Successful Adoptive Immunotherapy of Murine Poorly Immunogenic Tumor with Specific Effector Cells Generated from Gene-Modified Tumor-Primed Lymph Node Cells

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Successful Adoptive Immunotherapy of Murine Poorly Immunogenic Tumor with Specific Effector Cells Generated from Gene-Modified Tumor-Primed Lymph Node Cells

Hiroshi Tanaka,* Hirohisa Yoshizawa,2* Yoshifumi Yamaguchi,* Kazuhisa Ito,* Hiroshi Kagam,* Eiichi Suzuki,* Fumitake Gejyo,* Hirofumi Hamada, † and Masaaki Arakawa*

We previously reported that cytokine gene transfer into weakly immunogenic tumor cells could enhance the generation of precursor cells of tumor-reactive T cells and subsequently augment antitumor efficacy of adoptive immunotherapy. We investigated whether such potent antitumor effector T cells could be generated from mice bearing poorly immunogenic tumors. In contrast to similarly modified weakly immunogenic tumors, MCA102 cells, which are chemically induced poorly immunogenic fibrosarcoma cells transfected with cDNA for IL-2, IL-4, IL-6, IFN-γ, failed to augment the host immune reaction. Because priming of antitumor effector T cells in vivo requires two important signals provided by tumor-associated Ags and costimulatory molecules, these tumor cells were cotransfected with a B7-1 cDNA. Transfection of both IFN-γ and B7-1 (MCA102/B7-1/IFN-γ) resulted in regression of s.c. tumors, while tumor transfected with other combinations of cytokine and B7-1 showed progressive growth. Cotransfection of IFN-γ and B7-1 into other poorly immunogenic tumor B16 and LLC cells also resulted in the regression of s.c. tumors. Cells derived from lymph nodes draining MCA102/B7-1/IFN-γ tumors showed potent antitumor efficacy, eradicating established pulmonary metastases, but this effect was not seen with parental tumors. This mechanism of enhanced antitumor efficacy was further investigated, and T cells with down-regulated L-selectin expression, which constituted all the in vivo antitumor reactivity, were significantly increased in lymph nodes draining MCA102/B7-1/IFN-γ tumors. These T cells developed into potent antitumor effector cells after in vitro activation with anti-CD3/IL-2. The strategy presented here may provide a basis for developing potent immunotherapy for human cancers. The Journal of Immunology, 1999, 162: 3574–3582.

The T cell plays a crucial role in the host immune response to cancer. The adoptive immunotherapy of cancers with tumor-sensitized T cells has been well documented in several animal models (1–5). We previously reported that cells from lymph nodes (LN) draining progressively growing tumors could develop into mature effector cells after in vitro activation with anti-CD3 mAb and IL-2 (6–9). Transfer of these activated cells into mice bearing established tumors resulted in tumor eradication in a tumor-specific manner. Although numerous successful adoptive immunotherapy approaches have been reported in animal models, only a limited number of patients have responded to the therapy in clinical settings (10–16). The reason for the failure of adoptive immunotherapy of human cancer may be due in part to the weak immunogenicity of tumor cells and tumor-induced immunosuppression of the generation of immune effector cells. To enhance the immune response elicited in the host, exogenous expression of a variety of cytokines in tumor cells has been explored in animal models and has been shown to reduce or abrogate tumorigenicity (17–34). Although a vaccination approach is successful in the presence of a minimal tumor burden, combination therapies may widen the stage of tumors that can be effectively treated. For example, vaccination with cytokine gene-transduced tumor cells can be combined with adoptive immunotherapy. We reported that tumor cells genetically modified to secrete Th cytokines could enhance the in vivo priming of precursor cells of tumor-specific effector T cells and could subsequently augment the antitumor efficacy of anti-CD3/IL-2-activated cells (35).

Another approach to enhance the host immune response is the transfusion of tumor cells with cDNA-encoding costimulatory molecules. It is clear that Ag recognition alone is not sufficient for T cell activation to effector functions. Second signals such as co-ligation of auxiliary molecules are also critical for generating T cell-mediated immunity. Ag recognition in the absence of these second signals can lead to tolerance or anergy (36). One of a large number of costimulatory molecules, B7-1 (CD80) plays an important role in antitumor immunity (37, 38). The expression of B7-1 in some murine immunogenic tumor cells has been shown to induce tumor regression, whereas it has limited effects on poorly immunogenic tumors, suggesting that tumor immunogenicity is critical to the outcome (38–44).

Looking toward the treatment of human cancer, the present study aimed to establish a potent immunotherapy for tumors that lack apparent immunogenicity. MCA102, a poorly immunogenic fibrosarcoma of B6 origin, was transfected with cDNA for IL-2, IL-4, IL-6, IFN-γ, and/or B7-1. Tumor cells transfected with both B7-1 and...

