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Approaches to the treatment of brain tumors using cytokine-secreting allogeneic fibroblasts

Research Article

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Summary

The prognosis for patients with an intracerebral neoplasm is poor. Conventional treatments such as surgery, radiation therapy and chemotherapy have done little to affect long-term survival, and new methods of treatment are urgently needed. In this report approaches involving cytokine gene therapy in treatment of malignant brain tumors are reviewed and contrasted to a strategy developed in this laboratory involving the use of allogeneic cells genetically modified to secrete cytokines. In our studies, mice with an intracerebral glioma, melanoma or breast carcinoma treated solely by intratumoral injections with allogeneic cells genetically modified to secrete interleukin-2 were found to survive significantly longer than mice in various control groups. The anti-tumor response was mediated predominantly by T cell subsets ($CD8^+$ and NK/LAK cells). The injections resulted in the killing of only the neoplastic cells; non-neoplastic cells were unaffected. Experiments involving treatment of animals with intracerebral tumor using subcutaneous injections of cytokine secreting allogeneic cells in the presence of tumor antigens demonstrated no effect in prolonging survival in spite of the development of a vigorous systemic antitumor immune response. Of special interest, mice injected intracerebrally with the cytokine-secreting allogeneic cells alone exhibited no neurologic defect and there were no adverse effects on survival. The injection of cytokine-secreting allogeneic cells into the microenvironment of an intracerebral tumor is hypothesized to induce an anti-tumor immune response capable of prolonging survival. This preclinical animal data should directly translate into clinical treatments for patients with a malignant intracerebral tumor.

I. Introduction

The current prognosis for patients with malignant brain tumors remains poor (Mahaley et al, 1989). Malignant gliomas are the most common primary brain tumor. Despite treatment with surgery, radiation and chemotherapy, the 2-year survival remains less than 20%. One emerging strategy in the treatment of tumors involves stimulation of an immunologic response against the neoplastic cells. The hope is that the immune system can be called into play to destroy malignant cells. However, in most instances, proliferating tumors do not provoke anti-tumor cellular immune responses. The precise mechanisms that enable antigenic neoplasms to escape host immunity are incompletely understood. The cells appear to escape recognition by the immune system in spite of the fact that neoplastic cells form weakly immunogenic tumor associated antigens (TAAs).

Tumor cells may evade immune responses by losing expression of antigens or major histocompatiblity complex (MHC) molecules or by producing immunosuppressive cytokines. In addition T cells that recognize self-antigens may differentiate into suppressor or regulatory cells, which inhibit the activation and/or functions of effector cells. The inhibitory effects of suppressor cells may be mediated by cytokines. In particular interleukin-10 and TGF- are two examples of such cytokines. Successful methods to induce immunity to TAAs could lead to tumor cell destruction and prolong the survival of cancer patients.

A variety of strategies have been used to increase the immunogenetic properties of vaccine therapies for brain tumors. The immune response can be augmented by genetic modification of tumor cells to secrete cytokines including IL-2, GM-CSF and interferon- . One can also alter the MHC of the tumor cells to express allogeneic

determinants. Finally one can genetically modify the tumor cells to express co-stimulatory molecules such as B7. In some instances, objective evidence of tumor regression has been observed in patients receiving immunizations only with tumor cell immunogens, suggesting the potential effectiveness of this type of immunotherapy for malignant neoplasms. In addition modification of delivery techniques to treat intracerebral tumors has included intrathecal, intralymphatic, subcutaneous and intratumoral injections of treatment cells. We have utilized many of these techniques to enhance the immune response in the development of our cellular vaccine, as discussed below.

Recent advances in our understanding of the biology of the immune system have led to the identification of numerous cytokines that modulate immune responses (Kelso, 1989; Borden and Sondel, 1990; Gabrilove and Jakubowski, 1990). These agents mediate many of the immune responses involved in anti-tumor immunity. Several of these cytokines have been produced by recombinant DNA methodology and evaluated for their anti-tumor effects. In experimental clinical trials, the administration of cytokines and related immunomodulators has resulted in objective tumor responses in some patients with various types of neoplasms (Lotze et al, 1986; Rosenberg et al, 1988; Borden and Sondel, 1990).

