**In Vitro** Differentiation of T-Cells Capable of Mediating the Regression of Established Syngeneic Tumors in Mice

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**ABSTRACT**

Previous studies have shown that successful adoptive immunotherapy of a newly induced, weakly immunogenic murine sarcoma, MCA 105, can be achieved either with fresh noncultured immune spleen cells or with immune cells after *in vitro* stimulation and expansion. In this study, we utilized *in vivo* and *in vitro* depletions with monoclonal antibodies (mAb) of T-cell subpopulations expressing the L3T4 or Lyt-2 antigens to investigate the phenotype of the T-cells that mediate *in vivo* tumor regression. The efficiency of depletion was assessed by flow microfluorometric analysis and by the ability of specifically treated spleen cell populations to generate allogeneic cytotoxic T-lymphocytes. The therapeutic efficacy of adoptively transferred fresh noncultured MCA 105 immune cells was abrogated by *in vivo* administration of either L3T4 or Lyt-2 mAb to mice bearing 3-day established pulmonary metastases. *In vitro* treatment of fresh noncultured MCA 105 immune cells with either L3T4 or Lyt-2 mAb and complement also abrogated their antitumor efficacy confirming the initial findings. However, mixing L3T4 and Lyt-2 mAb and complement-treated MCA 105 immune cells reconstituted the antitumor efficacy indicating that cellular cooperation between these two lymphoid subpopulations was essential for the regression of established tumors. Unlike fresh noncultured immune cells, the antitumor efficacy of *in vitro* sensitized and expanded immune cells was abrogated by *in vivo* treatment with Lyt-2 but not with L3T4 mAb indicating Lyt-2 cells alone played a major role in mediating the regression of tumors. These findings provide evidence for an *in vitro*-induced differentiation of therapeutic T-lymphocytes. Our results thus suggest that the antitumor activities expressed by the two types of cells may represent T-cells at different stages of immunological differentiation.

**INTRODUCTION**

The adoptive transfer of specifically sensitized T-lymphocytes is capable of mediating the regression of established tumors in a variety of animal models (1–6). Successful therapy requires the availability of large numbers of immune effector cells. This requirement can be satisfied by developing *in vitro* methods for expanding lymphocytes with antitumor activity. The therapeutic efficacy of *in vitro*-stimulated tumor-immune T-lymphocytes expanded in IL-2 has been demonstrated in the highly immunogenic FBL-3 lymphoma model (6, 7).

Recently, we have developed and characterized two newly induced murine tumors in which adoptive immunotherapy was successfully demonstrated (3). Compared to the FBL-3 lymphoma, the MCA 105 and MCA 106 sarcomas are weakly immunogenic to their syngeneic hosts. Since these tumors are used within the first five *in vivo* passages, their tumor-associated transplantation antigens may be more representative of autochthonous tumors. Using the MCA 105 sarcoma, we have established culture conditions that allow the generation and expansion of therapeutically functional cells against experimentally induced MCA 105 pulmonary metastases (8).

Although IL-2 expanded, therapeutically effective lymphocytes were derived from tumor immune animals, these cells displayed considerable biological disparity compared to fresh noncultured immune lymphoid cells including altered *in vivo* traffic and homing patterns and changed response to exogenous IL-2 (9–12). These changes in characteristics of cultured lymphocytes have been assumed to be associated with the intrinsic properties of lymphoblasts similar to those of antigenically or mitogenically activated resting small lymphocytes. The *in vitro* expanded cells that were therapeutically effective were believed to be descendants of the cells that mediated antitumor immunity in fresh noncultured immune cell populations.

In the present study, the expression of T-cell differentiation antigens on the therapeutic lymphocytes was analyzed mainly by *in vivo* depletion with either Lyt-2 or L3T4 mAb. In some experiments, T-cell subpopulations were depleted *in vitro* by treatment with mAb and C. Our results demonstrate that the antitumor effect of fresh noncultured MCA 105 immune cells was attributed to the collaboration of L3T4* and Lyt-2* T-cell subpopulations. However, *in vitro* sensitization and expansion, Lyt-2* cells alone were sufficient to mediate the regression of established pulmonary metastases. These findings indicate that *in vitro* stimulation and expansion induced cellular differentiation of therapeutically functional cells.