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3 Abbreviations used in this paper: LN, lymph nodes; mIL, murine IL; hIL, human IL; CM, complete medium; LLC, Lewis lung carcinoma; PE, phycoerythrin.
Materials and Methods

Mice
Female C57BL/6J (B6) mice (Central Laboratory of Experimental Research, Tokyo, Japan) were used for experiments at the age of 10 wk or older. They were maintained in specific pathogen-free conditions.

Tumors
MCA102, MCA205, and MCA207 are antigenically distinct fibrosarcomas of B6 origin induced by intramuscular injection of 3-methylcholanthrene. LLC and B16F10 melanoma (B16) are also of B6 origin. These tumors were maintained in vivo in syngeneic mice by serial s.c. transplantation.

Expression vector
The eukaryotic cDNA expression vector, BCMGSNeo, conferring neomycin resistance, was kindly supplied by Dr. H. Karasuyama (Basil Institute for Immunology, Basel, Switzerland) (45). Mouse IL-2 (mIL-2), IL-4, and human IL-6 (hIL-6) cDNA-containing clones were transfected into MCA102, LLC, B16, and B16F10 cells using a lipofectin reagent (Life Technologies). These cDNA clones were introduced into the XhoI or NotI site of BCMGSNeo. The biological activities of hIL-6 in mice have been described previously (22, 23).

Gene transfection
BCMGSNeo/Neo, mIL-2 cDNA-containing BCMGSNeo/IL-2), mIL-4 cDNA-containing BCMGSNeo/IL-4), hIL-6 cDNA-containing BCMGSNeo/IL-6), mIFN-γ cDNA-containing BCMGSNeo/IFN-γ), and/or mB7-1 cDNA-containing BCMGSNeo (B7-1) vectors were transfected into MCA102, LLC, B16, and MCA205 cells using a lipofectin reagent (Life Technologies, Gaithersburg, MD). An MCA102 clone with relatively high MHC class I expression (MCA102H) was produced by a limiting dilution method and transfected with B7-1 (MCA102H/B7-1). These tumor cells (1 × 106 cells) were plated in a 35-mm tissue culture dish in 2 ml RPMI 1640 medium supplemented with 10% heat-inactivated FCS and cultured until the cells were confluent. The lipofectin-DNA complexes were overlaid onto the cells for a 12-h incubation period at 37°C in a CO2 incubator. After replacing the DNA-containing medium with RPMI 1640 medium containing 10% FCS, cells were incubated for an additional 48 h.

The transfected, MCA102Neo, MCA102IL-2, MCA102IL-4, MCA102IL-6, MCA102IFN-γ, MCA102B7-1, MCA102B7-1/IL-6, MCA102B7-1/IFN-γ, MCA102B7-1/IFN-γ, MCA205B7-1, MCA205B7-1/IFN-γ, LLC/IFN-γ, LLC/B7-1, LLC/B7-1/IFN-γ, B16/IFN-γ, B16B7-1, and B16B7-1/IFN-γ were selected by supplementation of the media for 14 days with 400–1000 μg/ml of the neomycin analogue, G418 (Life Technologies). These gene-modified tumor cells were maintained as monolayer cultures in complete medium (CM). CM consisted of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 10 U/ml penicillin, 50 μg/ml kanamycin, 50 μg/ml tritamine (all from Life Technologies), 0.5 μg/ml amphotericin-B (Fungizone; Life Technologies), and 5 × 10-5 M 2-ME (Sigma, St. Louis, MO).

Cytokine ELISA
Medium (1 ml) conditioned by 5 × 105 tumor cells for 24 h was assayed for mIL-2, mIL-4, hIL-6, or murine IFN-γ content by a quantitative “sandwich” enzyme immunoassay using a mIL-2 ELISA kit or a mIL-4 ELISA kit (Endogen, Boston, MA), a hIL-6 ELISA kit (Becton Dickinson Labware, Bedford, MA), or a mIFN-γ ELISA kit (Genzyme, Cambridge, MA).

mAb and flow cytometry
Hybridomas producing mAb against the murine CD3 chain (145-2C11), CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2), and the murine L-selectin (MEL-14) were obtained from the American Type Culture Collection (Manassas, VA). Anti-CD3 mAb was produced as a supernatant of an in vitro culture with hybridoma cells and then partially purified by 50% ammonium sulfate precipitation, and the IgG content was determined by ELISA. Anti-CD4 mAb, anti-CD8 mAb, and anti-L-selectin mAb were produced as ascites fluid from sublethally irradiated (500 rad) DBA/2 mice. For in vivo depletion of CD4+CD8+ T cells, mice were given i.v. injections of 0.15–0.2 ml of ascites fluid diluted to 1.0 ml with HBSS. This procedure has been previously shown to be effective in long-term T cell deletion (8). FITC-conjugated anti-B7-1 (16–10A), FITC-conjugated anti-H-2Kb (AF6-88.5), FITC-conjugated anti-H-2Dk (KH95), phycoerythrin (PE)-conjugated anti-I-Ak (AF6-120.1), FITC-conjugated anti-ICAM-1 (3E2), PE-conjugated anti-Thy1.2 (30–H12), PE-conjugated anti-CD3 (145-2C11), FITC-conjugated anti-CD4 (L3T4), FITC-conjugated anti-CD8 (Lyt-2), and PE-conjugated anti-L-selectin (MEL-14) were purchased from Pharmingen (San Diego, CA). Analyses of cell surface phenotypes were conducted by direct immunofluorescence staining of 0.5–1 × 106 cells with conjugated mAb. In each sample, 10,000 cells were analyzed by a FACScan flow microfluorometer (Becton Dickinson, Sunnyvale, CA).

