Importance of Lyt 2+ T-Cells in the Curative Effectiveness of a Low Dose of Melphalan for Mice Bearing a Large MOPC-315 Tumor

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ABSTRACT

We have previously demonstrated that the curative effectiveness of a low dose of melphalan (L-phenylalanine mustard; L-PAM) for mice bearing a large s.c. (approximately 20 mm in diameter) MOPC-315 tumor and extensive metastases requires the participation of T-cell-dependent antitumor immunity in tumor eradication (S. Ben-Elrair ef al., Cancer Immunol. Immunother., 15: 101-107, 1983). Here we show that the Lyt 2+ T-cells, and not the L3T4+ T-cells, participate in the cure of such tumor-bearing mice by a low dose of L-PAM. Specifically, depletion of Lyt 2+ T-cells from mice bearing a large MOPC-315 tumor by treatment with monoclonal anti-lyt 2.2 antibody abolished the curative effectiveness of the low dose of drug. In contrast, depletion of L3T4+ T-cells from mice bearing a large MOPC-315 tumor by treatment with monoclonal anti-L3T4 antibody did not significantly reduce the curative effectiveness of the low dose of drug. Histological examination of tumor nodules on various days following low-dose L-PAM therapy revealed widespread lymphocytic infiltration by Day 5 following the chemotherapy, and this infiltration was drastically reduced when the L-PAM-treated tumor bearers were treated either with anti-Thy 1.2 or anti-Lyt 2.2 antibody but not with anti-L3T4 antibody. The antitumor immunity exhibited by Lyt 2+ T-cells derived from mice which were in the process of eradicating a large MOPC-315 tumor following low-dose L-PAM therapy was exploited successfully to confer systemic antitumor immunity to mice bearing a barely palpable tumor. Specifically, the adoptively transferred Lyt 2+ splenic T-cells, in conjunction with a subcutaneous dose of L-PAM, brought about the cure of most mice. The Lyt 2+ splenic T-cells from L-PAM-treated MOPC-315 tumor bearers were also found to be capable of exerting a direct potent lytic effect against MOPC-315 tumor cells in an antigen-specific manner. Thus, it is conceivable that the direct cytotoxic activity of Lyt 2+ T-cells for MOPC-315 tumor cells is responsible, at least in part, for the ability of the Lyt 2+ T-cells from L-PAM-treated MOPC-315 tumor bearers to bring about the eradication of the tumor burden not eradicated through the direct antitumor effects of the low dose of drug.

INTRODUCTION

The therapeutic effectiveness of anticancer drugs can be facilitated by antitumor immunity (1-7). Consequently, situations may exist under which a low nonimmunosuppressive dose of drug can be therapeutically as effective as a high immunosuppressive dose of drug even though the lower dose of drug exerts a substantially weaker direct antitumor effect. Indeed, several investigators have shown that a low dose of drug in cooperation with host antitumor immunity can be as effective as, or even more effective than, a high dose of drug for mice bearing a barely palpable tumor. Specifically, Mathé et al. (2) have shown that a dose of 134 mg of CY per kg of body weight (mg/kg) was curative for 44% of mice bearing the L1210 leukemia, while a dose of 403 mg/kg was curative for just 5% of the tumor-bearing mice. Similarly, Hilgard et al. (6) have shown that a dose of 3 mg of CY per kg was curative for all rats bearing the L522 leukemia, while a dose of 20 to 100 mg/kg was not curative for any of the tumor-bearing rats.

The importance of antitumor immunity to the outcome of low-dose chemotherapy has been studied extensively in the MOPC-315 tumor system (5, 7, 10). These studies established that a low dose of either CY or L-PAM can cure mice bearing a large MOPC-315 tumor and extensive metastases only if T-cell-dependent antitumor immunity aids in tumor eradication. Accordingly, the therapeutic effectiveness of a low dose of either drug for mice bearing a large MOPC-315 tumor burden was abrogated if, at the time of the chemotherapy, the mice were depleted of T-cells by treatment with rat anti-mouse thymocyte serum (5, 7, 10).

In the MOPC-315 tumor model, as in some other tumor models (6, 11, 12), chemotherapeutic protocols which depend on the contribution of host antitumor immunity for their curative effectiveness were found to cure tumor-bearing rodents even at a stage of tumor growth when antitumor immunity is depressed by the inhibitory activity of suppressor cells (5, 7, 10, 13, 14). The effectiveness of the chemotherapy for such immunosuppressed tumor bearers was attributed to the immunomodulatory activity of the anticancer drugs which brought about the rapid appearance of antitumor immunity which, in turn, participated in tumor eradication (5, 10). Specifically, the anticancer drugs were shown to eliminate suppressor cell activity, thereby unmasking the existing concomitant antitumor immunity and facilitating the generation of additional antitumor immunity in response to stimulation by residual tumor cells (10, 13-15).

In the current study we have confirmed the importance of T-cell-dependent antitumor immunity to the outcome of low-dose L-PAM therapy for mice bearing a large MOPC-315 tumor by illustrating that depletion of Thy 1+ cells, by treating the tumor bearers with monoclonal anti-Thy 1.2 antibody [rather than with rat anti-mouse thymocyte serum, as we have done previously (7, 10)], abolishes the curative effectiveness of the low dose of drug. In addition, we have established that the subpopulation of T-cells which participates in the eradication of the tumor burden not eradicated by the direct antitumor effects of the drug is of the Lyt 2, and not the L3T4 phenotype. As part of this study, we determined the importance of the Lyt 2+ T-cells for the appearance of a massive lymphocytic infiltration in the tumor nodule of L-PAM-treated MOPC-315 tumor bearers. Finally, we determined whether Lyt 2+ T-cells from mice which are in the process of eradicating a large MOPC-315 tumor following low-dose L-PAM therapy can confer systemic antitumor immunity to other mice and whether the Lyt 2+ T-cells themselves can exert a direct and specific lytic effect for MOPC-315 tumor cells.