**MATERIALS AND METHODS**

**Mice.** Female C57BL/6J mice, 8–12 weeks old were obtained from The Jackson Laboratory, Bar Harbor, ME. Caged in groups of six or fewer, the animals were fed NIH laboratory chow and given water *ad libitum*.

**Tumor.** The MCA 105 is a methylncholanthrene-induced fibrosarcoma of C57BL/6 origin. It was maintained *in vivo* in syngeneic mice by serial s.c. transplantation of cryopreserved tumor samples as previously described (3, 9). The MCA 105 tumor used for the current study was in the 3rd to 5th transplantation generation.

**Immunization.** Single cell suspensions were prepared from solid tumors by enzymatic digestion as described (3). Mice were immunized by intradermal injections with a mixture of 1.5 × 10⁶ viable tumor cells and 100 μg of formalin-inactivated *Corynebacterium parum* (Burroughs Wellcome Co., Research Triangle Park, NC). This procedure resulted in less than 50% of animals capable of rejecting a 10⁶ viable tumor challenge (3). Immune spleens were obtained from tumor-free animals 2–4 weeks after such challenge.

**IL-2.** Human recombinant IL-2 was kindly supplied by the Cetus Corporation, Emeryville, CA. The biological and biochemical activities of the recombinant IL-2 have been described in detail (13). Purified material had a specific activity of 3–4 × 10⁶ U/mg protein (14).

**Spleen Cell Suspension.** Spleens from MCA 105 immune or normal mice were dissociated mechanically with the blunt end of a 10-ml plastic syringe plunger in HBSS. The resultant cell suspension was filtered through nylon mesh (100 gauge) and erythrocytes were lysed with ammonium chloride-potassium buffer (8.29 g NH₄Cl, 1.0 g KHCO₃, and 0.0372 g EDTA/liter, pH 7.4; Media Production Section, NIH, Bethesda, MD). The cells were washed and resuspended in HBSS for adoptive immunotherapy or in appropriate medium for culture.

**IVS.** Secondary IVS of MCA 105 immune spleen cells was carried
out in 24-well plates (3524; Costar, Cambridge, MA) as described previously (8). Briefly, 4 x 10^6 unfraccionated spleen cells were cultured with 10^6 2000 R-irradiated MCA 105 tumor cells in the presence of 1000 U/ml of recombinant IL-2 in complete medium. Complete medium was composed of RPMI 1640 (Biofluids Inc., Rockville, MD) with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY), 0.03% freshly prepared glutamine (Mediatech Production Section, NIH), 1 µM sodium pyruvate, 0.1 mM nonessential amino acids (Microbiological Associates, Walkersville, MD), 5 x 10^{-3} M 2-mercaptoethanol (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin (Shearing, Kenilworth, NJ), and 0.5 µg/ml fungizone (Flow Laboratories, McLean, VA). The method for IVS led to the growth of lymphoid cells that became confluent in 9–10 days. These IVS cells have been shown to be capable of mediating the regression of established pulmonary MCA 105 metastases (8).

Mixed Lymphocyte Culture and in Vitro Cytotoxic Assay. One-way mixed lymphocyte cultures were established as described previously (15). Briefly, 4 x 10^5 C57BL/6 responder spleen cells were cultured for 4 days in 2 ml of CM with 2 x 10^6 irradiated (3000 R) DBA/2 stimulator spleen cells. Responder cells were harvested and tested for cytotoxicity activity by a 4-h chromium-release assay as described previously (9, 15). Chromium-labeled P815 mastocytoma (syngeneic to DBA/2 mice) and EL-4 lymphoma (syngeneic to C57BL/6 mice) cells served as allogeneic and control targets, respectively. Cytotoxicity is expressed in percentage of lysis as follows: (experiment CPM − spontaneous CPM)/(maximal CPM − spontaneous CPM) x 100.

Adoptive Immunotherapy. Pulmonary metastases were established experimentally by i.v. infusion of 3–5 x 10^6 MCA 105 tumor cells. On day 3 when microscopic foci in the lung were evident, immune effector cells were transferred i.v. through the tail vein. Between 14 and 16 days after tumor inoculation, the mice were ear tagged, randomized, then sacrificed for enumeration of pulmonary tumor nodules as described previously (16). Metastatic foci too numerous to count were assigned an arbitrary value of 250 since this was the greatest number of nodules that could be reliably enumerated. In all experiments reported here, each group consisted of at least five animals.