Recombinant cytokines
Recombinant hIL-2 was kindly supplied by Shionogi Pharmaceutical (Osaka, Japan). Purified material had a sp. act. of 1.1 × 106 IU/mg protein. Recombinant mIFN-γ was kindly supplied by Otsuka Pharmaceutical (Tokyo, Japan). Purified material had a sp. act. of 1 × 106 IU/mg protein.

Lymphoid cell preparation and anti-CD3/IL-2 activation
Tumor growth was initiated by inoculating syngeneic B6 mice i.v. in the bilateral flank with 106 viable tumor cells. Ten to 12 days later, tumor-draining LNs were harvested and single-cell suspensions were prepared mechanically as described previously (15). The cells were stimulated in vitro by incubating ~106 cells in a 75-cm2 tissue culture flask containing 30 ml of CM with 2 μg/ml of anti-CD3 mAb. After 2 days of incubation at 37°C in a 5% CO2 atmosphere, activated cells were harvested, washed, and further cultured at a concentration of 6 × 103/ml in 30 ml of CM containing 40 U/ml of IL-2 for 3 days.

Adoptive immunotherapy
B6 mice were injected i.v. with 8 × 105 MCA102, 4 × 105 MCA205, 106 MCA207, or 8 × 105 LLC tumor cells in 1 ml of HBSS to initiate pulmonary metastases. On day 3, effector cells were given i.v. to each mouse. On day 14, the metastatic foci in all mice were enumerated as described previously (16). Metastatic foci too numerous to count were assigned an arbitrary number of 250. The significance of differences in numbers of pulmonary metastases between groups was determined by the Wilcoxon’s rank-sum test.

Fractionation of tumor-draining LN cells based on the expression of L-selectin
T cells in the LN cell suspension were concentrated by passage through nylon wool columns (Wako Pure Chemical Industries, Osaka, Japan). After a 45-min incubation at 37°C, the nonadherent elution contained 90–95% L-selectin–selective cells. These cells were further fractionated into two subpopulations based on the expression of L-selectin. Cells were first incubated for 20 min at 4°C with the L-selectin hybridoma ascites fluid at a 1:1000 dilution. The cells were washed of unbound Ab. A total of 3–4 × 107 cells in 4 ml CM were plated on a 25-cm2 tissue culture dish for 3 days. The morphology and in vitro proliferation of the transfectants were almost identical to those of parental MCA102 tumor cells (data not shown).

Results
Characteristics of gene-modified MCA102 tumor cells
The morphology and in vitro proliferation of the transfectants were almost identical to those of parental MCA102 tumor cells (data not shown).
The culture supernatants of MCA102 tumor cells transfected with mIL-2, mIL-4, hIL-6, or mIFN-γ contained ~400 U/ml of mIL-2, 180 μg/ml of mIL-4, 240 ng/ml of hIL-6, or 20 IU/ml of mIFN-γ, respectively. No cytokine was detected in the supernatants of parental or neomycin-resistant gene-transfected tumors. The expression of B7-1 in gene-modified MCA102 tumor cells was examined by flow cytometric analysis. B7-1 transfectants consistently expressed high levels of B7-1 (Fig. 1).

**Figure 1.** B7-1 expression on gene-transduced MCA102 tumor cells. Tumor cells were stained with FITC-labeled anti-B7-1 (16-10A) mAb. B7-1 expression was analyzed by flow cytometry. A total of 10⁶ cells was analyzed for each sample. Each frame consists of 10,000 cells.