MATERIALS AND METHODS

Tumors. We have used primarily the MOPC-315 plasmacytoma of BALB/c origin. Routinely, female BALB/c mice 7 to 10 wk old (Charles
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River Breeding Laboratories, Wilmington, MA) were inoculated s.c. with 1 × 10^6 viable tumor cells, a dose which is at least 300-fold the minimal lethal tumor dose, and it leads to the appearance of palpable tumors in 4 to 5 days. Thereafter, the tumors grow progressively, reaching 20 to 22 mm in diameter by Day 10 and killing the mice in about 18 days. For studies into the specificity of the cytolytic activity of spleen cells from L-PAM-treated MOPC-315 tumor bearers, we used an antigenically related syngeneic plasmacytoma (MOPC-104E) and two antigenically unrelated thymomas: the WEHI 22.1 of BALB/c origin and the EL4 of C57BL/6 origin (16). The MOPC-104E was maintained in BALB/c mice by serial weekly passage in an ascitic form, and the thymomas were maintained in vitro in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO).

Chemotherapy. A fresh stock solution of 10 mg of L-PAM (Burroughs Wellcome Co., Triangle Park, NC) per ml was prepared just prior to injection, as previously described (7), and was further diluted with Dulbecco's phosphate-buffered saline, pH 7.2 (Grand Island Biological Co.) to the desired concentration. In most experiments, a dose of 1.5 or 2.5 mg of L-PAM per kg (low dose) was administered i.p. to mice bearing a s.c. MOPC-315 tumor with a diameter of 20 to 22 mm. The mice were then monitored for tumor regression/progression as well as survival for a period of 60 days, and mice that survived the observation period and were tumor free were considered cured.

Hybridomas. As the source of monoclonal anti-Thy 1.2 antibody we used hybridoma 30-H12 (17), which was obtained from the American Tissue Culture Collection (Rockville, MD). As the source of monoclonal anti-L3T4 antibody we used hybridoma GK 1.5 (18), and as the source of monoclonal anti-Lyt 2.2 we used either hybridoma 3.155 (19) or hybridoma 2.43 (19) (all three hybridomas were available to us through the generosity of Dr. Frank Fitz at the University of Chicago, Chicago, IL). Among the monoclonal antibodies used, the anti-Thy 1.2, the anti-L3T4, and the anti-Lyt 2.2 produced by hybridoma 2.43 are rat anti-mouse immunoglobulins of the IgG2 subclass (17-19), while the anti-Lyt 2.2 produced by hybridoma 3.155 is a rat anti-mouse immunoglobulin of the IgM class (18, 19).

In Vivo Depletion of T-Cells or T-Cell Subsets. For the in vivo depletion of T-cells or T-cell subsets, we have prepared ascites containing monoclonal antibody in outbred nu/nu mice (Charles River Breeding Laboratories) as previously described (20, 21). The monoclonal antibody treatments were initiated on Day 7 or 8 following tumor inoculation (i.e., 2 or 3 days prior to the administration of low-dose L-PAM) and consisted of a single i.p. injection on each of 3 consecutive days followed by a fourth injection 2 days later.

Effectiveness and Selectivity of the in Vivo Depletion. The effectiveness and selectivity of the in vivo depletion of Lyt 1*, L3T4*, and Lyt 2* cells in the spleens of mice subjected to treatment with a given monoclonal antibody relative to that in the spleens of mice not subjected to the monoclonal antibody treatment. For this purpose, we determined the depletion of T-cells or T-cell subsets as previously described (8), by mechanical disruption between glass slides, and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 95%. In any individual experiment, the spleens for each group were obtained from at least 5 mice.

In Vitro Depletion of T-Cells and T-Cell Subsets. Spleen cell suspensions were depleted of T-cells or T-cell subsets as previously described (21) by treatment with the appropriate monoclonal antibody plus low-toxicity rabbit complement. The effectiveness and selectivity of the depletion were assessed by the use of immunofluorescent staining followed by analysis on the EPICS V. Treatment of spleen cells with monoclonal anti-Lyt 2.2 or anti-L3T4 antibody plus complement routinely led to more than 90% depletion in the corresponding subset of T-cells with essentially no decrease in the frequency of T-cells belonging to the other subset. Treatment of spleen cells with anti-Thy 1.2 antibody plus complement routinely led to more than 90% depletion of the Lyt 2* and the L3T4* T-cells.

Depletion of Phagocytic Cells. Spleen cell suspensions were depleted of phagocytic cells as previously described (16) by treatment with carbonyl iron and magnet. The effectiveness of the procedure in depleting macrophages was verified by determining the percentage of esterase-positive cells (16, 22) in the nonphagocytic cell fraction as compared to the percentage of macrophages in the unseparated spleen cell suspension. Depletion of phagocytic cells reduced the percentage of esterase-positive cells from approximately 8% to less than 1%.

Adoptive Transfer Assay. The ability of spleen cells to confer systemic antitumor immunity was assessed, as previously described (8, 23), by the ability of the spleen cells to cure MOPC-315 tumor bearers that had been treated with a subcurative dose of L-PAM. Briefly, mice inoculated with 1 × 10^6 MOPC-315 tumor cells and treated 4 days later with a subcurative dose of L-PAM (2.5 mg/kg) (7) served as recipients. One day later, these mice received 5 × 10^6 spleen cells i.v. The mice were monitored for tumor progression/regression and survival for a period of 60 days, and mice that survived the observation period and were tumor free were considered cured.