In Vivo and in Vitro Depletion of T-Cell Subpopulations by mAb. Two hybridomas producing rat IgG2b mAb against the L3T4 (GK 1.5) and Lyt-2 (2.43) T-cell antigens were obtained from American Type Culture Collection, Rockville, MD. The mAb were harvested as ascites fluid from subcutaneously irradiated (500 R) DBA/2 mice. For in vivo depletion, C57BL/6 mice were thymectomized 1 week before antibody administration. Treatment consisted of 2 i.v. injections, one week apart, of 0.2 ml of monoclonal ascitic fluid diluted to 1.0 ml with HBSS. This procedure has been shown to be effective at long-term depletion of T-cell subpopulations in vivo (17). In adoptive immunotherapy experiments, the first antibody injection was given within 1 h of cell transfer on day 3 after tumor inoculation. The second dose was given 1 week later on day 10. For in vitro depletion of T-cell subpopulations, immune spleen cells were suspended at a concentration of 10^7 cells/ml in cytoxicity medium containing 1% of mAb ascitic fluid. Cytotoxicity medium was composed of RPMI 1640 with 0.3% bovine serum albumin (Pathocyte 4; Miles Laboratories, Elkhart, IN) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. Cells were incubated for 45 min at 4°C, washed, and incubated with an excess of a mouse anti-rat kappa mAb (MAR 18.5; American Type Culture Collection, Rockville, MD), and 0.5 µg/ml fungizone (Flow Laboratories, McLean, VA). The method for IVS led to the growth of lymphoid cells that became confluent in 9–10 days. These IVS cells have been shown to be capable of mediating the regression of established pulmonary MCA 105 metastases (8).

RESULTS

Effect of Treatment with mAb on Splenic T-Lymphocyte Subpopulations. Treatment of thymectomized C57BL/6 mice with two doses of L3T4 or Lyt-2 mAb produced a dramatic reduction in splenic cells bearing the L3T4 or Lyt-2 phenotype, respectively. Flow microfluorometric analysis of nonfractionated spleen cells 26 days after the first antibody treatment revealed a >90% depletion of the specific T-cell subpopulation (Fig. 1). Analyses of splenic T-cell subpopulations 2 or 47 days after the first antibody treatment produced the same results as seen at 26 days (data not shown) indicating that in vivo depletion by antibodies of T-cell subpopulations was rapid and long-lasting, confirming previous observations (17).

Functionally, mice depleted of either L3T4+ or Lyt-2+ T-cells showed a diminished ability to generate CTL to allogeneic targets in vitro (Fig. 2). Mixing of the cells from the L3T4- and Lyt-2-depleted animals at the beginning of the culture reconstituted the response (Fig. 2). These findings are consistent with the results of fluorescence antibody analysis indicating that mice can be selectively depleted of functional T-cell subpopulations by mAb injections.

Effect of Treatment with mAb on Adoptive Immunotherapy Mediated by Fresh Noncultured Immune Spleen Cells. Our previous results indicated that systemic transfer of fresh noncultured MCA 105 immune spleen cells was capable of mediating the regression of established 3-day pulmonary MCA 105 metastases (8, 18). To investigate the phenotype of T-cells
mediating MCA 105 tumor regression, we treated the tumor-bearing recipients with monoclonal L3T4 or Lyt-2 antibodies on the day of and 7 days after the transfer of immune spleen cells. Since a long-term depletion of T-cell subpopulations by mAb was best achieved in thymectomized mice, all recipient mice were thymectomized 1 week before tumor inoculation. In two separate experiments (Table 1) $10^6$ viable fresh immune cells transferred to control animals that received rat immunoglobulin reduced the number of pulmonary metastases to an average of less than one per animal indicating the therapeutic efficacy of the immune spleen cells. However, the therapeutic efficacy of this dose of immune cells was abrogated in recipients that were also injected with mAb to either the L3T4 or Lyt-2 T-cell differentiation antigens. Since adoptive immunotherapy does not require the participation of host T-cells (3, 9), targets for the inoculated mAb are likely to be transferred donor cells. Sensitivity of fresh noncultured immune spleen cells to both L3T4 and Lyt-2 mAb indicates that tumor regression was mediated either by the collaboration of two separate T-cell subpopulations (L3T4* and Lyt-2* T-cells) or by the participation of a T-cell population expressing both the L3T4 and Lyt-2 antigens.