Tumor growth of gene-modified tumor cells in C57BL/6 mice

Confluent cultures of gene-modified and -unmodified tumor cells were harvested and inoculated s.c. in the right flank of B6 mice with 1 × 10⁶ viable tumor cells in 0.05 ml of HBSS. In vivo tumor growth of gene-modified and -unmodified MCA102 tumors are shown in Fig. 2. MCA102/B7-1/IFN-γ initially grew and then regressed. In contrast, other single or cotransfectants grew progressively (Fig. 2B). To assess the role of CD4⁺ or CD8⁺ T cells in the regression of MCA102/B7-1/IFN-γ tumors, mAb to the CD4⁺ or CD8⁺ determinant was administered 3 days before and 4 days after tumor inoculation. As shown in Fig. 3, the depletion of either CD4⁺ or CD8⁺ T cells abrogated the tumor regression, indicating that both CD4⁺ and CD8⁺ T cells were required for the regression of MCA102/B7-1/IFN-γ tumors. The regression induced by the cotransfection of these two genes was confirmed further in other tumor models. LLC and B16, poorly immunogenic tumors, and MCA205, a weakly immunogenic tumor of B6 origin, were also transfected in vitro with B7-1 and/or IFN-γ. In these tumor cells, cotransfection of B7-1 and IFN-γ resulted in tumor regression when injected s.c. (Fig. 4). Because only cotransfection of B7-1 and IFN-γ induced tumor regression, we examined whether vaccination with this gene-modified tumor could induce protective immunity against parental tumor challenge. Gene-modified or -unmodified MCA102 tumor cells were irradiated (4000 rad) and intradermally injected into B6 mice as a vaccination, and 2 wk after vaccination mice were challenged with parental tumors. Six of...
10 mice that were vaccinated with MCA102/B7-1/IFN-γ cells rejected parental tumor challenge in a tumor-specific manner (Table I). Although vaccination with MCA102/B7-1/IFN-γ cells could efficiently induce protective immunity against s.c. challenge, it could not mediate the regression of pulmonary metastases (data not shown).

Antitumor efficacy of effector cells generated from tumor-draining LN of gene-modified MCA102 tumors

We next examined the antitumor efficacy of cells generated from LN draining the gene-modified tumors by the anti-CD3/IL-2 method. We previously demonstrated that the activation of tumor-draining but not normal or bacterial adjuvant Corynebacterium parvum-stimulated LN cells with anti-CD3/IL-2 resulted in the generation of specific antitumor effector cells (6). To investigate the advantage of gene-modified tumor cells for adoptive immunotherapy, we used this anti-CD3/IL-2 activation method to generate effector cells. Cells from tumor-draining LN of s.c. MCA102 parental, MCA102/IFN-γ, MCA102/B7-1, or MCA102/B7-1/IFN-γ tumors were harvested and activated in vitro with anti-CD3/IL-2. There was no significant difference in in vitro cell proliferation between these cells (approximately threefold). The antitumor efficacy of cells generated from different LNs was adoptively transferred to mice bearing 3-day established pulmonary metastases (Table II). The coexpression of B7-1 and IFN-γ significantly enhanced the antitumor efficacy of anti-CD3/IL-2-activated cells when compared with that of cells generated from LNs draining parental MCA102, MCA102/IFN-γ, or MCA102/B7-1 tumors. In addition, the enhancing effect of IFN-γ was augmented further by the coexpression of B7-1. The adoptive transfer of fresh noncultured cells from MCA102/B7-1/IFN-γ tumor-draining LN failed to demonstrate antitumor efficacy (data not shown), indicating that the enhancing effect could be induced by promoting the precursor

Table I. Challenge of mice with parental MCA102 or MCA205 tumor cells after immunization with B7-1- and/or IFN-γ-transfected MCA102 tumor cells

<table>
<thead>
<tr>
<th>Immunization with</th>
<th>No. of Mice with Tumor/</th>
<th>No. of Mice Challenged with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCA102</td>
<td>MCA205</td>
</tr>
<tr>
<td>MCA102/IFN-γ</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>MCA102/B7-1</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>MCA102/B7-1/IFN-γ</td>
<td>4/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Control</td>
<td>10/10</td>
<td>10/10</td>
</tr>
</tbody>
</table>

B6 mice were immunized intradermally in the abdomen with 2 × 10^7 irradiated (4000 rad) tumor cells.

Two weeks after immunization, these mice were challenged with 10^7 parental MCA102 or MCA205 tumor cells. The number of mice with tumor growth was counted.

FIGURE 3. Effect of in vivo T cell subset depletion on MCA102/B7-1/IFN-γ tumor growth. Cells (10^6 in 0.05 ml of HBSS) were injected s.c. into the right flank of mice, and tumor size was measured serially. A 200-μl volume of ascites fluid containing mAb or 0.25 mg of rIg diluted to 1.0 ml of HBSS was administered i.v. 3 days before and 4 days after tumor inoculation. The results are expressed as mean diameter (in millimeters) of tumors from groups of five mice each.

FIGURE 4. In vivo tumor growth of B7-1- and/or IFN-γ-transfected tumor cells in B6 mice. Cells (10^6 in 0.05 ml of HBSS) were injected s.c. into the right flank of mice, and tumor size was measured serially. The results are expressed as mean diameter (in millimeters) of tumors from groups of five mice each.
response. To investigate the T cell subset populations that participated in tumor eradication, we depleted a CD4⁺ or CD8⁺ T cell subset population in vivo with mAb. As shown in Table III, depletion of either a CD4⁺ or CD8⁺ T cell subset resulted in abrogation of the antitumor effect, indicating that both populations of T cells participated in mediating the tumor eradication.

