Prolonged survival of mice with glioma injected intracerebrally with double cytokine–secreting cells

Terry Lichtor, M.D., Ph.D., Roberta P. Glick, M.D., Tae Sung Kim, Ph.D., Roger Hand, M.D., and Edward P. Cohen, M.D.

Division of Neurosurgery, Cook County Hospital and Hektoen Institute for Medical Research, Chicago, Illinois; Departments of Anatomy and Cell Biology, Microbiology and Immunology, and Medicine, University of Illinois College of Medicine, Chicago, Illinois; and Department of Neurosurgery, Loyola University Medical Center, Chicago, Illinois

A novel approach toward the treatment of glioma was developed in a murine model. The genes for both interleukin-2 (IL-2) and interferon-γ (IFN-γ) were first transfected into a mouse fibroblast cell line that expresses defined major histocompatibility complex (MHC) determinants (H-2k). The double cytokine–secreting cells were then cotransplanted intracerebrally with the GI261 murine glioma cell line into syngeneic C57BL/6 mice (H-2b) whose cells differed at the MHC from the cellular immunogen. The results indicate that the survival of mice with glioma injected with the cytokine-secreting allogeneic cells was significantly prolonged, relative to the survival of mice receiving equivalent numbers of glioma cells alone. Using a standard 51Cr-release assay, the specific release of isotope from labeled GI261 cells coincubated with spleen cells from mice injected intracerebrally with the glioma cells and the cytokine-secreting fibroblasts was significantly higher than the release of isotope from glioma cells coincubated with spleen cells from nonimmunized mice. The cellular antglioma response was mediated by natural killer/lymphokine-activated killer and Lyt-2,2+ (CD8+) cells. The increased survival of mice with glioma and the specific immunocytotoxic responses after immunization with fibroblasts modified to secrete both IL-2 and IFN-γ indicate the potential of an immunotherapeutic approach to gliomas with cytokine-secreting cells.

KEY WORDS • glioma • interleukin-2 • interferon-γ • gene therapy

The prognosis for patients with primary malignant glioma remains poor, in spite of a variety of different forms of treatment. Surgery, irradiation, and chemotherapeutic treatments rarely succeed in curing the disease.

An emerging strategy in the treatment of various neoplasms involves the stimulation of an immune response against the malignant cells. Cytokines such as interleukin-2 (IL-2) or interferon-γ (IFN-γ) have been used in both experimental animals and patients to treat malignant disease with inconsistent success. Interleukin-2 has no direct toxic effect on cancer cells; its antitumor activity is mediated by modulation of the host's immunological response to the neoplasm. Interferon-γ induces the expression of major histocompatibility complex (MHC) class I determinants and augments the sensitivity of tumor cells to cytotoxic T lymphocyte–mediated lysis. Whether cytokine treatment is effective in the treatment of intracerebral neoplasms has remained uncertain.

To evaluate the potential of a novel form of cytokine treatment of glioma, in a prior study we first modified a mouse fibroblast cell line, LM, which expresses defined MHC determinants (H-2k) to secrete both IL-2 and IFN-γ. The cytokine-secreting cells (LM–IL-2/IFN-γ) were then cotransplanted subcutaneously with GI261 murine glioma cells (H-2b) into syngeneic C57BL/6 mice (H-2b) whose cells differed at the MHC from the cytokine-secreting cells. The period of survival in the tumor-bearing mice treated with the IL-2/IFN-γ double–secreting allogeneic cells was significantly prolonged relative to the survival of untreated mice with glioma, or that of mice with glioma treated with nonsecreting LM cells.

At the present time, we report the effects of intracerebral injection of LM–IL-2/IFN-γ cells on the survival of mice bearing glioma. The results indicate that the survival of mice injected with a mixture of glioma cells and the double cytokine–secreting cells was significantly pro-
Antiglioma response using cytokine-secreting cells

longed, relative to the survival of mice injected with an equivalent number of tumor cells alone. The antitumor immunity was mediated predominantly by natural killer (NK) and lymphokine-activated killer (LAK) cells.