To further elucidate the nature of the primary T-cells capable of mediating the regression of MCA 105 tumors, we have attempted to treat immune cell donors in vivo with the two mAb before harvesting their spleens for adoptive immunotherapy. Although spleen cells from Lyt-2 antibody treated immune

Table 1  Effect of in vivo monoclonal antibody treatment on adoptive immunotherapy of established MCA 105 pulmonary metastases with fresh noncultured immune spleen cells

<table>
<thead>
<tr>
<th>Source of spleen cells</th>
<th>mAb for in vivo treatment</th>
<th>Mean no. of metastases</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Rlg</td>
<td>223 (27)*</td>
<td>170 (51)</td>
<td></td>
</tr>
<tr>
<td>MCA 105 immune</td>
<td>Rlg</td>
<td>149 (47)</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>MCA 105 immune</td>
<td>Lyt-2 (L3T4)</td>
<td>120 (33)</td>
<td>116 (40)</td>
<td></td>
</tr>
<tr>
<td>MCA 105 immune</td>
<td>Lyt-2 (L3T4)</td>
<td>157 (24)</td>
<td>188 (41)</td>
<td></td>
</tr>
</tbody>
</table>

*Thymectomized C57BL/6 mice were injected i.v. with $3 \times 10^6$ MCA 105 tumor cells in 1.0 ml of HBSS. On day 3, $10^6$ freshly harvested spleen cells in 1.0 ml of HBSS were given i.v.

Monoclonal ascitic fluid (0.2 ml) or 0.25 mg Rlg diluted to 1.0 ml in HBSS was administered as two i.v. injections, 1 week apart, begun within 1 h of cell transfer on day 3.

Lungs were harvested and metastases counted on day 14.

Rlg, rat immunoglobulin.

*Numbers in parentheses, mean ± SE.
Fig. 3. Effect of in vitro depletion with mAb and C of MCA 105 immune spleen cells on the ability to generate allogeneic CTL in vitro. Antibody depleted cells were cultured with irradiated DBA/2 stimulator cells for 4 days. *Cyt-labeled P815 served as specific allogeneic targets. Cytotoxicity activity to syngeneic targets (EL-4) was <5% for all cells.

Table 2. Effect of depleting fresh noncultured immune cells of L3T4* and Lyt-2* cells in vitro with monoclonal antibodies and complement on adoptive immunotherapy of established MCA 105 pulmonary metastases

<table>
<thead>
<tr>
<th>Group</th>
<th>Source of spleen cells*</th>
<th>In vitro treatment*</th>
<th>Mean no. of metastases*</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal</td>
<td></td>
<td>212 (19)*</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>MCA 105 immune</td>
<td>C</td>
<td>199 (23)</td>
<td>184 (43)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>MCA 105 immune</td>
<td>α-L3T4 + C</td>
<td>2 (1)</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>MCA 105 immune</td>
<td>α-Lyt-2 + C</td>
<td>184 (19)</td>
<td>200 (50)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>MCA 105 immune</td>
<td>α-Lyt-2 + C</td>
<td>157 (9)</td>
<td>113 (53)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Mixture of D and E</td>
<td></td>
<td>7 (2)</td>
<td>17 (11)</td>
<td></td>
</tr>
</tbody>
</table>

* Irradiated (500 R, Experiment 1) or normal (Experiment 2) C57BL/6 mice were injected i.v. with 3 or 5 x 10^5 MCA 105 tumor cells in 1.0 ml HBSS, respectively. On day 3, mice were treated i.v. with 6 x 10^6 viable spleen cells after depletion.
* Mice were injected i.v. with 3 x 10^7 viable L3T4*-depleted cells admixed with 3 x 10^7 viable Lyt-2*-depleted cells.
* Lungs were harvested and metastases counted on day 14.
* Numbers in parentheses, mean ± SE.