**Expression of MHC class I molecule on B7-1 and/or IFN-γ gene-modified MCA102 tumor cells**

To clarify the mechanisms of enhanced antitumor response induced by MCA102/B7-1/IFN-γ cells, we analyzed the expression of cell surface molecules on the gene-modified tumors by flow cytometry. As shown in Fig. 5, up-regulation of MHC class I molecules (Kb and Dp) was observed in both MCA102/IFN-γ and MCA102/B7-1/IFN-γ tumors, but not in MCA102/B7-1 or parental MCA102 tumors. No expression of MHC class II molecule

### Table II. Adoptive immunotherapy with anti-CD3/IL-2-activated cells generated from LN-draining B7-1- and/or IFN-γ-transfected MCA102 tumor cells

<table>
<thead>
<tr>
<th>No. of Cells Transferred (× 10⁵)</th>
<th>Mean No. of Pulmonary Metastases (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCA102</td>
</tr>
<tr>
<td>3</td>
<td>199 (41)</td>
</tr>
<tr>
<td>1</td>
<td>214 (36)</td>
</tr>
<tr>
<td>0.3</td>
<td>250</td>
</tr>
<tr>
<td>0</td>
<td>250</td>
</tr>
</tbody>
</table>

- Ingual LN cells obtained from mice bearing s.c. MCA102, MCA102/B7-1, MCA102/IFN-γ, or MCA102/B7-1/IFN-γ tumors for 12 days were activated by the anti-CD3/IL-2 method. These cells were given i.v. to mice with 3-day established pulmonary MCA102 metastases.
- Lungs were harvested and metastases were counted 14 days after tumor i.v. inoculation. Significant differences were detected from groups receiving: *, no treatment; †, activated MCA102 tumor-draining LN cells; ‡, activated MCA102/B7-1 tumor-draining LN cells; §, activated MCA102/IFN-γ tumor-draining LN cells.

### Table III. Effect of in vivo T cell subset depletion on therapeutic efficacy of anti-CD3/IL-2-activated cells generated from LN-draining MCA102/B7-1/IFN-γ tumors

<table>
<thead>
<tr>
<th>No. of Cells Transferred (× 10⁵)</th>
<th>mAb for In Vivo Depletion</th>
<th>Mean No. of Pulmonary Metastases (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>rgf</td>
<td>168 (15)</td>
</tr>
<tr>
<td>3</td>
<td>Anti-CD4</td>
<td>150 (26)</td>
</tr>
<tr>
<td>3</td>
<td>Anti-CD8</td>
<td>141 (25)</td>
</tr>
</tbody>
</table>

- Cells from LN-draining s.c. MCA102/B7-1/IFN-γ tumors for 12 days were activated by the anti-CD3/IL-2 method. These cells were given i.v. to mice with 3-day established pulmonary MCA102 metastases.
- A 200-μl volume of ascites fluid containing mAb or 0.25 mg of rIg diluted to 1.0 ml of HBSS was administered i.v. within 1 h of cell transfer.
- Lungs were harvested and metastases were counted 14 days after tumor i.v. inoculation. Significant differences were detected from groups receiving: *, no treatment; †, activated MCA102 tumor-draining LN cells; ‡, activated MCA102/B7-1 tumor-draining LN cells; §, activated MCA102/IFN-γ tumor-draining LN cells.

### Table IV. Specificity of adoptive immunotherapy mediated by anti-CD3/IL-2-activated cells generated from LN-draining MCA102/B7-1/IFN-γ tumors

<table>
<thead>
<tr>
<th>Adoptive Immunotherapy</th>
<th>Mean No. of Pulmonary Metastases (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCA102</td>
</tr>
<tr>
<td>−</td>
<td>250</td>
</tr>
<tr>
<td>+</td>
<td>23 (10)*</td>
</tr>
</tbody>
</table>

- Cells from LN-draining s.c. MCA102/B7-1/IFN-γ tumors for 12 days were activated by the anti-CD3/IL-2 method. B6 mice were sublethally irradiated (500 rad) and injected with 8 × 10⁵ MCA102, 4 × 10⁵ MCA205, 10⁵ MCA207, or 8 × 10⁵ LLC tumor cells to initiate pulmonary metastases. On day 3, activated LN cells (3 × 10⁷/mouse) were given i.v. to each mouse.
- Lungs were harvested and metastases were counted 14 days after tumor i.v. inoculation.
- Significantly different from group receiving no treatment.