Interleukin-2 (IL-2) is an important cytokine in the generation of anti-tumor immunity (Rosenberg et al, 1988). In response to tumor antigens, the helper T-cell subset of lymphocytes secretes small quantities of IL-2. This IL-2 acts locally at the site of tumor antigen presentation to activate cytotoxic T-cells and natural killer cells that mediate systemic tumor cell destruction. Intravenous, intralymphatic or intralesional administration of IL-2 has resulted in clinically significant responses in several types of cancer (Lotze et al, 1986; Pizza et al, 1988; Rosenberg et al, 1988; Gandolfi et al, 1989; Sama et al, 1990). However, severe toxicities (hypotension and edema) limit the dose and efficacy of intravenous and intralymphatic IL-2 administration (Lotze et al, 1986; Sama et al, 1990). The toxicity of systemically administered cytokines is not surprising since these agents mediate local cellular interactions, and they are normally secreted in quantities too small to have systemic effects. To circumvent the toxicity of systemic IL-2 administration, several investigators have examined intralesional injection of IL-2 (Bubenik et al, 1988; Gandolfi et al, 1989). This approach eliminates the toxicity associated with systemic IL-2 administration. However, multiple intralesional injections are required to optimize therapeutic efficacy (Bubenik et al, 1988; Gandolfi et al, 1989). These injections will be impractical for many patients without potential significant morbidity, particularly when tumor sites are not accessible for direct injection.

Cytokine gene transfer has resulted in significant anti-tumor immune responses in several animal tumor models (Tepper et al, 1989; Watanabe et al, 1989; Fearon et al, 1990; Gansbacher et al, 1990). In these studies, the transfer of cytokine genes into tumor cells has reduced or abrogated the tumorigenicity of the cells after implantation into syngeneic hosts. The transfer of genes for IL-2 (Fearon et al, 1990; Gansbacher et al, 1990), gamma interferon (IFN-) (Watanabe et al, 1989), and IL-4 (Tepper et al, 1989) significantly reduced or eliminated the growth of several different histological types of murine tumors. Other cytokines capable of producing similar results include granulocyte-macrophage colony-stimulating factor (GM-CSF) (Yu et al, 1997) and interleukin-12 (Ehtesham et al, 2002). In the studies employing IL-2 gene transfer, the treated animals also developed systemic anti-tumor immunity and were protected against subsequent tumor challenges with the unmodified parental tumor (Fearon et al, 1990; Gansbacher et al, 1990). Similar inhibition of tumor growth and protective immunity were also demonstrated when immunizations were performed with a mixture of unmodified parental tumor cells and genetically modified tumor cells engineered to express the IL-2 gene. No toxicity associated with expression of the cytokine transgenes was reported in these animal tumor studies (Tepper et al, 1989; Watanabe et al, 1989; Fearon et al, 1990; Gansbacher et al, 1990). An alternative strategy is to genetically modify tumor cells to express an antisense gene to TGF- , which is a cytokine highly expressed in glioma cells that acts to inhibit the function of cytotoxic T cells (Fakhrai et al 1996).

Previous immunotherapy stategies have utilized classical immunologic cell types including activated lymphocytes and LAK cells. More recently, a variety of cells have been investigated for their usefulness in tumor oncology including tumor cells themselves (syngeneic or allogeneic), Dendritic cells or fibroblasts (syngeneic or allogeneic). Although syngeneic tumor cells have the advantage that they express most of the appropriate antigens needed for targeted therapy, many types of tumors are difficult to establish in culture. In addition cytokine gene therapies requiring the transduction of autologous tumor cells may not be practical for many cancer patients. Modification of neoplastic cells taken directly from tumor-bearing patients may be difficult. In particular a primary tumor cell line, required for retroviral modification has to be established. An alternative cell type that can be used for therapeutic immunizations is the Dendritic cell (DC), which is a specialized antigen presenting cell. Pre-clinical studies have indicated that immunizing either mice or rats with DC pulsed using tumor cell antigens can stimulate a cytotoxic T cell response that is tumor-specific and that engenders protective immunity against CNS tumor in the treated animals (Ashley et al, 1997; Heimberger et al, 2002). It is also conceivable that a subpopulation of the primary tumor, selected for its capacity to grow *in vitro*, may not reflect the tumor cell population as a whole especially since tumors such as glioma are known to be heterogeneous.