Antitumor Cytotoxicity Assay. The level of antitumor cytotoxicity was determined, as previously described (24), by the 3.5-h 51Cr release assay. Briefly, 5 × 10^5 51Cr-labeled target cells were incubated with various numbers of fresh spleen cells in 12- x 75-mm plastic tubes. At the end of the incubation period, the cells were pelleted, and both supernatants (Sup) and pellets (Pel) were counted in an Auto-Gamma scintillation counter. The percentage of 51Cr release for each sample was calculated as follows.

\[
\% \text{ of } 51\text{Cr release} = \frac{\text{cpm in Sup}}{\text{cpm in Sup} + \text{cpm in Pel}} \times 100
\]

Subsequently, the percentage of specific 51Cr release was calculated by the following formula

\[
\% \text{ of specific } 51\text{Cr release} = \frac{T - C}{M - C} \times 100
\]

where T is the percentage of release with test lymphocytes, C is the percentage of spontaneous release (which was 10 to 15% with EL4 as well as WEHI 22.1 target cells, and 20 to 22% with MOPC-315 as well.
as MOPC-104E target cells), and \( M \) is the percentage of maximal release obtained by 3 cycles of freezing and thawing (which was 85 to 90% for all four target cells). Routinely, we carried out each determination in triplicate. Variations in the percentage of \(^{31}\text{Cr} \) released between individual samples rarely exceeded 5%. However, some variation was noted in the level of antitumor cytotoxicity between different experiments. Still, the pattern of the results remained consistent. The level of antitumor cytotoxicity presented is the mean \( \pm \) SE of \(^{31}\text{Cr} \) release of at least 3 different experiments.

**Statistical Analysis.** To determine whether the differences in the fraction of mice surviving following different treatments are significant, the \( G \) test of independence (25) was used. To determine whether the differences in the percentage of \(^{31}\text{Cr} \) released by different populations of spleen cells are significant, the Student \( t \) test was used. A \( P \) value of <0.05 was considered significant in both tests.

**RESULTS**

**Effect of Depleting T-Cells or Their Subsets on the Curative Effectiveness of Low-Dose L-PAM for Mice Bearing a Large MOPC-315 Tumor.** Initially, we confirmed the importance of T-cell-dependent antitumor immunity to the outcome of low-dose L-PAM therapy for mice bearing a large MOPC-315 tumor by demonstrating that treatment of such mice with monoclonal anti-Thy 1.2 antibody abolishes almost completely the curative effectiveness of the low dose of drug (Fig. 1). Subsequently, we determined which subset of T-lymphocytes is responsible for the curative effectiveness of the low dose of L-PAM for MOPC-315 tumor bearers. This was done by determining the effect of depleting the Lyt 2* or the L3T4* T-cells from mice bearing a large MOPC-315 tumor, by treatment with the corresponding monoclonal antibody, on the outcome of the low-dose chemotherapy. As can be seen from Fig. 1, treatment of mice bearing a large MOPC-315 tumor with anti-Lyt 2.2 antibody (derived from hybridoma 3.155), like treatment with monoclonal anti-Thy 1.2 antibody, abolished almost completely the curative effectiveness of the low dose of L-PAM (i.e., reduced the cure rate from 93% to 4% versus from 93% to 7%, respectively). In fact, the rate of death following treatment of mice bearing a large MOPC-315 tumor with L-PAM and anti-Lyt 2.2 antibody did not differ from that observed following treatment of mice bearing a large MOPC-315 tumor with L-PAM and anti-Thy 1.2 antibody. In contrast, treatment of mice bearing a large MOPC-315 tumor with monoclonal anti-L3T4 antibody did not reduce significantly the curative effectiveness of the low dose of L-PAM nor did it alter the rate of death of the mice. In the initial studies the monoclonal anti-Lyt 2.2 antibody used (produced by hybridoma 3.155) was of the IgM class, while the monoclonal anti-Thy 1.2 and anti-L3T4 antibodies were of the IgG2b subclass. In subsequent experiments we established that a monoclonal anti-Lyt 2.2 antibody of the IgG2b subclass (produced by hybridoma 2.43), like the anti-Lyt 2.2 of the IgM class, can abolish the curative effectiveness of the low dose of drug for mice bearing a large MOPC-315 tumor under conditions in which anti-L3T4 antibody of the IgG2b subclass fails to do so (Fig. 2). Thus, the curative effectiveness of a low dose of L-PAM for mice bearing a large MOPC-315 tumor depends on the contribution of the Lyt 2* T-cells and not the L3T4* T-cells for tumor eradication.

**Effect of Low-Dose L-PAM Therapy on Lymphocytic Infiltration into the s.c. Tumor Nodule of Mice Bearing a Large MOPC-315 Tumor.** We evaluated histological sections of tumor nodules obtained from MOPC-315 tumor bearers on various days following low-dose L-PAM therapy. These sections were contrasted with sections of tumor nodules obtained from mice treated with a high dose of L-PAM (15 mg/kg), since the high dose of L-PAM, unlike the low dose of L-PAM, does not require the contribution of antitumor immunity to bring about the eradication of a large tumor burden (7). For each determination, sections from at least 6 mice were examined. Representative photomicrographs are provided to illustrate the changes occurring within the tumor nodule as a consequence of the chemotherapy. As can be seen from Fig. 3A, sections of untreated MOPC-315 tumors obtained just prior to the chemotherapy showed homogeneous tissue architecture with sheets of anaplastic plasmacytoid tumor cells with prominent nucleoli and coarse chromatin. In addition, there was little if any lymphocytic infiltration into the tumor site. Sections taken from tumors on various days following low-dose L-PAM therapy showed a progression of tumor damage and lymphocytic infiltration. For example, on Day 3 after the low-dose chemotherapy, each tumor nodule contained widespread areas of necrosis as well as widespread areas of viable tumor cells and minimal lymphocytic infiltration (data not shown). By Day 5 following low-dose chemotherapy, the lymphocytic infiltration was very prominent (Fig. 3B) and represented at least 50% of the cells.