Comparison of antitumor efficacy of cells generated from LN-draining MCA102/B7-1 and MCA102/B7-1/IFN-γ tumors

To examine whether up-regulation of MHC class I could be the sole cause of the enhanced precursor response by IFN-γ, the antitumor efficacy of cells generated from MCA102/B7-1 or MCA102/B7-1/IFN-γ tumors was compared in adoptive immunotherapy for established pulmonary metastases. Cells from LN-draining s.c. MCA102/B7-1 or MCA102/B7-1/IFN-γ tumors were harvested and activated in vitro with anti-CD3/IL-2. As shown in Table IV, the antitumor efficacy of activated MCA102/B7-1/IFN-γ tumor-draining LN cells was significantly superior to that of activated MCA102/B7-1 tumor-draining LN cells. These results suggest that the enhancement effect of IFN-γ from tumor cells could not be explained solely by the up-regulation of MHC class I expression.

Phenotypic analysis of gene-modified or unmodified tumor-primed LN cells before and after anti-CD3/IL-2 activation

Phenotypic analysis of cells from gene-modified or unmodified tumor-draining LN for CD3, CD4, CD8, or the homing molecule L-selectin are shown in Fig. 7. Although LN draining these tumors consisted of almost identical fractions of CD4⁺ and CD8⁺ T cells, LN-draining MCA102/B7-1/IFN-γ tumors had an increased proportion of cells with down-regulation of L-selectin (L-selectin⁻). Approximately 25% of cells from LN-draining MCA102/B7-1/IFN-γ tumors were L-selectin⁻ cells. These L-selectin⁻ cells consisted of almost identical fractions of CD4⁺ and CD8⁺ T cells (data not shown).
Antitumor efficacy of adoptive immunotherapy mediated by activated L-selectin<sup>−</sup> cells derived from LN-draining MCA102/B7-1/IFN-γ tumors

To determine whether the increased proportion of cells with downregulated L-selectin may reflect the efficient precursor response in tumor-draining LN, cells purified according to the expression of L-selectin were activated by the anti-CD3/IL-2 method, and the antitumor efficacy of these activated cells were analyzed in adoptive immunotherapy. On activation by the anti-CD3/IL-2 method, L-selectin<sup>−</sup> cells proliferated more vigorously than L-selectin<sup>+</sup> cells and unfractionated LN cells. L-selectin<sup>−</sup> cells increased approximately eightfold, compared with a threefold increase observed in L-selectin<sup>+</sup> cells and unfractionated LN cells. These activated cells were adoptively transferred to mice bearing 3-day established pulmonary metastases. As shown in Table VI, the transfer of $0.3 \times 10^7$ L-selectin<sup>−</sup> cells were therapeutically equally effective as $1 \times 10^7$ unfractionated cells, whereas the transfer of $1 \times 10^7$ L-selectin<sup>+</sup> cells did not demonstrate any antitumor efficacy. Because activated L-selectin<sup>−</sup> cells generated from LN-draining MCA102/B7-1/IFN-γ tumors efficiently eliminated microscopic 3-day established pulmonary metastases, we further investigated whether adoptive transfer of the cells could prolong the survival. Mice that received $2 \times 10^7$ L-selectin<sup>−</sup> cells had an equivalent median survival time to untreated mice. However, the transfer of $2 \times 10^7$ L-selectin<sup>−</sup> cells resulted in long-term survival and indicated more efficient tumor eradication than unfractionated cells (Fig. 8). These results indicate that the antitumor efficacy of activated L-selectin<sup>−</sup> cells is far greater than that of activated L-selectin<sup>+</sup> or unfractionated cells and that the precursor response in LN-draining MCA102/B7-1/IFN-γ is exclusively augmented.

Table V. Adoptive immunotherapy with anti-CD3/IL-2-activated cells generated from LN cells draining MCA102H/B7-1 or MCA102/B7-1/IFN-γ tumors

<table>
<thead>
<tr>
<th>No. of Cells Transferred&lt;sup&gt;a&lt;/sup&gt; (× 10&lt;sup&gt;7&lt;/sup&gt;)</th>
<th>Mean No. of Pulmonary Metastases (SEM)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA102H/B7-1</td>
<td>MCA102/B7-1/IFN-γ</td>
</tr>
<tr>
<td>3</td>
<td>75 (13)*</td>
</tr>
<tr>
<td>1</td>
<td>115 (9)*</td>
</tr>
<tr>
<td>0.3</td>
<td>187 (17)</td>
</tr>
<tr>
<td>0</td>
<td>213 (14)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells from LN-draining s.c. MCA102H/B7-1 or MCA102/B7-1/IFN-γ tumors for 12 days were activated by the anti-CD3/IL-2 method. These cells were given i.v. to mice with 3-day established pulmonary MCA102 metastases.