Table 3. Effect of in vivo monoclonal antibody treatment on adoptive immunotherapy of established MCA 105 pulmonary metastases with secondary in vitro sensitized immune spleen cells

<table>
<thead>
<tr>
<th>Source of spleen cells for IVS*</th>
<th>mAb for in vitro treatment*</th>
<th>Mean no. of metastases*</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Rlg</td>
<td>229 (13)*</td>
<td>ND*</td>
<td>160 (43)</td>
<td></td>
</tr>
<tr>
<td>MCA 105 immune</td>
<td>Rlg</td>
<td>213 (37)</td>
<td>119 (53)</td>
<td>173 (51)</td>
<td></td>
</tr>
<tr>
<td>MCA 105 immune</td>
<td>α-L3T4</td>
<td>&lt;1 (1)</td>
<td>1 (1)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MCA 105 immune</td>
<td>α-Lyt-2</td>
<td>&lt;1 (1)</td>
<td>&lt;1 (1)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MCA 105 immune</td>
<td>α-Lyt-2 + C</td>
<td>250</td>
<td>250</td>
<td>201 (21)</td>
<td></td>
</tr>
</tbody>
</table>

*Thymectomized C57BL/6 mice were injected i.v. with 5 x 10^5 MCA 105 tumor cells in 1.0 ml of HBSS. On day 3, IVS cells (1.8 x 10^7 for Experiment 1 and 2.4 x 10^7 for Experiments 2 and 3) from initial 9-day culture suspended in 1.0 ml of HBSS were given i.v.
* Monoclonal ascitic fluid (0.2 ml) or 0.25 mg of Rlg diluted to 1.0 ml in HBSS was administered as two i.v. injections, 1 week apart, begun within 1 hour of cell transfer on day 3.
* Lungs were harvested and metastases counted on days 16 or 17.
* Numbers in parentheses, mean ± SE.

DISCUSSION

It has been well established that peripheral T lymphocytes representing distinct functional lineages are associated with the expression of certain surface antigens defined by mAb (19–27). Identification of the T-cell subpopulations mediating the regression of established tumors would contribute to an understanding of the mechanisms underlying tumor eradication. The selection of purified murine T-cell subpopulations in many studies has been based on the expression of Lyt antigens (9, 28–30). While the expression of the Lyt-2 antigen has been identified to be associated with T-cell functions such as cytotoxicity and suppression, the association between the expression of the Lyt-1 antigen and the T-cell helper function has become obscure in

with the monoclonal Lyt-2 but not with the L3T4 antibodies administered after cell transfer. These results indicate that the primary effectors in IVS immune cell populations capable of mediating the regression of MCA 105 tumors belonged to Lyt-2+ T-cell subpopulation. The lack of effect of cell depletion by the L3T4 antibody also indicates that tumor regression proceeded without the participation of L3T4* T-cells.

Phenotypic Expression on Immune Cells Before and After Secondary IVS. Flow microfluorometric analysis of MCA 105 immune spleen cells before and after secondary IVS revealed a significant shift in their expression of the L3T4 and Lyt-2 antigens (Fig. 4). Nonfractionated fresh noncultured immune cells displayed a normal distribution of Thy-1* (37%), L3T4* (21%), and Lyt-2* (16%) T-cells. After 9 days of culture in the presence of MCA 105 tumor cells and recombinant IL-2, all cells were positive for Thy-1.2 staining. However, the majority of the cells (82%) displayed the Lyt-2 phenotype while there were no detectable L3T4* T-cells. These results, therefore, are consistent with the finding that after IVS the therapeutic immune effector cells expressed only the Lyt-2 T-cell differentiation antigen.

Fig. 4. Flow microfluorometric analysis of MCA 105 immune spleen cells before and after 9 days of IVS expansion for the expression of the L3T4 and Lyt-2 antigens.
view of the demonstration that all T-cells express the Lyt-1 antigen albeit at different levels (31). Indeed, treatment of Lyt-2+ CTL generated from mixed lymphocyte-tumor cultures with the Lyt-1 antibody and C resulted in up to a 50% loss of cytotoxic activity (9, 28). Therefore, the results obtained by using the Lyt-1 antibody are difficult to interpret.

In the present study, we have used a new mAb, a-L3T4, reactive with a subpopulation of murine T-cells. Compared with Lyt-1, the expression of L3T4 appears to be more closely associated with T-cells of "helper/inducer" function. Recent work has indicated that this T-cell subpopulation can be more precisely defined by the ability to recognize class II histocompatibility antigens on antigen-presenting cells (26). This antibody has been shown to react with the mouse equivalent of human Leu 3 or OKT4 and rat W3/25 antigens (25). More importantly, the expression of L3T4 and Lyt-2 phenotypes by murine splenic T-cells are generally considered to be mutually exclusive (26).