![Fig. 1. Effect of depleting T-cells or their subsets on the curative effectiveness of low-dose L-PAM (2.5 mg/kg) for mice bearing a large s.c. (≥20 mm) MOPC-315 tumor. Mice bearing a large MOPC-315 tumor were treated with either saline (C) or monoclonal anti-Thy 1.2 ( ), anti-Lyt 2.2 ( ), or anti-L3T4 ( ) antibody prior to receiving low-dose L-PAM therapy. The monoclonal anti-Thy 1.2 (30-H12) and anti-L3T4 ( ) antibody used (produced by hybridoma 3.155) were of the IgM subclass, while the anti-Lyt 2.2 (3.155) antibody was of the IgG class. The numbers in parentheses indicate the number of tumor-free mice/total mice inoculated. *, statistically significant relative to the survival data for mice treated with L-PAM and saline (\( P < 0.001 \)). The survival data for the other group were not significantly different from those for mice treated with L-PAM and saline.](image1)

![Fig. 2. Curative effectiveness of low-dose L-PAM (1.5 mg/kg) for MOPC-315 tumor bearers that had been subjected to treatment with anti-Lyt 2.2 (2.43) (Δ) or anti-L3T4 ( ) antibody of the same immunoglobulin class and subclass (IgG2b). As a control, we provide the survival data for mice treated with low-dose L-PAM and saline ( ). The numbers in parentheses indicate the number of tumor-free mice/total mice inoculated. *, statistically significant relative to the survival data for mice treated with L-PAM and saline (\( P < 0.001 \)). The survival data for the other group were not significantly different from those for mice treated with L-PAM and saline.](image2)
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Fig. 3. Histological sections of primary s.c. tumor nodules either prior to or following L-PAM therapy of mice bearing a large (>20 mm) MOPC-315 tumor. A, untreated tumor-bearing mice; B and C, two different fields of the same section on Day 5 after low-dose (1.5 mg/kg) L-PAM therapy; D, Day 7 after low-dose L-PAM therapy; and E, Day 5 after high-dose (15 mg/kg) L-PAM therapy. Original magnification, × 520.

Thus, the cure of mice bearing a large MOPC-315 tumor by a low dose of L-PAM, but not by a high dose of L-PAM, is associated with the appearance of a significant lymphocytic infiltration within the s.c. tumor nodule.

Importance of T-Cells and Their Subsets for the Low-Dose L-PAM-induced Lymphocytic Infiltration into the s.c. Tumor Nodule of MOPC-315 Tumor Bearers. Experiments were performed to determine the effect of anti-Thy 1.2, anti-Lyt 2.2, or anti-L3T4 antibody treatment on the ability of low-dose L-PAM therapy to bring about the appearance of a significant lymphocytic infiltration into the tumor nodule of MOPC-315 tumor bearers (Fig. 4). On Day 3 following low-dose L-PAM therapy of mice bearing a large MOPC-315 tumor, there was no difference in the appearance of tumor nodules regardless of whether

present within the tumor nodule. However, each tumor nodule still contained distinct foci of viable tumor cells (Fig. 3C). By Day 7 following low-dose chemotherapy, almost all viable tumor cells disappeared, and the tumor nodules consisted almost exclusively of lymphocytes and macrophages within granulation tissue (Fig. 3D). In contrast to the massive lymphocytic infiltration observed in the tumor nodule following low-dose L-PAM therapy, no significant infiltration was observed following high-dose chemotherapy. As can be seen from Fig. 3E, by Day 5 after high-dose chemotherapy essentially no viable tumor cells were left within the primary tumor nodule and, at the same time, essentially no lymphocytic infiltration was present. Specifically, the tumor cells showed bizarre nuclei, karyorrhexis with nuclear debris, nuclear ballooning, and cellular necrosis.
or not the tumor-bearing mice were also treated with monoclonal antibody. Specifically, sections of tumor nodules from mice treated with low-dose L-PAM alone or low-dose L-PAM plus any of the monoclonal antibodies showed little if any lymphocytic infiltration (data not shown). However, by Day 5 following low-dose chemotherapy, a difference was observed between sections taken from mice treated with L-PAM only and sections taken from mice treated with L-PAM plus either monoclonal anti-Thy 1.2 or anti-Lyt 2.2 antibody. Specifically, while sections of tumor nodules derived from mice treated with L-PAM only showed a few foci of viable tumor cells (Fig. 3C) and prominent lymphocytic infiltrate (Fig. 3B), sections of tumor nodules derived from mice treated with L-PAM plus either anti-Thy 1.2 (Fig. 4A) or anti-Lyt 2.2 (Fig. 4B) antibody showed large areas of viable tumor cells and no significant lymphocytic infiltration. In contrast to the appearance, on Day 5 after chemotherapy, of tumor nodules derived from mice treated with low-dose L-PAM plus either anti-Thy 1.2 or anti-Lyt 2.2 antibody, tumor nodules derived from mice treated with low-dose L-PAM plus anti-L3T4 antibody resembled those from mice treated with low-dose L-PAM only. Specifically, sections of tumor nodules derived from mice treated with low-dose L-PAM plus anti-L3T4 antibody contained a few foci of viable tumor cells and had a prominent lymphocytic infiltrate (Fig. 4C). On Day 7 after the low-dose chemotherapy, the tumor nodules of mice treated with L-PAM plus either anti-Thy 1.2 or anti-Lyt 2.2 antibody consisted almost exclusively of viable tumor cells which displayed frequent mitotic figures (data not shown). At the same time, tumor nodules from mice treated with L-PAM plus anti-L3T4 antibody, like tumor nodules from mice treated with low-dose L-PAM only (Fig. 3D), consisted almost exclusively of lymphocytes and macrophages and almost no viable tumor cells (data not shown). Thus, essentially all the lymphocytic infiltration occurring following low-dose L-PAM therapy of mice bearing a large MOPC-315 tumor can be prevented by treating the mice with anti-Thy 1.2 or anti-Lyt 2.2 antibody but not with anti-L3T4 antibody.