Materials and Methods

Cell Lines and Experimental Animals

The GI261 is a glial tumor cell line of C57BL/6 mouse origin (H-2b). The tumor cells were serially transferred in histocompatible C57BL/6 female mice, which were maintained and treated according to National Institutes of Health guidelines for the care and use of laboratory animals. Nine animals were used in each group. Interleukin-2–dependent CTLL-2 cells used for assay were grown in RPMI-1640 medium; supplemented with fetal bovine serum (FBS), 2 × 10⁻³ M 2-mercaptoethanol, 2 mM glutamine, and antibiotics; and 100 U/ml IL-2 at 37°C in a 7% CO₂/air atmosphere. Additional mouse cell lines—LM fibroblasts, B16F1 melanoma cells, EL4 lymphoma cells and LL2 Lewis lung carcinoma cells—were all maintained under the same conditions.

Preparation of IL-2– and/or IFN-γ-Secreting Mouse Fibroblasts

The IL-2–secreting mouse fibroblasts were prepared in a manner described previously. In brief, a retroviral vector (pZipNeoSV-IL-2) that carries the gene for human IL-2 was transduced into mouse fibroblasts. The vector also contained a gene (neo) that conferred resistance to the aminoglycoside antibiotic, G418. Helper-free stocks of recombinant retroviruses, generated from the GP+env Am12 packaging cell line, were used to transduce the LM cells. For this purpose, virus-containing supernatants were added to the cells, followed by overnight incubation at 37°C in growth medium (Dulbecco’s minimal essential medium (DMEM) containing 10% FBS and antibiotics) in the presence of 5 μg/ml polybrene. Afterward, the cells were selected in growth medium containing 1 mg/ml G418.

To prepare IL-2/IFN-γ double cytokine–secreting cells, IL-2 secretion was confirmed and the cells were then cotransfected (lipofectin-mediated) with deoxyribonucleic acid (DNA) from pZipNeoSVIFN-γ and DNA from pHyg, as previously described. The plasmid conferred resistance to hygromycin. Interferon-γ secretion by the hygromycin-resistant cells was assayed using an IFN-γ enzyme-linked immunosorbent assay kit.

Histopathological Examination of Tumors

The brain from each animal was removed for histological examination. The tissue was fixed in 10% formalin, prepared for sectioning after paraffin embedding, and 4-μm sections were stained with hematoxylin and eosin according to standard procedures.

Spleen Cell–Mediated Cytotoxicity by ⁵¹Cr-Release Assay

Spleen cell suspensions were prepared using spleens from mice injected intracerebrally with the cytokine-secreting cells. The spleens were forced through 40-gauge stainless steel screens in a small quantity of growth medium. The mononuclear cells were obtained using a Ficoll–Hypaque gradient as previously described. Spleen cell–mediated cytotoxicity measurements were performed using a standard 4-hour ⁵¹Cr-release assay. Briefly, 5 × 10⁶ tumor cells were labeled with chromium-51 by incubation for 1 hour at 37°C in growth medium containing 100 μCi of sodium chromium-51. After three washes with DMEM, 10⁴ ⁵¹Cr-labeled GI261 cells were incubated with the nonplastic-adherent population of spleen cells from the immunized mice at varying target:effector ratios. The percent specific cytosis was calculated as:

Experimental ⁵¹Cr Release − Spontaneous ⁵¹Cr Release × 100.