In all previous studies, purified T-cell subpopulations were prepared in vitro either by the C-mediated antibody lysis or by fractionation on a flow microfluorometer before adoptive transfer of cells to tumor-bearing hosts (2, 9, 28-30, 32, 33). Interpretation of these studies was complicated by possible incomplete elimination of a particular T-cell subpopulation and by the possible role of host T-cells in tumor rejection. Recently, several reports have indicated that systemic administration of mAb of certain isotypes was effective at selectively depleting functional T-cell population in vivo (17, 34-36). We therefore chose this approach to analyze T-cell phenotypes associated with the regression of established pulmonary metastases.

The efficacy of in vivo depletions of T-cell subpopulations with the two mAb was assessed directly by immunofluorescence staining as well as by the ability of the spleen cells from treated mice to generate allogenic CTL in a 4-day one-way mixed lymphocyte culture. At 26 days after the first injection of mAb, selective depletion of the two major T-cell populations could be demonstrated by the flow microfluorometric analysis. A 92 and 95% specific reduction was seen in L3T4 and Lyt-2 antibody-treated groups respectively compared with thymectomized controls (Fig. 1). At 47 days, we still observed a 90 and 100% specific depletion respectively (data not shown) in L3T4 and Lyt-2 antibody-treated groups confirming previous published results that unmodified mAb could be effective at depleting T-cells in vivo (17). All recipients for antibody treatment were thymectomized. This procedure was necessary for achieving a stable, long-term depletion since antibody-treated normal euthymic mice showed an initial depletion of a specific T-cell subpopulation that lasted approximately 20 days. By 40 days, in thymectomized mice, the composition of the two major T-cell subpopulations was indistinguishable from that of normal untreated mice (data not shown). It is possible, however, that periodic injections of antibody would result in a prolonged depletion in normal mice (34-36). Functionally, spleen cells from mice depleted of either the L3T4 or Lyt-2 subpopulation showed diminished ability to generate allogenic CTL (Fig. 2). Since the generation of allogenic CTL depends on the presence of both helper (L3T4*) and cytotoxic precursor (Lyt-2*) cells (37), mixing of the two cell populations at the beginning of culture reconstituted the response (Fig. 2). These findings thus indicate that under these experimental conditions, specific depletion of T-cell subpopulations in vivo with the two antibodies was effective.

By in vivo mAb depletion of T-cell subpopulations, we found that the primary effector T lymphocytes from fresh noncultured immune spleens capable of mediating the regression of MCA 105 tumors were sensitive to both L3T4 and Lyt-2 mAb treatment (Table 1). This finding indicates that tumor regression was mediated either through a cooperative effect of the two major T-cell subpopulations or required a distinct T-cell population bearing both L3T4 and Lyt-2 markers. To distinguish these two possibilities, we have initially attempted to treat tumor-bearing mice with a mixture of spleen cells from thymectomized, mAb treated, MCA 105 immune animals. However, this approach was not feasible because spleen cells from L3T4 antibody-treated immune animals retained a significant level of antitumor activity when transferred (data not shown). It is possible that a long-lasting depletion of L3T4+ cell functions required a constant presence of the antibody in vivo since the presence of L3T4 antibody was sufficient to inhibit all helper functions without the lysis of its targets. Alternatively, we used antibody and C to deplete lymphoid subpopulations. Information generated by using in vitro depleted immune cells confirmed our initial observation and also demonstrated that by mixing an equal number of L3T4+ and Lyt-2+-depleted immune spleen cells, the antitumor activity was reconstituted to that of C-treated control immune cells (Table 2) indicating the participation of both T-cell subpopulations.

In one of the experiments summarized in Table II (Experiment 2), tumor-bearing mice were immunocompetent normal animals. Adoptive immunotherapy with either L3T4 or Lyt-2 mAb and C-treated immune spleen cells was ineffective in the reduction of pulmonary metastases. Since the host's T-cells were intact, both L3T4+ and Lyt-2+ T-cells must have been present at a normal level. Demonstration of adoptive immunotherapy in T-cell depleted "B" mice also indicates the phenomenon is independent of host T-cells (2, 9). Thus, the requirement of both L3T4+ and Lyt-2+ cells of donor origin to reconstitute the antitumor efficacy in vivo indicates the collaboration between the two cell populations of previously sensitized T-cells.