Importance of Lyt 2+ T-Cells for the Ability of Spleen Cells from L-PAM-treated MOPC-315 Tumor Bearers to Adoptively Transfer Systemic Antitumor Immunity. Initially, we determined how soon following low-dose L-PAM therapy do spleen cells from mice bearing a large MOPC-315 tumor acquire the ability to confer systemic antitumor immunity (Fig. 5). Specifically, spleen cells (5 x 10^7) derived from MOPC-315 tumor bearers either prior to, or 1, 3, 7, or 10 days following low-dose chemotherapy, were adoptively transferred into mice bearing a Day 5 (barely palpable) MOPC-315 tumor that had been treated 1 day earlier with a subcurative dose of L-PAM (2.5 mg/kg). The ability of the adoptively transferred spleen cells to bring about the cure of the recipient mice was used as a measure of the ability of the spleen cells to confer systemic antitumor immunity. As expected (8, 23), spleen cells from either normal mice or untreated MOPC-315 tumor bearers were ineffective in conferring systemic antitumor immunity. As expected (8, 23), spleen cells from either normal mice or untreated MOPC-315 tumor bearers were ineffective in conferring systemic antitumor immunity and did not facilitate the therapeutic outcome of L-PAM therapy for mice bearing a barely palpable tumor. However, by Day 3 following low-dose chemotherapy, the MOPC-315 tumor bearer spleen cells acquired the ability to confer potent systemic antitumor immunity, and this ability was evident at least through Day 10 following the low-dose chemotherapy. Thus, as a consequence
of low-dose L-PAM therapy, spleen cells from MOPC-315 tumor bearers rapidly acquire the ability to confer systemic antitumor immunity. In fact, the ability of spleen cells from L-PAM-treated MOPC-315 tumor bearers to display systemic antitumor immunity is evident at a time following low-dose L-PAM therapy when the mice are still engaged in tumor eradication.

Experiments were then performed to determine the phenotype of the cells that are responsible for the ability of the spleen cells from L-PAM-treated MOPC-315 tumor bearers to confer systemic antitumor immunity. For this purpose spleen cells were obtained on Day 5 or 6 after treatment of mice bearing a large MOPC-315 tumor with a low dose of L-PAM, i.e., when the mice were still engaged in tumor eradication. The spleen cells were treated in vitro with monoclonal antibody plus complement to deplete either all T-cells or just the Lyt 2$^+$ or L3T4$^*$ cells were treated in vitro with monoclonal antibody plus complement to deplete either all T-cells or just the Lyt 2$^+$ or L3T4$^*$ cells. The survival data for the other groups were not significantly different from those for mice treated with L-PAM and normal spleen cells.

Table 1 Level of anti-MOPC-315 cytotoxicity exhibited by spleen cells from mice which are in the process of eradicating a large MOPC-315 tumor following low-dose L-PAM therapy

<table>
<thead>
<tr>
<th>Source of spleen cells</th>
<th>Antitumor cytotoxicity (% of specific $^{51}$Cr release) at an effector/target cell ratio of</th>
</tr>
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<tbody>
<tr>
<td>400/1</td>
<td>200/1</td>
</tr>
<tr>
<td>L-PAM-treated TuB$^*$</td>
<td>51.8 ± 8.0$^c$</td>
</tr>
<tr>
<td>Untreated TuB$^*$</td>
<td>55.1 ± 6.7$^d$</td>
</tr>
<tr>
<td>Normal mice</td>
<td>45.8 ± 9.0$^e$</td>
</tr>
<tr>
<td></td>
<td>$$\text{mean} \pm \text{SE}$ of 3 different experiments.</td>
</tr>
<tr>
<td></td>
<td>$$^c P &lt; 0.02$ for lytic activity of spleen cells from L-PAM-treated tumor bearers relative to spleen cells from normal mice when evaluated at the same effector/target cell ratio.</td>
</tr>
<tr>
<td></td>
<td>$$^d P &lt; 0.01$ for lytic activity of spleen cells from L-PAM-treated tumor bearers relative to spleen cells from normal mice and spleen cells from untreated tumor bearers when evaluated at the same effector/target cell ratio.</td>
</tr>
<tr>
<td></td>
<td>$$^e Mice bearing a 20-mm MOPC-315 tumor.</td>
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<tr>
<td></td>
<td>ND, not done.</td>
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Importance of Lyt 2$^+$ T-Cells for the Ability of Spleen Cells from L-PAM-treated MOPC-315 Tumor Bearers to Lyse MOPC-315 Tumor Cells in Vitro. Initially we determined whether spleen cells from MOPC-315 tumor bearers, which as a consequence of low-dose L-PAM therapy acquire the ability to confer systemic antitumor immunity to other mice, also acquire the ability to lyse MOPC-315 tumor cells in vitro. For this purpose, spleen cells derived from MOPC-315 tumor bearers that had been treated 7 days earlier with a low dose of L-PAM were evaluated for their direct lytic activity against MOPC-315 tumor cells by the 3.5-h $^{51}$Cr release assay (Table 1). As a reference point we assessed the anti-MOPC-315 lytic activity of normal spleen cells and of spleen cells from untreated MOPC-315 tumor bearers (Table 1). As expected (24), spleen cells from normal mice or untreated MOPC-315 tumor bearers were unable to lyse MOPC-315 tumor cells in vitro. In contrast, spleen cells from L-PAM-treated MOPC-315 tumor bearers exhibited a potent lytic activity against MOPC-315 tumor cells when evaluated at an effector/target cell ratio ranging from 400/1 to 50/1. Thus, as a consequence of low-dose L-PAM therapy, spleen cells from MOPC-315 tumor bearers acquire the ability to lyse MOPC-315 tumor cells in vitro in a short-term assay.