Determination of Classes of Effector Cells Activated for Antiglioma Cytotoxicity

Antibody depletion studies using monoclonal antibodies (mAbs) for T cells, T-cell subsets, or NK/LAK cells were used to identify the predominant cell types activated for antitumor cytotoxicity in mice immunized with the cytokine-secreting cells. Spleen cell suspensions from the immunized mice were prepared, as described above, and incubated at 4°C for 1 hour with the relevant mAb and low-toxicity rabbit complement before a standard 4-hour ⁵¹Cr-release assay toward the GI261 cells was performed. Anti-Lyt-2.2 (CD8+) or anti–asialo GM1 (NK/LAK) was added to respective spleen cell suspensions. Anti–asialo GM1 mAb was previously found to inhibit cell-mediated cytotoxicity toward YAC-1 cells, an NK/LAK–sensitive cell line. Immunofluorescent staining was used to determine the efficiency of the depletion of CD8+ or NK/LAK cells after treatment with mAbs and complement, as determined by cytofluorometric analysis of cells stained with serially diluted antibodies. Treatment of spleen cells with anti–Lyt-2.2 or anti–asialo GM1 mAbs completely eliminated the corresponding T-cell subset without any change in the frequency of the other T-cell subsets. The antibodies were titrated to the extent that the concentrations used were five times the amount required to saturate the binding of specific cell types from naive C57BL/6 mice.

Statistical Analyses

Statistical analyses were performed using commercially available software. Statistical significance was taken at the level of p < 0.05 unless otherwise indicated. For the analyses of variance (ANOVA) a posteriori multiple comparisons between groups were made using Tukey’s test, for which the level of statistical significance was p < 0.012 rather than 0.05. For the regression analyses, the various cell types and antibody treatments were assumed to be nominal variables. They were therefore converted into dummy independent variables to be run against the continuous dependent variable of ⁵¹Cr release. The ANOVAs and regression analyses were subjected to a series of diagnostic tests that assured that the input data did not violate the assumptions of independence and normality.

Sources of Supplies and Equipment

The GI261 cell line was obtained originally from J. Mayo, National Cancer Institute, Frederick, MD, and the C57BL/6 mice from Jackson Laboratory, Bar Harbor, ME. Interleukin-2 was purchased from Genzyme, Cambridge, MA, and the IL-2–dependent CTLL-2 cells were obtained from A. Finnegan, Rush Medical College, Chicago, IL. The LM fibroblasts, B16F1 melanoma cells, EL4 lymphoma cells, and LL2 Lewis lung carcinoma cells were obtained from the American Type Culture Collection, Rockville, MD.

The retroviral vectors pZipNeoSV–IL-2 (originally made available by T. Taniguchi, Institute for Molecular and Cellular Biology, Osaka University, Japan) and pZipNeoSVIFN-γ were obtained from M. K. L. Collins, Institute of Cancer Research, London, England. The GP+env Am12 packaging cell line was provided by A. Finnegan, Rush Medical College, Chicago, IL. The LM fibroblasts, B16F1 melanoma cells, EL4 lymphoma cells, and LL2 Lewis lung carcinoma cells were obtained from the American Type Culture Collection, Rockville, MD.

Mononuclear cells used in the spleen cell suspensions were obtained using a Ficoll–Hypaque gradient provided by Pharmacia, Piscataway, NJ. The sodium chromium-51 was obtained from Amersham, Arlington Heights, IL. Low-toxicity rabbit complement was provided by Pel Freeze, Rogers, AR. Anti–Lyt-2.2 (CD8+) was obtained from Pharmingen, San Diego, CA, and anti–asialo GM1 (NK/LAK) from Wako Chemical Co., Dallas, TX. Cytofluorometric analysis of cells stained with serially diluted antibodies was performed using the EPICS V, Coulter Electronics Inc., Hialeah, FL. Statistical analyses were performed using MINITAB.
The number of glioma cells used (10^5) was sufficient to maintain glioma cells from C57BL/6 mice bearing subcutaneous tumors. Cells injected intracerebrally. The glioma cells were obtained from Gl261 glioma secreting cells were determined against Gl261 gliomaous culture. When the cells were reassayed after 6 months of continuous culture, equivalent amounts of the relevant cytokines were secreted by cells modified to secrete either cytokine alone. Cells were secreted cytokine-secreting fibroblasts (LM–IL-2/IFN-γ) led to the most significant therapeutic benefit in mice with intracerebral glioma. The brain from each of the animals in both the treated and untreated groups was removed shortly after death for cytoytic activities of spleen cells from C57BL/6 mice injected intracerebrally with a mixture of glioma and cytokine-secreting cells*. Days after injection of tumor cells