We have chosen an allogeneic fibroblast cell line as a cellular vaccine for a number of reasons. Fibroblasts obtained from established allogeneic fibroblast cell lines may be readily cultured *in vitro* and genetically modified

to express and secrete cytokines (Kim et al, 1992; Kim et al, 1994; Tahara et al, 1994; Fakhrai et al, 1995; Sobol et al, 1995). The cells can be genetically modified to secrete cytokines and subsequently injected directly into the tumor bed. The use of *allogeneic* rather than *syngeneic* cells was initially based upon evidence that allogeneic MHC determinants augment the immunogenic properties of the tumor vaccine (Kim et al, 1992; Kim et al, 1994; Tahara et al, 1994). Application of genetically modified fibroblasts in therapeutic vaccines facilitates titration of single or multiple cytokine doses independent of tumor cell doses. Like other allografts, the allogeneic cytokine-secreting cells are rejected. Furthermore, the number of cells can be expanded as desired for multiple rounds of therapy. In addition, the slow continuous release of cytokines and the eventual rejection of the allograft may be a useful advantage in the treatment of brain tumors where long-term secretion of high concentrations of certain cytokines may be associated with increased morbidity. Thus, an allogeneic cytokine secreting vaccine is readily available, easily expanded, possibly less toxic and more immunogenic. These considerations provide the rationale for examining the use of allogeneic fibroblasts genetically modified to secrete cytokines as a means of enhancing anti-tumor immune responses in treatment of malignant intracerebral tumors (Kim et al, 1992; Kim et al, 1994; Tahara et al, 1994; Fakhrai et al, 1995; Lichtor et al, 1995; Sobol et al, 1995; Lichtor et al 2002).

II. Materials and methods

A. Cell lines and experimental animals

Gl261 is a malignant glial tumor syngeneic in C57BL/6 mice. The tumor was originally obtained from Dr. J. Mayo (DCT, DPT, National Cancer Institute, Frederick, MD); it was maintained by serial transfer in histocompatible C57BL/6 mice. SB-5b cells are a breast adenocarcinoma that formed spontaneously in a C3H/He mouse. These cells were grown by *in vivo* passage in female C3H/He mice. B16F1 cells are a highly malignant melanoma cell line derived from a melanoma arising spontaneously in C57BL/6 mice (from I. Fidler, M.D. Anderson, Houston, TX). LM cells, a fibroblast cell line of C3H/He mouse origin, were from the American Type Culture Collection (Manassas, VA). The B16F1 and LM cells were maintained at 37^0C in a humidified 7% CO_2/air atmosphere in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Sigma, St Louis, MO) and antibiotics (Life Technologies) (growth medium).

The animals used were eight to ten-week-old pathogen-free C57Bl/6 (H-2^b) or C3H/He (H-2^k) mice obtained from Charles River Breeding Laboratories (Portage, MI). The mice were maintained in the animal care facilities of the University of Illinois, according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. They were 8-12 weeks old when used in the experiments.

B. Preparation of cytokine (IL-2 and/or IFN-) secreting mouse fibroblasts

IL-2 secreting mouse fibroblasts were prepared as described previously (Kim et al, 1992). The gene for IL-2 was transduced into LM fibroblasts with a retroviral plasmid (pZipNeoSV-IL-2) (obtained originally from T. Taniguchi, Institute for Molecular and Cellular Biology, Osaka University, Japan) (Yamada et al, 1987). The plasmid contains a human IL-2 cDNA and a gene (<u>neo^r</u>) that confers resistance to the aminoglycoside antibiotic, G418 (Colbere-Garapin et al, 1981) used for selection.