Experiments were then performed to determine whether T-cells are responsible for the anti-MOPC-315 cytotoxicity of
Spleen cells from L-PAM-treated MOPC-315 tumor bearers, and if so, which subset of T-cells is responsible for this activity. For this purpose, we determined initially the effect of depletion of T-cells (by treatment with monoclonal antibody and complement) or macrophages (by treatment with carbonyl iron and magnet) from spleen cell suspensions of L-PAM-treated MOPC-315 tumor bearers on the ability of the spleen cells to lyse MOPC-315 tumor cells (Table 2). Depletion of T-cells among spleen cells from L-PAM-treated MOPC-315 tumor bearers completely abolished the ability of the spleen cells to lyse MOPC-315 tumor cells. At the same time, depletion of phagocytic cells failed to reduce the anti-MOPC-315 cytotoxicity. Thus, T-cells are responsible for the anti-MOPC-315 cytotoxicity of spleen cells from L-PAM-treated MOPC-315 tumor bearers. The T-cell subset(s) responsible for the anti-MOPC-315 cytotoxicity of spleen cells from L-PAM-treated MOPC-315 tumor bearers was then identified by assessing the effect of treating the spleen cells with anti-Lyt 2.2 or anti-L3T4 antibody plus complement on their ability to lyse MOPC-315 tumor cells (Table 2). While depletion of Lyt 2+ T-cells completely abrogated the anti-MOPC-315 cytotoxicity of spleen cells from L-PAM-treated MOPC-315 tumor bearers, depletion of L3T4+ T-cells failed to reduce the anti-MOPC-315 cytotoxicity of the spleen cells. Thus, the ability of spleen cells from L-PAM-treated MOPC-315 tumor bearers to lyse MOPC-315 tumor cells resides in the Lyt 2+ and not L3T4+, T-cells.

Specificity of the Lytic Activity of Spleen Cells from L-PAM-treated MOPC-315 Tumor Bearers. Experiments were performed to determine whether spleen cells from L-PAM-treated MOPC-315 tumor bearers are cytotoxic only for MOPC-315 tumor cells or also for a different, antigenically related plasmacytoma, and even for a thymoma. For this purpose we assessed the lytic activity of spleen cells from L-PAM-treated MOPC-315 tumor bearers against the syngeneic MOPC-104E plasmacytoma as well as against the syngeneic EL4 thymoma and the allogeneic ELA thymoma (Table 3). Spleen cells from L-PAM-treated MOPC-315 tumor bearers which exhibited a potent anti-MOPC-315 cytotoxicity were somewhat cytotoxic also for the antigenically related MOPC-104E plasmacytoma. However, these spleen cells were not cytotoxic for either the EL4 or the WEHI 22.1 thymus. Thus, the lytic activity exhibited by spleen cells from mice which are still engaged in the eradication of a large MOPC-315 tumor following low-dose L-PAM therapy is specific for MOPC-315-associated antigens.

Table 2 Effect of depleting phagocytic cells, T-cells, or T-cell subsets on the anti-MOPC-315 cytotoxic activity of spleen cells from mice which are in the process of eradicating a large MOPC-315 tumor following low-dose L-PAM therapy

<table>
<thead>
<tr>
<th>Treatment of spleen cells*</th>
<th>Antitumor cytotoxicity (% of specific 51Cr release)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium only</td>
<td>51.2 ± 10.6*</td>
</tr>
<tr>
<td>Carbonyl iron and magnet</td>
<td>55.2 ± 8.8*</td>
</tr>
<tr>
<td>Anti-Thy 1.2 plus complement</td>
<td>1.3 ± 1.9*</td>
</tr>
<tr>
<td>Anti-Lyt 2.2 plus complement</td>
<td>2.6 ± 2.3*</td>
</tr>
<tr>
<td>Anti-L3T4 plus complement</td>
<td>49.4 ± 6.6*</td>
</tr>
</tbody>
</table>

* Spleen cells derived from mice which were treated with 2.5 mg/kg of L-PAM 7 days earlier when they bore a 20-mm MOPC-315 tumor.  
* Determined as an effector/target cell ratio of 100/1.  
* Mean ± SE of 4 different experiments.  
* Normal spleen cells evaluated under the same conditions led to 0.2 ± 0.2% Cr release.  
* ND, not done.  
* Mean ± SE of 3 different experiments.  
* P < 0.05 for the lytic activity against MOPC-315 relative to the lytic activity against MOPC-104E and P < 0.01 relative to the lytic activity against EL4.

Table 3 Assessment of the lytic activity of spleen cells from L-PAM-treated MOPC-315 tumor bearers (mice which were treated with 2.5 mg/kg of L-PAM 7 days earlier when they bore a 20-mm MOPC-315 tumor) against the MOPC-104E plasmacytoma as well as against the ELA and WEHI 22.1 thymomas

<table>
<thead>
<tr>
<th>At an effector/target cell ratio of</th>
<th>Against</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPC-315</td>
<td>MOPC-104E</td>
</tr>
<tr>
<td>100/1</td>
<td>4.6 ± 4.6</td>
</tr>
<tr>
<td>49.4 ± 9.0*</td>
<td>12.8 ± 7.8</td>
</tr>
<tr>
<td>55.1 ± 6.7*</td>
<td>13.1 ± 7.3</td>
</tr>
<tr>
<td>44.2 ± 6.8*</td>
<td>5.7 ± 3.3</td>
</tr>
</tbody>
</table>

* Mean ± SE of 3 different experiments.  
* P < 0.05 for the lytic activity against MOPC-315 relative to the lytic activity against MOPC-104E and P < 0.01 relative to the lytic activity against EL4.  
* ND, not done.  
* P < 0.01 for lytic activity against MOPC-315 relative to the lytic activity against the other 3 target cells.  
* Mean of triplicate samples in a single experiment.

DISCUSSION

The studies presented here illustrate that the curative effectiveness of a low dose of L-PAM for mice bearing a large MOPC-315 tumor depends on the participation of Lyt 2+ T-cell-mediated antitumor immunity in tumor eradication. The Lyt 2+ T-cells are responsible for the substantial lymphocytic infiltration into the tumor site which occurs following low-dose L-PAM therapy, and the tumor-infiltrating lymphocytes are most likely responsible for the eradication of the tumor burden not eradicated through the direct antitumor effects of the low dose of drug. The tumor burden controlled by the Lyt 2+ T-cells following low-dose L-PAM therapy is quite large since, in the absence of the contribution of antitumor immunity, the tumoricidal/tumoristatic effects of the low dose of drug alone are not sufficient even for the eradication of a very small (nonpalpable) MOPC-315 tumor (7, 23). The Lyt 2+ T-cells from mice which are in the process of eradicating a large MOPC-315 tumor are shown here to be responsible for the ability of spleen cells from such mice to: (a) confer curative systemic antitumor immunity to MOPC-315 tumor bearers that had been treated with a subcurative dose of L-PAM; and (b) exert a direct specific lytic effect against MOPC-315 tumor cells.