**Fig. 1.** Graph showing the survival rate of mice injected intracerebrally with a mixture of glioma cells and cytokine-secreting fibroblasts. The C57BL/6 mice (eight mice/group) were injected intracerebrally with a mixture of 10^6 cells of one of the cell types and 10^5 Gl261 glioma cells. The median lengths of survival were as follows (in days): mice with nonimmunized glioma cells, 16.9 ± 1.9; glioma plus LM cells, 20.0 ± 4.5; glioma plus LM–interferon-γ (IFN-γ) cells, 18.0 ± 1.8; glioma plus LM–interleukin-2 (IL-2) cells, 23.4 ± 6.8; glioma plus LM–IL-2/IFN-γ cells, 28.1 ± 5.8. Probability values were: nonimmunized vs. LM–IL-2, p < 0.025; nonimmunized or LM vs. LM–IL-2/IFN-γ, p < 0.005; LM–IL-2 vs. LM–IL-2/IFN-γ, p < 0.05.

### Results

#### Survival of Mice Injected Intracerebrally With Gl261 Glioma and LM–IL-2/IFN-γ Cells

The LM mouse fibroblasts were genetically modified by retroviral gene transfer for the secretion of both IL-2 and IFN-γ. The cells formed approximately 8000 U of IL-2 per 10^6 cells and 120 U of IFN-γ per 10^6 cells in 48 hours. Equivalent quantities of IL-2 or IFN-γ were secreted by cells modified to secrete either cytokine alone. Nontransduced LM cells did not form detectable quantities of IL-2 or IFN-γ. Periodic passage of the cells in medium containing G418 (300 μg/ml) resulted in the formation of equivalent amounts of the relevant cytokines when the cells were reassayed after 6 months of continuous culture.

The immunotherapeutic effects of the double cytokine-secreting cells were determined against Gl261 glioma cells injected intracerebrally. The glioma cells were obtained from C57BL/6 mice bearing subcutaneous tumors. The number of glioma cells used (10^5) was sufficient to cause death within 28 days in 100% of animals injected intracerebrally. To determine the effect of LM–IL-2/IFN-γ cells on the survival of C57BL/6 mice with intracerebral glioma, 10^5 glioma cells were mixed with 10^6 cytokine-secreting cells, and the cell mixtures were then injected at a depth of 3 mm in a volume of 4 μl into the right frontal lobes of naive C57BL/6 mice. For use as controls, other naive C57BL/6 mice were injected with equivalent numbers of either tumor cells alone or tumor cells and non-cytokine-secreting fibroblasts (LM). The results (Fig. 1) indicate that mice injected with glioma and LM–IL-2/IFN-γ cells survived significantly longer (p < 0.001) than mice injected with glioma cells alone or mice injected with a mixture of glioma cells and noncytokine-secreting fibroblasts (LM) (p < 0.005). The survival time of mice injected with glioma cells and single cytokine-secreting LM–IL-2 cells was significantly (p < 0.05) shorter than that of mice injected with glioma and LM–IL-2/IFN-γ cells (Fig. 1) and significantly longer than that of mice injected with glioma cells alone (p < 0.025). The survival time of mice injected with a mixture of glioma cells and LM–IFN-γ cells was not significantly different from that of mice injected with glioma cells alone (p > 0.1). The C57BL/6 mice that were injected intracerebrally with equivalent numbers of LM–IL-2 cells (but no glioma cells) lived for over 3 months without obvious signs of neurological deficit. Thus, a combination of IL-2 and IFN-γ secretion led to the most significant therapeutic benefit in mice with intracerebral glioma.