To prepare the IL-2/IFN- double cytokine-secreting cells, the IL-2 secreting cells were co-transfected (lipofectin-mediated; Gibco BRL, Grand Island, NY) with DNA from pZipNeoSVIFN- (obtained from M.K.L. Collins, Institute of Cancer Research, London, England) along with DNA from pHyg (obtained from L. Lau, University of Illinois, Chicago, Illinois), as previously described (Kim et al, 1995). The plasmid confers resistance to hygromycin (Sugden et al, 1985) used for selection.

IFN- single cytokine-secreting cell-lines were prepared by co-transfection of LM cells with DNA from pZipNeoSVIFN- along with DNA from pHyg, as previously described (Kim et al, 1995). The cells were maintained for 14 days in growth medium containing 300 µg/ml hygromycin. To maintain cytokine-secretion, every third passage the cells were routinely placed in the relevant selection medium.

C. Modification of LM or LM-IL-2 fibroblasts (H-2k) to express H-2Kb class I-determinants

A plasmid (pBR327H-2K^b from Biogen Research Corp, Cambridge, MA) encoding MHC H-2K^b determinants was used to modify LM or LM-IL-2 fibroblasts to express H-2K^b determinants. Ten µg of PBR327H-2K^b and 1 µg of pBabePuro was mixed with Lipofectin (Gibco BRL), according to the supplier's instructions. The plasmid pBabePuro (obtained from M.K.L. Collins, University College, London, England) conferring resistance to puromycin, was used for selection. The plasmid-mixture was added to $1x10^6$ LM or LM-IL-2 cells in 10 ml of DMEM, without FBS. For use as a control, an equivalent number of LM or LM-IL-2 cells were transfected with 1µg of pBabePuro alone. The cells were incubated for 18 hrs at 37^0C in a CO_2/air atmosphere, washed with DMEM, followed by the addition of growth medium. After incubation for 48 hrs, the cell cultures were divided and replated in growth medium supplemented with 3.0 µg/ml puromycin (Sigma; St Louis, MO) followed by incubation at 37^0C for 7 additional days. The surviving colonies were pooled and tested by staining with specific FITC-conjugated antibodies for the expression of H-2K^b-determinants. One hundred percent of non-transfected fibroblasts maintained in growth medium containing puromycin died during the seven-day period of incubation.

D. Assays for cytokine secretion

IL-2 secretion by the G418-resistant cells was assayed with the use of the IL-2-dependent cell-line CTLL-2, as previously described (Gillis et al, 1978). One unit of IL-2 gave half-maximal proliferation of CTLL-2 cells under these conditions (Gillis et al, 1978). IL-2 and IFN- secretion by the transfected cells were assayed by the use of a human IL-2 or a mouse IFN- ELISA kit (Genzyme, Cambridge, MA).

E. The detection of mRNAs specifying IL-2 or IFN- by transfected LM cells by the reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used as a further confirmation of the expression of the transferred cytokine genes. Total cellular RNA

was prepared from the relevant cell types (Chomczynski et al, 1987) and then transcribed into cDNA and amplified, as previously described (Kim et al, 1995).

F. Spleen cell-mediated cytotoxicity by 51Cr-release assay

Mononuclear cells from the spleens of C57BL/6 mice immunized with the various cell constructs were used as sources of effector cells for the cytotoxicity studies using a standard 4 hour cromium release assay, as previously described (Kim et al, 1995).

G. In vitro determination of the classes of effector cells activated for the anti-glioma cytotoxicity

The effect of monoclonal antibodies (mAbs) for T-cell subsets or NK/LAK cells on the anti-tumor response was used to identify the predominant cell-types activated for anti-tumor cytotoxicity in mice immunized with the cytokine-secreting cells.

H. Statistical analysis

Student's t test was used to determine the statistical differences between the survival of mice in various experimental and control groups. A *P* value below 0.05 was considered significant.