<sup>b</sup> Lungs were harvested and metastases were counted 14 days after tumor i.v. inoculation. Significant differences were detected from groups receiving: *, no treatment; or †, activated MCA102H/B7-1 tumor-draining LN cells.
Discussion

In many animal models, tumors transduced with genes for cytokine such as IL-2 (17–19), IL-4 (20, 21), IL-6 (22), IFN-γ (24–28), TNF (29–31), granulocyte-CSF (32), or granulocyte-macrophage-CSF (33, 34) may have reduced tumorigenicity with an augmented host-immune response. Although vaccination with the gene-modified tumors resulted in the rejection of parental tumor challenge, this strategy is not thought to be always directly applicable for treatment of human cancers, which often lack apparent immunogenicity.

We previously reported that vaccine therapy with gene-modified tumor cells could be used to augment the antitumor efficacy of adoptive immunotherapy (35). Although vaccination therapy with tumor cells modified to secrete cytokines alone did not mediate the tumor regression of established visceral metastases, vaccination could enhance the in vivo priming of antitumor precursor T cells in tumor-draining LN and subsequently augment the antitumor efficacy of adoptive immunotherapy. Cells derived from LN draining these gene-modified tumors showed potent antitumor efficacy in the eradication of pulmonary metastases. We further demonstrated that the suppressed function of CD4+ T cells by tumor burden was restored by the local secretion of the Th cytokines from tumor cells and subsequently facilitated the priming of precursor lymphocytes of effector cells (35).

In the current study, we first investigated whether such genetic modifications could enhance the host-immune reaction against tumors that lacked apparent immunogenicity. Although transfection of cytokines successfully enhanced the generation of antitumor effector T cells when relatively immunogenic tumors were used, our results indicated that transfection of cytokines alone failed to induce a host-immune response, which led to the eradication of poorly immunogenic tumors and priming of tumor-reactive T cells (Fig. 2 and Tables I and II). Transfection of costimulatory molecules such as B7-1 have been demonstrated to reduce or abrogate tumorigenicity by eliciting a specific antitumor response against parental tumors (38–44). The transfection of B7-1 enhanced the host-immune reaction, and the expression on weakly immunogenic MCA205 tumor cells reduced tumorigenicity of these cells when injected s.c. (Fig. 4). In contrast, B7-1 transfection into poorly immunogenic tumors did not induce tumor regression or precursor

Table VI. Adoptive immunotherapy with anti-CD3/IL-2-activated cells generated from LN cells draining MCA102/B7-1/IFN-γ tumors separated based on L-selectin expression

<table>
<thead>
<tr>
<th>Cells a</th>
<th>No. of Cells Transferred (× 10⁷)</th>
<th>Mean No. of Pulmonary Metastases (SEM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>1</td>
<td>56 (8)</td>
</tr>
<tr>
<td>L-selectin+</td>
<td>1</td>
<td>210 (26)</td>
</tr>
<tr>
<td>L-selectin−</td>
<td>0.3</td>
<td>62 (10)c</td>
</tr>
</tbody>
</table>

a Cells from LN-draining s.c. MCA102/B7-1/IFN-γ tumors for 12 days were separated based on L-selectin expression and activated by the anti-CD3/IL-2 method. These cells were given i.v. to mice with 3-day established pulmonary MCA102 metastases.

b Lungs were harvested and metastases were counted 14 days after tumor i.v. inoculation.

c Significantly different from group receiving no treatment.
response in the tumor-draining LN (Fig. 4 and Table II). These data are consistent with the past observation that tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity (41). Considering these facts, we speculated that dysfunction of CD4+ T cells, which is observed in a weakly immunogenic tumor system, may preclude the generation of effector T cells facilitated by B7-1 expression on poorly immunogenic tumor cells. Therefore, we investigated whether cotransfection of the Th cytokines and B7-1 could enhance the host antitumor immunity.

The coexpression of IFN-γ and B7-1 induced the regression of s.c. tumors, but combinations of other cytokines and B7-1 had no effect (Fig. 2B). This particular combination also induced tumor regression of two other poorly immunogenic tumors (Fig. 4). The regression was mediated by a host-immune reaction, because mice that were given sublethal irradiation were incapable of rejecting the cotransfected tumor cells (data not shown). Mice vaccinated with MCA102/B7-1/IFN-γ cells rejected parental tumor challenge, indicating that vaccination could induce systemic tumor immunity (Table I). Furthermore, cells from MCA102/B7-1/IFN-γ tumor-draining LN acquired potent antitumor efficacy after in vitro activation with anti-CD3 and IL-2. Adoptive transfer of fresh tumor-draining LN cells did not mediate antitumor efficacy (data not shown), indicating that the vaccination could enhance priming of antitumor precursor T cells in tumor-draining LN and subsequently augment the antitumor efficacy of adoptive immunotherapy. These effector cells were tumor-specific because the cells derived from LN-draining MCA102/B7-1/IFN-γ tumors had no antitumor reactivity against MCA207, LLC, or B16 tumors (Table IV).