The brain from each of the animals in both the treated and untreated groups was removed shortly after death for

### Table 1

<table>
<thead>
<tr>
<th>Cell Types†</th>
<th>mAb Treatment</th>
<th>% Cytolysis at E/T Ratio of 100:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>glioma</td>
<td>—</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>anti-Lyt-2.2+ + C</td>
<td>3.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>−1.4 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>−7.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>glioma + LM</td>
<td>—</td>
<td>10.3 ± 3.9</td>
</tr>
<tr>
<td>anti-Lyt-2.2+ + C</td>
<td>5.8 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>−1.9 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>−7.8 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>glioma + LM–IL-2</td>
<td>—</td>
<td>19.4 ± 1.6§</td>
</tr>
<tr>
<td>anti-Lyt-2.2+ + C</td>
<td>17.7 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>5.2 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>−6.6 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>glioma + LM–IL-2/IFN-γ</td>
<td>—</td>
<td>40.5 ± 12.4§</td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>38.3 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>20.4 ± 11.9</td>
<td></td>
</tr>
<tr>
<td>anti–asialo GM1 + C</td>
<td>−4.6 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

* Abbreviations: C = complement; E/T = effector-to-target cells; IFN-γ = interferon-γ; IL-2 = interleukin-2; LAK = lymphokine activated killer; LM = mouse fibroblast cell line; mAbs = monoclonal antibodies; NK = natural killer; — = no treatment.

† C57BL/6 mice received a single intracerebral injection of glioma cells (10^6) together with one of the modified fibroblast cell types (10^6) cells. Three weeks after the injection, mononuclear cells from the spleens of the immunized mice obtained through Ficoll–Hypaque centrifugation were used for the 51Cr-release assay. All values represent the mean ± standard deviation of triplicate determinations.

‡ p < 0.005 relative to 51Cr release for spleen cells from animals immunized with glioma.

§ p < 0.05 relative to 51Cr release for spleen cells from animals immunized with glioma + LM cells.

¶ p < 0.025 relative to 51Cr release for spleen cells from animals immunized with glioma.

** p < 0.05 relative to 51Cr release for spleen cells from animals immunized with glioma + LM–IL-2 cells.

---

T. Lichtor, et al.
Antiglioma response using cytokine-secreting cells

**Table 2**

<table>
<thead>
<tr>
<th>Cell Type†</th>
<th>Nonimmunized</th>
<th>LM–IL-2</th>
<th>LM–IL-2/IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI261</td>
<td>2.2 ± 0.9</td>
<td>44.6 ± 0.8</td>
<td>63.3 ± 7.2</td>
</tr>
<tr>
<td>B16F1</td>
<td>−0.2 ± 2.0</td>
<td>14.9 ± 1.2</td>
<td>15.4 ± 1.3</td>
</tr>
<tr>
<td>EL4</td>
<td>4.1 ± 1.3</td>
<td>46.3 ± 4.8</td>
<td>37.8 ± 1.5</td>
</tr>
<tr>
<td>LL/2</td>
<td>10.1 ± 1.0</td>
<td>19.3 ± 1.4</td>
<td>15.1 ± 1.4</td>
</tr>
</tbody>
</table>

* Abbreviations: E/T = effector-to-target cells; IFN-γ = interferon-γ; IL-2 = interleukin-2; LM = mouse fibroblast cell line.
† C57BL/6 mice received a single intracerebral injection of glioma cells (2.0 × 10⁶) together with one of the modified fibroblast cell types (10⁶ cells). Two weeks after the injection, mononuclear cells from the spleens of the immunized mice obtained through Ficoll–Hypaque centrifugation were used for the ⁵¹Cr-release assay. All tumor cells are H-2b haplotype. All values represent the mean ± standard deviation of triplicate determinations.

Histological examination. The tumors ranged in size from 3 to 5 mm. The presence of tumor tissue was apparent in each instance, with typical findings of a glioblastoma including areas of infiltrating anaplastic tumor cells along with necrosis. Lymphocytes were present in tumors from mice co-injected with either LM–IL-2 or LM–IL-2/IFN-γ cells.

