$ populations. These and the unfractionated cultures containing $W3/25^+$ and $W3/25^-$ populations were tested for cytotoxicity against target MST-1 in ^{51}Cr release assays. Fig. 7 gives the results of these experiments and represents the mean of three trials, each trial carried out in triplicate; the standard error of the mean did not exceed 10% for these experiments. $W3/25^+$ cells were poorly cytotoxic; cytotoxicity ranged from 11 to 13% specific ^{51}Cr release. $W3/25^-$ cells were more effective, displaying 22–50% specific ^{51}Cr release. The unfractionated mixed population from which $W3/25^+$ and $W3/25^-$ were derived, under similar experimental conditions, yielded specific ^{51}Cr release of 37–62% at ratios of 20:1 to 50:1. Also, removal of T lymphocytes by treatment with RART and C decreased in vitro cytotoxicity to less than 10%. $W3/13^+$ cells were cytotoxic against MST (average 70% ^{51}Cr release at an E:T of 50:1).

Cytotoxicity of unfractionated immune spleen cells, incubated for 7 d with mitomycin C-treated MST (MST_{Mit}) was augmented to 60–70% ^{51}Cr release; at the time of removal from donor rats, immune spleen cells showed a 10% ^{51}Cr release against MST (1). Control spleen cells, immune spleen cells cultured without antigenic stimulation, and immune spleen cells cultured with an unrelated tumor (BC5), showed no augmentation of ^{51}Cr release after 7 d in culture, and were ineffective in vitro and in vivo against MST (1). These results indicate that in vitro culture with the specific tumor antigens is necessary to augment effector function of infused cells.

Discussion

There has been a plethora of reports describing either protection, prevention, or elimination in vivo of syngeneic or allogeneic tumor by injection of cytotoxic effector

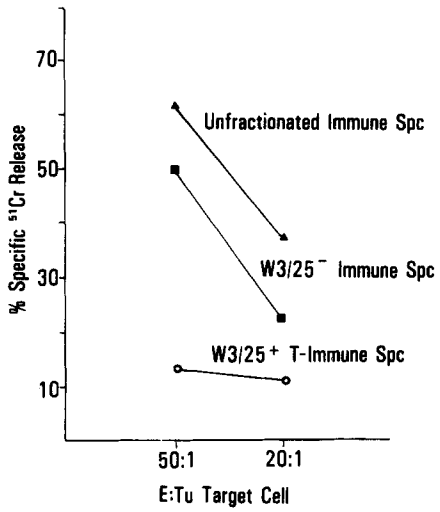

FIG. 7. In vitro cytotoxicity of $W3/25^+$ and $W3/25^-$ immune spleen cells of 7-d MLTC and the unfractionated population from which these were derived. Immune spleen cells and control spleen cells were tested in a ^{51}Cr release assay against MST-1. Day 7 control spleen cells expressed negative cytotoxicity (data not shown). Each point is the mean of three trials in three separate experiments (nine observations) at E:T ratios of 20:1 and 50:1. E/Tu, spleen (E):tumor cells (Tu).

cells, i.e., immune lymphoid cells (9–73). The tumors have been lymphomas, leukemias, plasma cell tumors, fibrosarcomata, mammary, and lung carcinomas, and they have been induced by RNA C-type viruses, DNA simian virus 40, and chemical carcinogens. The test species have included mice, rats, and guinea pigs (we are excluding human trials in this report). In most instances, malignant tumors have been prevented from growing by administration of immune lymphoid cells before or simultaneously with the inoculation of transplantable tumor or inducing virus. However, a number of papers described the elimination of tumors after they have been inoculated into test animals, usually 3–5 d before administration of effector cells (14, 15, 17, 19, 20, 23–26, 28, 31, 46, 56, 57, 62, 70, 73) when the tumors ranged from 0.5 to 2.0 cm in Diam. In some models combined immuno- and chemotherapy have been applied (19, 22, 24–26, 30, 31, 48, 50, 51, 59, 62, 63).

In our model, established sarcomas of large size (1–7 cm) and relatively long duration (18 d) were completely and permanently eliminated, by remote infusion of syngeneic effector T lymphocytes that were stimulated a second time by the specific antigen, i.e., by MST-1 tumor. Several rough calculations reveal that a 5-cm in Diam 5-g tumor is about 3% of the body weight (165 g) of the test rats bearing the tumors. If translation to human proportions is permissible, this represents a 2-kg neoplasm in a 70-kg adult. In addition, tumors were expanding for 18 d before treatment was started; in 100-d-old test rats, this is 18% of their lives, or in human terms, about 3.5 yr of a 20-yr-old adult. Regression of these large tumors (5–6 g), which we estimate to contain not less than 5×10^8 viable tumor cells, was visible after infusion of 7.5×10^8 effector cells, which is about two times the number of cells that can be recovered from an adult BN spleen. The W3/25$^+$ effector population was about 4×10^8 cells.