In a previous study (9) with the same tumor model using in vitro depletion techniques, we have found that the regression of established MCA 105 intradermal tumors could not be demonstrated by the transfer of a mixture of Lyt-1 and Lyt-2 antibody and C-treated immune spleen cells. This finding was interpreted to mean that cells of Lyt-1+,2+ phenotype played a major role in tumor eradication. Compared to our current demonstration that both L3T4+ and Lyt-2+ cells participated in tumor eradication, this contradiction is likely due to a significant elimination of Lyt-2+ immune cells by in vitro treatment of the Lyt-1 antibody and C. The presence of the Lyt-1 antigen on Lyt-2+ T-cells has been demonstrated and at times, tumor-bearing mice with the Lyt-1 antibody and C in vitro could lead to the elimination of some Lyt-2+ cells (31, 37). It is also possible, however unlikely, that the difference reflects the histological locations of tumors, i.e., intradermal versus pulmonary.

Our findings of the phenotypic expression of T-cells that are capable of mediating the regression of established tumors are different from several other reports employing different tumor models (28, 32, 33). It is conceivable that the differences in identification of the effector cell phenotype may reflect differences in the structural nature of tumor-associated transplantation antigens expressed on the tumor cells. The MCA 105 sarcoma we used for the current analysis differs from most available animal tumor models. It is weakly immunogenic and was used within the fifth transplantation passage. Therefore, its tumor-associated transplantation antigens are less likely to have been subjected to in vitro or in vivo modification. However,
that the therapeutic effector cells generated after secondary IVS expressed only the Lyt-2 T-cell differentiation antigen. This phenotype is typical of that associated with T-cell functions such as cytotoxic activity against allogeneic or virus-infected target cells. In addition, cells of this subpopulation exert substantial antigen-specific suppressive effects to both humoral and cell-mediated immune responses. Therefore, it is possible that tumor regression mediated by the transfer of IVS immune cells reflects a direct cytotoxicity by these cells and is characteristically different from that mediated by transfer of fresh noncultured immune cells. Since the culture system also induces nonspecific cytotoxic activity (8), it is not clear whether the generation of MCA 105 specific cytotoxic effector cells is in association with their in vivo antitumor activity. However, in the absence of IL-2, the generation of specific MCA 105 cytotoxic cells has been demonstrated by culturing MCA 105 immune spleen cells with heavily irradiated MCA 105 tumor stimulation cells (9). It is also not clear whether the in vitro differentiation we have described here is paralleled by a similar differentiation in vivo. The feasibility of in vivo T-cell depletion with mAb affords a means to analyze the sequence of events following transfer of immune cells.

As indicated in previous results (8, 9), the in vivo antitumor efficacy of IL-2-cultured but not fresh, noncultured immune cells can be improved by the administration of a dose of exogenic IL-2 that itself has no antitumor effect. In view of the fact that therapeutic effector cells generated by secondary IVS are T-cells of the Lyt-2 phenotype, the responsiveness of these cells to IL-2 in the absence of L3T4 helper cells may reflect their inability to produce a sufficient amount of IL-2 in vivo. Analogous to our findings, a recent report (38) indicates that in the adaptive chemoimmunotherapy of disseminated FBL-3 leukemia, a purified Lyt-2+ T-cell population specifically cytotoxic to the tumor provided with exogenous IL-2 could mediate tumor regression.

Our results provide direct evidence of an in vitro-induced differentiation of therapeutic immune lymphocytes. Considering the functional aspects of T-cells expressing the L3T4 and Lyt-2 molecules, it may be postulated that L3T4+ T-cells provide a "helper" mechanism for the differentiation or maturation of presensitized Lyt-2+ effector cells similar to the cellular cooperation involved in induction of allogeneic CTL. However, in this situation, the reaction represents a secondary immune response since it required both T-cells from previously immunized animals. It has been demonstrated that IL-2 can substitute for the helper function of L3T4+ cells in inducing in vitro and in vivo differentiation and proliferation of CTL (39–41). It is thus conceivable that the requirement for L3T4 cells for the in vivo antitumor activity or for the in vitro generation of Lyt-2 effector cells could be circumvented by administration of sufficient amounts of IL-2. Such experiments are presently underway.

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IN VITRO DIFFERENTIATION OF ANTITUMOR T-CELLS


In Vitro Differentiation of T-Cells Capable of Mediating the Regression of Established Syngeneic Tumors in Mice

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