**Induction of Anti-GI261 Glioma Immunocytotoxic Responses in Tumor-Bearing Mice Injected Intracerebrally With LM–IL-2/IFN-γ Double–Secreting Cells**

A standard ⁵¹Cr-release assay was used to detect the presence of spleen cell–mediated immunity toward GI261 glioma in mice injected intracerebrally with glioma and LM–IL-2/IFN-γ cells. Spleen cells were obtained from mice sacrificed 3 weeks after the implantation of tumor and modified LM fibroblasts. For comparison, spleen cells were obtained according to the same schedule from mice injected intracerebrally with GI261 cells alone or with glioma cells and nontransfected LM cells. The magnitude of the cytotoxic response toward the glioma cells (Table 1) was significantly higher in spleen cell populations from mice injected intracerebrally with glioma and LM–IL-2/IFN-γ cells than from animals injected intracerebrally with glioma cells alone (p < 0.025), or with glioma cells and nonsecreting LM cells (p < 0.05). Mice injected with glioma and LM–IL-2 cells had lesser cytolytic responses than mice injected with LM–IL-2/IFN-γ cells (p < 0.05).

The antiglioma cytolytic response in spleen cell populations from mice injected intracerebrally with LM–IL-2 cells and glioma cells was greater than that found in spleen cell populations from animals injected with glioma cells alone (p < 0.005), or with glioma cells and nontransfected LM cells (p < 0.05). A one-way ANOVA confirmed that the differences between the four groups were significant (F = 12.6, p = 0.002). Thus, intracerebral immunization of mice with IL–2/IFN-γ double cytokine–secreting fibroblasts stimulated a generalized immunocytotoxic antiglioma response that exceeded the response found in nonimmunized mice with glioma or mice with glioma injected with IL–2–secreting LM cells.

To determine if C57BL/6 mice with glioma injected intracerebrally with LM–IL-2/IFN-γ or LM–IL-2 cells exhibited cytotoxicity toward other tumor cell types, spleen cells from the injected mice were tested against ⁵¹Cr-labeled B16F1 melanoma, EL-4 lymphoma, and LL-2 Lewis lung carcinoma cells, all of which are of C57BL/6 mouse origin (H–2b). The results (Table 2) showed the presence of cytotoxicity toward glioma cells as well as toward each of the other tumors. The magnitude of the cytotoxic responses was significantly higher in the mice injected with glioma and LM–IL-2/IFN-γ cells.

**Classes of Immune-Effecter Cells Activated for Antiglioma Cytotoxicity in Mice Injected Intracerebrally With LM–IL-2/IFN-γ Cells**

Monoclonal antibodies for CD8+ or NK/LAK cells were used to determine certain classes of effector cells involved in the antiglioma response. Naïve C57BL/6 mice were injected intracerebrally with glioma and the double cytokine–secreting cells. Twenty-one days later, mononuclear cells from the spleens of these animals were treated with excess quantities of mAbs (five times the amount required to saturate the relevant binding sites of 100% of the cells), as determined by specific immunofluorescence of Lyt-2.2 or asialo GM1 mAbs and complement before they were tested for cytotoxic activity toward ⁵¹Cr-labeled GI261 glioma cells. As indicated (Table 1), treatment of spleen cells from mice injected with glioma and LM–IL-2/IFN-γ cells with asialo GM1 mAb reduced the cytotoxic activity toward GI261 cells to “background,” that is, to a level equivalent to that present in a population of spleen cells from nonimmunized mice, or of spleen cells from mice injected with noncytokine-secreting LM cells. By ANOVA, the differences were significant (F = 11.0, p = 0.003). Similar results were obtained in mice injected intracerebrally with glioma and LM–IL-2 cells. Treatment of spleen cells from mice injected with glioma and LM–IL-2 or LM–IL-2/IFN-γ cells with Lyt-2.2 mAb reduced the cytotoxic activity toward GI261 cells by 73.2% and 49.7% respectively. By ANOVA, the differences were significant (F = 110.1, p < 0.001).

A multivariate balanced ANOVA indicated that treatment with LM–IL-2/IFN-γ or LM–IL-2 cells and treatment with the antibody used to inactivate NK/LAK or CD8+ cells each had a significant effect on ⁵¹Cr release (F = 23.4, p < 0.001 and F = 24.2, p < 0.001, respectively). This explained 78% of the variation between treatment groups (R² = 0.777). Overall, 77% of the variation was explained by the model. These data indicate that activation of NK/LAK and CD8+ cells was primarily responsible for the antiglioma response in mice immunized with LM–IL-2 or LM–IL-2/IFN-γ cells.