III. Results

A. Simultaneous intracerebral injection of glioma and cytokine secreting allogeneic cells

We measured the survival of C57Bl/6 mice injected intracerebrally (i.c.) with a mixture of Gl261 glioma cells and cytokine secreting LM cells. Gl261 cells are a glioma cell-line of C57Bl/6 mouse origin (H-2^b). LM fibroblasts are derived from C3H/He mice and express H-2^k determinants. We initially evaluated the immunotherapeutic effects of single cytokine-secreting LM-IL-2 cells and double cytokine-secreting LM-IL-2/interferon- cells in mice bearing an i.c. glioma. A mixture of G1261 cells and the single or double cytokine-secreting cells were injected i.c. into the right frontal lobe of C57BL/6 mice, syngeneic with G1261 cells (**Figure 1**). Mice injected i.c. with the mixture of glioma and LM-IL-2 cells survived significantly longer (P<0.025) than control mice injected i.c. with an equivalent number of glioma cells alone. Somewhat more dramatic results were obtained for mice injected i.c. with a mixture of glioma cells and LM-IL-2/interferon- double cytokine-secreting cells. In addition, the survival of this group was statistically prolonged relative to either untreated mice with glioma or those animals injected with Gl261 cells and LM-IL-2 cells. The survival time of mice injected with a mixture of glioma cells and LM-Interferon- cells was not significantly different from that of mice injected with glioma cells alone (P>0.1). Of special interest, mice injected i.c. with an equivalent number of LM-IL-2 cells alone lived for more than three months and showed no evidence of ill effects or neurologic deficit. Immunocytotoxic studies demonstrated a significantly elevated cromium release from Gl261 cells co-incubated with spleen cells from mice injected i.c. with glioma cells and the cyotkine secreting fibroblasts (**Table 1**).

Thus, therapy with an immunogen that combined the expression of allogeneic antigens and the secretion of cytokines led to the most significant benefit in mice with an intracerebral glioma.

B. Specificity of the immune response

The specificity of the immunocytotoxic response was evaluated against a variety of tumor cell lines (**Table 2**). Only spleen cells from immunized animals demonstrated an immunocytotoxic response. The response, although somewhat non-specific when tested against a variety of tumor cell lines, was markedly enhanced when tested against the same tumor cells with which the animal was initially injected.

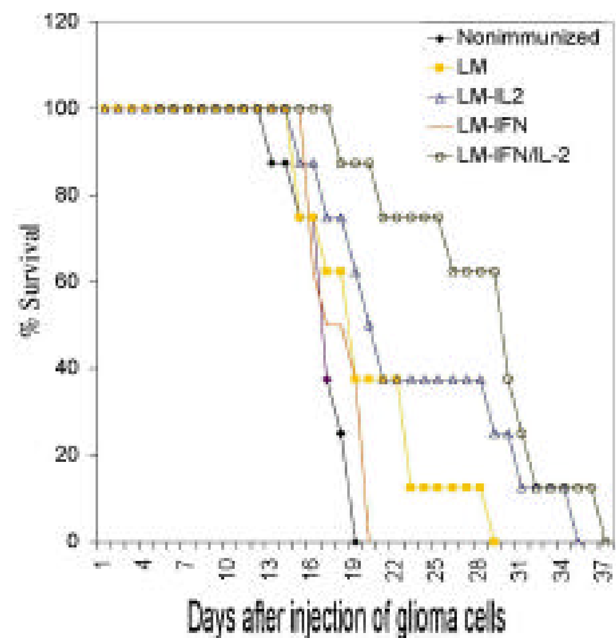

Figure 1. Graph showing the survival rate of mice injected i.c. with a mixture of glioma cells and fibroblasts (LM cells) engineered to secrete cytokines. The C57Bl/6 mice (8 per group) were injected i.c. with a mixture of 10^6 cells of one of the cell types and 10^5 Gl261 glioma cells. The median lengths 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-IL-2 cells, 23.4 ± 6.8; glioma plus LM-IFN- cells, 18.0 ± 1.8; glioma plus LM-IL-2/IFN- cells, 28.1 ± 5.8. Probability values were: nonimmunized vs. LM-IL-2, p < 0.025; nonimmunized or LM vs LM-IL-2/IFN- , p < 0.005; LM-IL-2 vs LM-IL-2/IFN- (, p < 0.05.