The subset associated with elimination of established tumors was defined by W3/25$^+$, W3/13$^+$ blast cells. However, in vitro this subset was poorly cytotoxic for MST-1. These disparate results suggested that the (W3/25$^+$, W3/13$^+$) T cell functioned as a helper or amplifier cell in the tumor-bearing host and that elimination of MST-1 required participation by the host. In support of this was the following observation. There was a time lag between infusion of effector cells and noticeable regression of tumor. If W3/25$^+$, W3/13$^+$ cells were directly cytotoxic in vivo, one might have expected an immediate or almost immediate effect on tumor size. Instead, there was continued tumor expansion for 10–14 d after effector cell infusion. In addition, White et al. (74) have provided some evidence that the W3/25$^+$ cell functions as a helper cell. However, we cannot exclude the possibility that the W3/25$^+$ cell may be converted to a cytotoxic effector cell after transfer to the assay animal, or that a small number of cytotoxic cells contained in the effector cell population expand to kill the tumor.

The biological significance of the surface membrane markers on the cells of the effector subset is still undefined. In the MLTC, a T cell subset was expanded in response to tumor antigens and transformed into blast elements. With these changes there was an increase in the W3/25 antigen on the cell surface and a decrease of W3/13 antigen. We have not determined whether the W3/25$^+$ antigen is obligatory for helper function and whether its presence determines function or vice versa. Ia antigens, at least the ones detected by the antiserum used, seem not to be related to the in vitro function of W3/25$^+$ cells. In preliminary experiments when immune spleen cells of 7-d cultures were depleted of Ia-positive cells by anti-Ia antibody plus C or when Ia

antigens were stripped from the lymphocyte membrane by capping, in vitro cytotoxicity was the same as that observed with populations that were Ia^+. Several recent reports have emphasized that Ia antigens of the I-A region of major histocompatibility complex are associated with amplifier T cells (75, 76); other reports indicate that Ia antigens, particularly those related to the I-J region, are associated with cytotoxic T cells (77–79). Ia antigens may merely be a differentiation marker or a marker for cell activation (75, 80–83). In our model, the acquisition of Ia during MLTC appears to have a positive relationship with cell activation because cell cultures from normal spleens, from spleens derived from rats with progressive tumors, and from regressor donor spleens that failed to become cytotoxic and to eliminate tumors in vivo, contained only 3–5% Ia^+ T cells and also very few T-blast forms. The $W3/25^-$ population was associated with enhancement of MST-1 in vivo and with moderate cytotoxicity for MST-1 in vitro. The $W3/25^-$ population was a melange of cells that included ($W3/13^+$, $W3/25^-$) T cells (about 14% of this population), null cells, Ig^+ cells, and 1–3% macrophages. Suppressor cells were likely contained in the $W3/25^-$ population, as is suggested by the tumor enhancement in vivo and by the failure of small numbers of unfractionated cells (containing a mixture of $5–7 \times 10^6$ $W3/25^+$ and $5–7 \times 10^6$ $W3/25^-$) to eliminate tumor as shown in Fig. 6. However, this possible inhibitory effect would be overcome when higher numbers of unfractionated cells are used (Fig. 1). The phenotype of the suppressor element has not been identified. Suppressor T cells have been shown to operate in a model in vivo with established tumors (73). We also have not ascertained the cytotoxic cell contained in $W3/25^-$ population. The in vitro cytotoxicity results suggest that one of the cytotoxic cells is a $W3/13^+$ T cell. A null cell with antibody to viral antigens might also be operative (E. Fernandez-Cruz and J. D. Feldman. Unpublished observations.).

Our observations in this in vivo tumor model suggest that successful elimination of an expanding tumor depends upon a balance of helper cell activity overriding suppressor cell activity with participation of the host to provide the cytotoxic elements. If there are insufficient helper cells or too many suppressor elements, there will be no participation by the host and no provision of cytotoxic cells. The manipulations and use of immune effector cells in this rat model are applicable to human neoplastic conditions. With methods available to grow T cell lines with specific reactivities (81, 84–87), it is conceivable that a T lymphocyte subset could be isolated from peripheral blood or draining lymph nodes of humans bearing tumors and could be cultivated in vitro into permanent cell lines. These T cell lines, selected by their specific anti-tumor reactivities, could be returned to the tumorous patient in any number of effector cells needed.

Summary

Established subcutaneous Moloney sarcomas (MST-1) of large size and long duration were eliminated from syngeneic rats by intravenous infusion of varying numbers of specific syngeneic effector T lymphocytes. Spleen cells from BN rats in which tumor had regressed were cultured in an in vitro mixed lymphocyte tumor cell culture (MLTC) to augment cytotoxicity of effector cells. In the MLTC a T cell subset was expanded in response to MST-1 antigens and transformed into blast elements. With these changes, there was an increase in the W3/25 antigen on the T cell surface, a decrease of W3/13 antigen, and an increase in the number of T cells

with Ia antigens. The subset associated with elimination of established tumors was a blast T cell W3/25$^+$, W3/13$^+$, as detected by monoclonal antibodies to rat T antigens. The W3/25$^+$ subset was poorly cytotoxic in vitro for MST-1 and apparently functioned in vivo as an amplifier or helper cell in the tumor-bearing host. The W3/25$^-$ population was a melange of cells that included (W3/13$^+$, W3/25$^-$) T cells, null cells, Ig$^+$ cells, and macrophages, and was associated with enhancement of tumor in vivo, suggesting the presence of suppressor cells.

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