**Cytotoxicity Assays**

To investigate whether the observed cytotoxicity toward unrelated tumor cell lines in mice injected with cells secreting IL-2 was secondary to the activation of nonspecific antitumor NK/LAK cells, an additional experiment was performed in which the C57BL/6 mice received subcutaneous injections of LM–IL-2 cells without receiving...
injections of any tumor cells. Spleen cells from these mice were then tested for cytotoxic activity toward either 51Cr-labeled Gl261 glioma or EL4 lymphoma cells. The cytotoxicity assays were also performed after pretreatment of the spleen cells with mAbs for CD8+ or NK/LAK. Spleen cells obtained from the animals that received subcutaneous injections of LM–IL-2 cells but no tumor cells demonstrated cytotoxicity against both Gl261 glioma and EL4 lymphoma cells (Table 3). Furthermore, the antitumor response in this assay was mediated entirely by NK/LAK cells, as antigen-specific Lyt-2.2+ T cells were not involved. In final experiments designed to determine whether the secretion of IL-2 stimulated cytotoxicity against normal cellular antigens, normal spleen cells were used as target cells in the 51Cr-release assay. Spleen cells from mice injected subcutaneously with LM–IL-2 cells did not elicit any cytotoxic response against normal spleen cells, and normal spleen cells did not stimulate any cytotoxic activity against B16F1 melanoma cells (Table 4). In this experiment spleen cells from mice injected subcutaneously with only LM–IL-2 cells did demonstrate cytotoxicity against B16F1 melanoma cells.

**Discussion**

The survival of mice injected intracerebrally with a mixture of glioma cells and LM fibroblasts genetically engineered to secrete both IL-2 and IFN-γ was significantly prolonged, relative to the survival of mice receiving equivalent numbers of glioma cells alone. Similar results were obtained if mice were injected intracerebrally with a mixture of glioma and LM cells modified to secrete only IL-2, although in this instance survival time was clearly less than that of mice with glioma treated with cells modified to secrete both IL-2 and IFN-γ. Animals injected intracerebrally with glioma cells and cells modified to secrete IFN-γ alone died in shorter intervals. The animals’ resistance to tumor growth correlated with specific immunocytotoxic responses that were mediated predominantly by NK/LAK and CD8+ cells.

In patients with advanced metastatic melanoma or renal cell carcinoma, systemic biological therapy with IL-2 resulted in significant antitumor effects. Inoculation of various cytokines to enhance the immune response, although attempts against gliomas have involved the intrathecal injection of autologous lymphocytes and/or administration of various cytokines to enhance the immune response, with inconsistent results. The systemic administration of IL-2 has been attempted, but the results of these studies have failed to indicate a substantial tumoricidal effect or prolongation of life.

Interleukin-2 is required for the growth of cytotoxic T lymphocytes and it enhances NK/LAK activity. Interferon-γ induces the expression of MHC class I determinants and augments the sensitivity of tumor cells to cytotoxic T lymphocyte–mediated lysis. Although IFN-γ has been found to inhibit growth of glioma cell lines in vitro, analogous effects have not been observed in the case of gliomas proliferating in vivo. In light of the fact that IL-2 stimulates NK/LAK activity, which generates an antitumor response against a variety of tumor cell types, it is not surprising that cytotoxicity against other target tumor cells was observed in the 51Cr-release assay. The best cytotoxic responses as well as survival data in this study were noted in mice immunized intracerebrally with glioma tumor cells in addition to LM–IL-2/IFN-γ fibroblasts. From this we can infer that activation of nonspecific antitumor NK/LAK activity in addition to antigen-specific CD8+ cells provides the optimum opportunity for tumor rejection.