Table 1

The effect of mAbs against T cell subsets or NK/LAK cells on the anti-glioma cytotoxic activities of spleen cells from C57BL/6 mice injected i.c. with a mixture of glioma and the cytokine(s)-secreting cells

Cell-types for immunization[a]	Mab-treatment	% Cytolysis at E:T ratio of 100:1
Glioma	--	3.6 ± 1.2
	Anti-Lyt-2.2	-1.4 ± 2.5
	Anti-asialo GM1	-7.1 ± 2.8
Glioma + LM	--	5.8 ± 2.8
	Anti-Lyt-2.2	-1.9 ± 4.2
	Anti-asialo GM1	-7.8 ± 1.8
Glioma + LM-IL-2	--	17.7 ± 0.7[b,c]
	Anti-Lyt-2.2	5.2 ± 2.0
	Anti-asialo GM1	-6.6 ± 2.3
Glioma + LM-IL-2/IFN-γ	--	38.3 ± 4.4[c,d,e]
	Anti-Lyt-2.2	20.4 ± 11.9
	Anti-asialo GM1	-4.6 ± 0.8

[a]C57BL/6 mice received a single i.c. injection of (10^5) glioma cells 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 ^{51}Cr-release assay. All values represent the mean ± SD of triplicate determinations.

[b]$P < 0.005$ relative to ^{51}Cr release for spleen cells from animals immunized with glioma.

[c]$P < 0.05$ relative to ^{51}Cr release for spleen cells from animals immunized with glioma + LM cells.

[d]$P < 0.025$ relative to ^{51}Cr release for spleen cells from animals immunized with glioma.

[e]$P < 0.05$ relative to ^{51}Cr release for spleen cells from animals immunized with glioma + LM-IL-2 cells.

Table 2

Cytotoxicity toward various tumor cell-types in spleen cells from C57BL/6 mice injected i.c. with a mixture of glioma and the cytokine(s)-secreting cells

Cell type[a]	% cytolysis at E:T ratio of 100:1		
	Non-immunized	LM-IL-2	LM-IL-2/IFN-γ
Gl261	2.2 ± 2.9	44.6 ± 0.8	63.3 ± 7.2
B16F1	-0.2 ± 2.0	14.9 ± 1.2	15.4 ± 1.3
EL4	4.1 ± 1.3	46.3 ± 4.8	37.8 ± 1.5
LL/2	10.1 ± 1.0	19.3 ± 1.4	15.1 ± 1.4

[a]C57BL/6 mice received a single i.c. injection of (2.0 X 10^5) Gl261 glioma cells together with one of the modified fibroblast cell-types (10^6 cells). Two weeks after the injection, mononuclear cells from the spleens of the immunized mice obtained through Ficoll-Hypaque centrifugation were used for the ^{51}Cr-release assay using 4 different ^{51}Cr-labeled cell types as tumor targets including Gl261 glioma, B16F1 melanoma, EL-4 lymphoma and LL/2 Lewis lung carcinoma cells. All tumor cells are of C57Bl/6 origin (H-2^b haplotype). All values represent the mean ± SD of triplicate determinations.