A deficiency in the presentation of endogenous Ag may be one reason for the lack of tumor immunogenicity (47), and the up-regulation of MHC class I expression by IFN-γ enables weakly immunogenic tumors to express endogenous Ag (27). Tumor cells modified to secrete IFN-γ have reduced immunogenicity, and this phenomenon was explained by the up-regulation of MHC class I Ag expression on tumor cells (24–28). Because IFN-γ is a potent inducer of MHC class I, class II Ag, and other molecules such as ICAM-1, we analyzed the expression of these molecules on IFN-γ-transfected MCA102 tumor cells. MHC class I expression was up-regulated on IFN-γ-transfected MCA102 tumor cells, but neither MHC class II nor ICAM-1 was expressed on IFN-γ-transfected tumor cells (Fig. 5).

To examine this correlation between the up-regulation of MHC class I expression on tumor cells and the augmented antitumor reactivity, we selected one clone from the parental tumor cells that had a high level of MHC class I Ag expression (MCA102H). B7-1 transfection of MCA102H resulted in tumor regression, indicating that MHC class I expression plays an important role in the regression of tumors (Fig. 6). However, interestingly the antitumor efficacy of activated MCA102/B7-1/IFN-γ tumor-draining LN cells was far superior to that of activated MCA102H/B7-1 tumor-draining LN cells (Table V). This significant difference was not attributed to the difference in mean intensity of B7-1 and MHC class I Ag on tumor cells, indicating that enhanced priming of precursor lymphocytes of antitumor effector T cells in MCA102/B7-1/IFN-γ tumor-draining LN could not be explained solely by the up-regulation of MHC class I expression. Our data is consistent with previous studies that demonstrated that IFN-γ-transfected tumor cells were more effective in the immunotherapy of tumor-bearing mice than MHC class I Ag gene-transfected tumor cells (28) and that up-regulation of MHC class I expression by low IFN-γ secretors was insufficient to decrease tumorigenicity (26).

IFN-γ may influence the outcome of an immune response in several distinct ways, and its importance in tumor immunology has been demonstrated by numerous reports (24–28). We and others previously demonstrated that the function of the CD4+ T cell subset, especially CD4+ Th cells, is depressed in a tumor-bearing host, and the intensity of the suppressive effect is correlated to the tumor burden (35, 48, 49). Among the many cytokines expressed by CD4+ Th cells, a recent study revealed down-regulation of IFN-γ but not IL-2 levels of CD4+ T cells in a particular tumor model (50). This selective down-regulation of cytokine expression may also occur in our tumor model, because coexpression of IFN-γ but not IL-2 abrogated tumorigenicity and enhanced the precursor response.

In our tumor model, there was no expression of MHC class II or ICAM-1 on IFN-γ-transfected tumor cells (Fig. 5). Nevertheless, both CD4+ and CD8+ T cells appeared to be required for antitumor efficacy, because in vivo depletion of either CD4+ or CD8+ T cell subsets by mAb abrogated the tumor regression (Fig. 3). This result may suggest an important role for host APC as an MHC class II Ag presenter to prime CD4+ T cells. IFN-γ from tumor cells could act primarily on macrophages and dendritic cells through up-regulation of MHC class II expression, thereby enhancing the Ag presentation to CD4+ T cells. This could be an alternative explanation for the enhancing effect by IFN-γ.

We have confirmed the enhanced generation of precursor cells of antitumor effector T lymphocytes by analyzing the antitumor efficacy of in vitro activated tumor-draining LN cells in adoptive immunotherapy. Recently, it was demonstrated that the down-regulation of the homing molecule L-selectin could serve as a surrogate marker for identifying specific tumor-sensitized T cells (51, 52). Using this method, we further confirmed the generation of precursor T cells in the tumor-draining LN. Phenotypic analysis of tumor-draining LN cells revealed an increased population of cells with down-regulated L-selectin expression in MCA102/B7-1/IFN-γ but not in MCA102H/B7-1 tumor-draining LN (Fig. 7). Cells with down-regulated L-selectin expression constituted all the antitumor reactivity, indicating that the coexpression of B7-1 and IFN-γ of tumor cells induced an efficient precursor response in the tumor-draining LN (Table VI, Fig. 8).

In conclusion, the cotransfection of poorly immunogenic murine tumors with B7-1 and IFN-γ could enhance the in vivo priming of precursor cells of tumor-specific T cells in the tumor-draining LN. MHC class I up-regulation on tumor cells by IFN-γ alone could not account for the enhanced antitumor immunity, indicating that this particular combination of transfection could facilitate a suitable microenvironment for the priming of tumor-reactive T cells. Our observation implies the therapeutic utility of gene-modification of tumors for the treatment of tumors that lack apparent immunogenicity. The strategy presented here will help us to understand the mechanisms of induction of host antitumor immunity as well as to establish more effective immunotherapy for human cancer.

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References
ADOPTIVE IMMUNOTHERAPY OF MURINE POORLY IMMUNOGNOM TUMOR