Cytokine secretion by tumor cells themselves was attempted in immunotherapeutic protocols. The introduction and expression of genes specifying various cytokines into malignant cells resulted in an augmentation of their immunogenic properties. The secretion of IFN-γ by human glioma cells led to an inhibition of tumor growth in vivo when the malignant cells were cocultivated with LAK cells. Furthermore, significant inhibition of growth of a human glioma cell line in nude mice was observed when the cells were injected subcutaneously along with a plasmacytoma cell line modified to secrete IL-4. A simi-
ilar effect was also observed when the glioma cells and IL-4–secreting cells were injected intracerebrally. The subcutaneous injection of inbred rats with 9L gliosarcoma cells modified for IL-2 secretion resulted in a dramatic eosinophil infiltration into re-
mancytoma cells modified for IL-4 secretion implanted in treatment of a malignancy of the central nervous system.

J. Neurosurg. / Volume 83 / December, 1995

however, no effect on tumor growth occurred if the cells were injected intracerebrally.\textsuperscript{5,6}

A major concern relating to the immunological treat-
ment of brain neoplasms is the effect of the blood-brain barrier on the development of a host–immune response. In the studies reported here, spleen cell–mediated antglioma responses were generated following intracerebral injections of glioma and LM cells that had been modified to secrete both IL-2 and IFN-\gamma. Recent studies\textsuperscript{3} using plas-
macytoma cells modified for IL-4 secretion implanted in the brains of nude mice along with a human glioma cell line resulted in a dramatic eosinophil infiltration into re-

regions of necrotic tumor, indicating that an immune re-
sponse had taken place against a tumor of the central ner-
vous system in situ. Alternatively, a systemic immune response may have developed, which was followed by an accumulation of eosinophils migrating into the tumor.

The use of fibroblasts for cytokine administration is an attractive method of therapy because many of the side effects of high-dose administration have not been ob-

served. The intracerebral injection of the cytokine-se-
creting cells alone did not lead to significant long-term side effects. Allogeneic rather than syngeneic cells were chosen for local cytokine administration because of the known adjuvant effects of foreign MHC determinants on the antitumor response and the likelihood that the cells would be rejected by cellular immune mechanisms.\textsuperscript{5,6,10,11,15,16,21,22} The data presented here raise the possibility that injection of cytokine-secreting fibroblasts directly into an intracerebral neoplasm may be a useful approach in the treatment of a malignancy of the central nervous system.

Acknowledgments

We would like to thank Dr. James L. Stone for his continued encouragement and support of this work. We also thank Drs. George Pappas, Department of Anatomy and Cell Biology, University of Illinois at Chicago, and O. Howard Reichman, Department of Neurosurgery, Loyola University, for their thoughtful advice and careful review of the manuscript.

References


4. Eberlein TJ, Rosenstein M, Rosenberg SA: Regression of a dis-

eminated syngeneic solid tumor by systemic transfer of lym-


sion of anchorage-independent growth and tumorigenicity in

immunodeficient mice by transfection of the H-2 class I gene H-2L\textsuperscript{d} into a human colon cancer cell line (HCT). Proc Natl Acad Sci USA 85:8543–8547, 1988


10. Hämmerling GJ, Klar D, Katzav S, et al: Manipulation of me-
tastasis and tumor growth by transfection with histocompati-


termined classes of anti-melanoma effector cells. J Immunother 


14. Kim TS, Collins MKL, Cohen EP: Independent cell types are involved in the induction of antitumor responses of C57BL/6 mice immunized with interleukin-2-secreting al-

genous mouse fibroblasts expressing melanoma-associated anti-

15. Kim TS, Russell SJ, Collins MKL, et al: Immunity to B16 mel-

anoma in mice immunized with IL-2-secreting allogeneic mouse fibroblasts expressing melanoma-associated antigens. Int J Cancer 51:283–289, 1992


established pulmonary metastases by the systemic administra-


Manuscript received November 23, 1994. Accepted in final form June 1, 1995. This study was supported by the U.S. Department of Health and Human Services Grants CA 55651-03 awarded to Dr. Cohen and National Institutes of Health Grant K17 NS01777-01 awarded to Dr. Glick. Address reprint requests to: Terry Lichtor, M.D., Ph.D., Division of Neurosurgery, 1835 West Harrison Street, Chicago, Illinois 60612.