Many investigators have stressed the importance of T-cell-dependent antitumor immunity for the therapeutic effectiveness of a variety of anticancer drugs in a variety of tumor models (3–8, 10). The most common approach has been to illustrate that treatment of tumor bearers with anti-thymocyte serum reduces the curative effectiveness of the anticancer drugs. Although some investigators have attempted to identify the subset of T-cells which is responsible for the curative effectiveness of the anticancer drugs, these studies focused on identifying the subset of T-cells among spleen cells of tumor-bearing hosts that, as a consequence of the chemotherapy, displayed the ability to adoptively transfer antitumor immunity (11, 21, 26, 27). Moreover, these studies relied primarily on identifying the T-cell subset which is important for the neutralization of tumor growth when admixed with tumor cells prior to inoculation into normal recipients, thus bypassing the need for the tumor-immune T-cells to migrate into the tumor site. The studies presented here identify the subset of splenic T-lymphocytes from mice bearing a large MOPC-315 tumor which, as a consequence of low-dose L-PAM therapy, acquires the ability not only to bring about the destruction of tumor cells present in their vicinity, but also to migrate into the tumor site. Specifically, the studies presented here illustrate that i.v. administration of Lyt 2+ splenic T-cells from mice which are in the process

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of eradicating a large MOPC-315 tumor following low-dose L-PAM therapy can, in conjunction with a subcurative dose of drug, bring about the cure of mice bearing a s.c. tumor.

Although the ability of a splenic T-cell subset to confer systemic antitumor immunity indicates that the T-cell subset can migrate into the tumor site and bring about killing of tumor cells, the value of the information may be limited as it relates to the identification of the T-cell subset responsible for the curative effectiveness of anticancer drugs in the original tumor-bearing animals. In other words, information obtained from studies with isolated lymphoid organs, such as the spleen, provides insight as to the phenotype of cells with antitumor reactivity present within that particular lymphoid organ regardless of whether this cell type plays any role in tumor eradication in the original animal (28, 29). Thus, in order to gather information as to the cell type responsible for the ability of a low dose of L-PAM to cure mice bearing a large MOPC-315 tumor, it is essential to address this question in the original mouse. In the current study, we attempted to accomplish this goal by manipulating in vivo the immune response of tumor-bearing mice and determining the effect of the in vivo manipulation on the therapeutic outcome of low-dose L-PAM therapy. Specifically, we determined the effect of in vivo depletion of Lyt 2+ or L3T4+ T-cells on the therapeutic outcome of low-dose L-PAM therapy for mice bearing a large MOPC-315 tumor. Our studies revealed the dependency of the curative effectiveness of low-dose L-PAM therapy for mice bearing a large MOPC-315 tumor on the participation of Lyt 2+ T-cells in tumor eradication.

The inability of monoclonal anti-L3T4 antibody to reduce substantially the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers is not likely the result of the nature of the monoclonal antibody used, nor the result of the inability of the monoclonal antibody to deplete most L3T4+ T-cells, but rather the result of a lack of substantial participation of L3T4+ T-cells in the cure of the mice following low-dose L-PAM therapy. Accordingly, the monoclonal anti-L3T4 antibody used was of the same class and subclass of immunoglobulin (i.e., IgG2b) as the anti-Thy 1.2 antibody and the anti-Lyt 2.2 antibody (produced by hybridoma 2.43) that were effective in abolishing the curative effectiveness of the low dose of L-PAM. In addition, the monoclonal anti-L3T4 antibody treatment was effective in depleting the L3T4+ T-cells from MOPC-315 tumor bearers if judged from the magnitude of reduction in frequency of such cells in the spleens of the mice. The validity of using the magnitude of depletion of L3T4+ T-cells from the spleen as an indicator for the efficiency of the depletion in other sites is supported by observations of other investigators regarding the equal efficiency of the protocol of anti-L3T4 antibody treatment used here (namely, i.p. administration of monoclonal anti-L3T4 antibody produced by hybridoma GK 1.5) in depleting the L3T4+ T-cells from the spleens and from the peripheral blood and lymph nodes of BALB/c mice (30).

We have recently established the importance of Lyt 2+ T-cells for the resistance of L-PAM-cured MOPC-315 tumor bearers to a challenge with MOPC-315 tumor cells (21). In addition, we have shown that the Lyt 2+ T-cells do not require help from L3T4+ T-cells in order to endue the cured mice with resistance to the tumor challenge. However, although the L3T4+ T-cells were not required for the resistance of the L-PAM-cured MOPC-315 tumor bearers to challenge with MOPC-315 tumor cells, it does not necessarily mean that this subset of T-cells would not be required for the cure of MOPC-315 tumor bearers following low-dose L-PAM therapy. The possibility exists that the activity of the Lyt 2+ T-cells present in L-PAM-cured MOPC-315 tumor bearers may have developed with help from L3T4+ T-cells after the administration of the low-dose L-PAM to MOPC-315 tumor bearers (e.g., during the active eradication of the tumor burden not eradicated by the direct antitumor effects of the anticancer drug). Accordingly, L3T4+ T-cells may be required for the curative effectiveness of low-dose L-PAM for mice bearing a large MOPC-315 tumor if the development of antitumor immunity had been arrested, as a consequence of tumor progression, at a stage which still requires the participation of L3T4+ T-cells for the generation of fully active Lyt 2+ T-cells (31). Moreover, it is not warranted to conclude from the fact that Lyt 2+ T-cells are required for the resistance of the cured mice to tumor challenge, that Lyt 2+ T-cells are also required for the curative effectiveness of low-dose L-PAM for MOPC-315 tumor bearers. In this regard it should be pointed out that Bateman et al. (32) have recently shown that, although the Lyt 2+ T-cells and not the L3T4+ T-cells are required for the resistance of mice to a secondary challenge with Moloney sarcoma virus-induced tumors, the L3T4+ T-cells and not the Lyt 2+ T-cells are required for the resistance of the mice to primary sarcoma growth. Here we show that, in the MOPC-315 tumor model, the Lyt 2+ T-cells without help from L3T4+ T-cells are sufficient not only to endue the L-PAM-cured MOPC-315 tumor bearers with resistance to tumor challenge (21), but also to bring about the cure of mice bearing a large MOPC-315 tumor following low-dose chemotherapy.

As a consequence of low-dose L-PAM therapy, a substantial lymphocytic infiltrate was evident within the primary tumor nodule at a time following the chemotherapy when the animals were still engaged in active tumor eradication. The potential role of the lymphocytic infiltrate in eradicating the tumor burden not eliminated through the direct antitumor effects of the low dose of L-PAM is suggested by the fact that no such infiltration was seen at any time following high-dose chemotherapy which, unlike low-dose chemotherapy, does not depend on the contribution of antitumor immunity for its curative effectiveness (7). Moreover, treatment of mice bearing a large MOPC-315 tumor with monoclonal anti-Lyt 2.2 antibody abolished the curative effectiveness of the low dose of L-PAM and prevented the lymphocytic infiltration, while treatment with monoclonal anti-L3T4 antibody did not reduce significantly the curative effectiveness of the low-dose L-PAM nor the magnitude of lymphocytic infiltration.

Cells of the same phenotype as the ones responsible for the curative effectiveness of low-dose L-PAM therapy for mice bearing a large MOPC-315 tumor are responsible for the ability of spleen cells from the treated mice to mediate in vivo tumor eradication upon adoptive transfer to other mice. This observation raises the question of whether the Lyt 2+ T-cells present in the spleens of mice that are in the process of eradicating a large MOPC-315 tumor following low-dose L-PAM therapy are important for the therapeutic outcome of the low dose of drug. In this regard, it should be pointed out that, prior to low-dose L-PAM therapy, spleens from mice bearing a large MOPC-315 tumor contain a large number of metastatic tumor cells (33), and the in situ eradication of the metastatic tumor cells, following low-dose chemotherapy, may be mediated by the Lyt 2+ T-cells present in the spleens of the tumor-bearing mice.

The mechanism through which the Lyt 2+ T-cells from MOPC-315 tumor-bearing mice which are in the process of eradicating a large MOPC-315 tumor following low-dose L-
PAM therapy mediate their in vivo antitumor effect is unknown. The fact that splenic Lyt 2+ T-cells from such mice can confer curative systemic antitumor immunity to MOPC-315 tumor bearers that have been treated with a subcurative dose of L-PAM as well as exert in vitro a CTL-type lytic effect against MOPC-315 tumor cells suggests that, in vivo, too, the Lyt 2+ T-cells may lyse MOPC-315 tumor cells directly in an antigen-specific manner. However, the CTL-type lytic activity is not necessarily the only mechanism through which the Lyt 2+ T-cells can bring about tumor eradication in vivo. Accordingly, the Lyt 2+ T-cells may also bring about lysis of MOPC-315 tumor cells through an antigen-nonspecific mechanism. For example, the Lyt 2+ T-cells may secrete, following in vivo interaction with MOPC-315 tumor cells, lymphokines which in turn recruit and activate other cells (such as macrophages) for killing (34, 35).

Using monoclonal antibodies to deplete T-cell subsets from intact mice, Awad and North (36) have recently demonstrated the importance of Lyt 2+ T-cells in the rejection of a Day 9 L5178Y tumor. In their tumor model, treatment of mice with monoclonal anti-L3T4 antibody resulted in the complete regression of a 10-mm s.c. tumor as a consequence of the removal of L3T4+ suppressor T-cells that down-regulated the tumor-eradicating activity of Lyt 2+ T-cells (36). It would be interesting to determine whether anti-L3T4 antibody treatment can also facilitate the tumor-eradicating activity of the Lyt 2+ T-cells in the MOPC-315 tumor model, thereby leading to the cure of mice bearing a large tumor. At present, however, it is difficult to assess the likelihood that treatment with anti-L3T4 antibody will unmask the reactivity of the Lyt 2+ T-cells since it is not known whether L3T4+ T-cells play any role in the suppressed antitumor immunity of mice bearing a large MOPC-315 tumor. Regardless of the nature of the suppressor cells that operate in mice bearing a large MOPC-315 tumor, as a consequence of low-dose L-PAM therapy, such mice rapidly acquire the ability to reject a large tumor burden (7, 8). It should be pointed out at this stage that even if low-dose L-PAM leads to the acquisition of tumor-eradicating activity in Lyt 2+ T-cells by interfering with the suppressive activity of L3T4+ T-cells, it does not necessarily mean that anti-L3T4 antibody treatment will be as effective therapeutically for MOPC-315 tumor bearers as low-dose L-PAM since, in addition to unmasking the antitumor immunity of the Lyt 2+ T-cells, the drug may also lead to: (a) reduction in tumor burden (37); (b) enhancement in tumor cell immunogenicity (38); (c) appearance of immunopotentiating reactivity (15, 39, 40); and (d) possibly even to enhancement in the susceptibility of the tumor cells to immunomediated lysis (41).

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IMPORTANCE OF Lyt 2* T-CELLS IN TUMOR ERADICATION


Importance of Lyt 2+ T-Cells in the Curative Effectiveness of a Low Dose of Melphalan for Mice Bearing a Large MOPC-315 Tumor


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