C. Intracerebral survival and toxicity of the cytokine-secreting allogeneic cells

The toxicity of the allogeneic cell based cytokine gene therapy for tumors is likely to depend in part on the ability of the genetically modified cells to survive in the CNS. The intracerebral distribution and survival of the cytokine secreting cells was investigated using both allogeneic C57BL/6 and syngeneic C3H/He mice. As a means of assessing survival of the allogeneic cells in the CNS, PCR analysis was performed to identify the presence of the neomycin gene in the brain sections at various time intervals (2-60 days). In brief, high molecular weight DNA was isolated using techniques described previously (Gillis et al, 1978). PCR amplification of the DNA was subsequently performed in a reaction mixture consisting of 0.4 μM of primer for the Neo^r gene, 3-5 μl of the DNA samples, 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, and 2.0 U Taq polymerase (Gibco). The sequences of the *Neo* gene primers are as follows: 5' primer, 5'GCTGTGCTCGACGTTGTCAC3'; 3' primer, 5'CTCTTCGTCCAGATCATCCTG3'. The reactions were run for 38 cycles of 94°C (1 min), 55°C (1 min), 72°C (1 min) using a Perkin-Elmer Cetus thermal cycler. After amplification, 5 μl of the reaction mixture was removed and analyzed by electrophoresis in a 2.0% agarose gel. DNA sequences specific for the neomycin gene were found in DNA isolated from allogeneic mice on days 8, 14, but were no longer detected on days 28 and 60 (**Figure 2**). Similar experiments in syngeneic mice detected DNA sequences specific for the neomycin gene at 55 days. DNA sequences specific for the neomycin gene were not found in control mice injected with LM (non-cytokine secreting) fibroblasts (data not shown). Thus, modified allogeneic cells fail to survive in the CNS beyond 14 days as evidenced by PCR. The animals implanted with the genetically modified cells were observed daily for evidence of neurologic deficit and other morbidity or mortality for over 60 days, and at no time did the mice exhibit neurologic deficits or adverse effects on survival.

D. Evaluation of the therapeutic benefits of LM cells modified to secrete interleukin-2 in mice with an established pre-existing glioma

To determine if the cytokine secreting cells could be effective in treating a clinically relevant model of mice with an established glioma, naïve C57Bl/6 mice bearing cannulas were first injected with Gl261 glioma followed two days later with injection of either non-IL-2-secreting allogeneic LM fibroblasts or syngeneic/allogeneic LM-IL-$2/K^b$ cells. The animals received two more injections of the same type of cells as first injected through the cannulas at weekly intervals for a total of three injections. The animals with an established glioma treated with IL-2 secreting syngeneic/allogeneic fibroblasts survived significantly longer in comparision to either untreated animals ($P < 0.05$) or animals treated with allogeneic LM fibroblasts ($P < 0.025$) (**Figure 3**). This experiment was repeated one additional time with similar results.

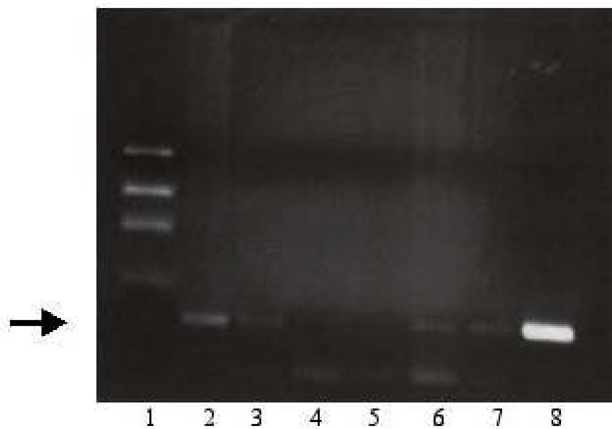

Figure 2. PCR anaylsis for the survival of modified fibroblasts in the CNS. PCR analysis was performed for the presence of the neomycin resistance gene in brain sections taken at various time intervals (0-60 days) after implantation of modified fibroblasts into the CNS in allogeneic and syngeneic mice. DNA sequences for the neomycin resistance gene were observed on Days 8 and 14 but not on Days 28 or 60 after implantation in allogeneic mice, and up to 55 days in syngeneic mice. Lane 1, low-mass molecular marker (Life Technologies); Lane 2, 8 days after injection into allogeneic mice; Lane 3, 14 days after injection into allogeneic mice; Lane 4, 28 days after injection into allogeneic mice; Lane 5, 60 days after injection into allogeneic mice; Lane 6, 55 days after injection into syngeneic mice; Lane 7, 10^3 LM-IL-2 cells; Lane 8, pZipNeo plasmid. *Arrow* indicates the location of the 249 – base pair Neo^r gene.

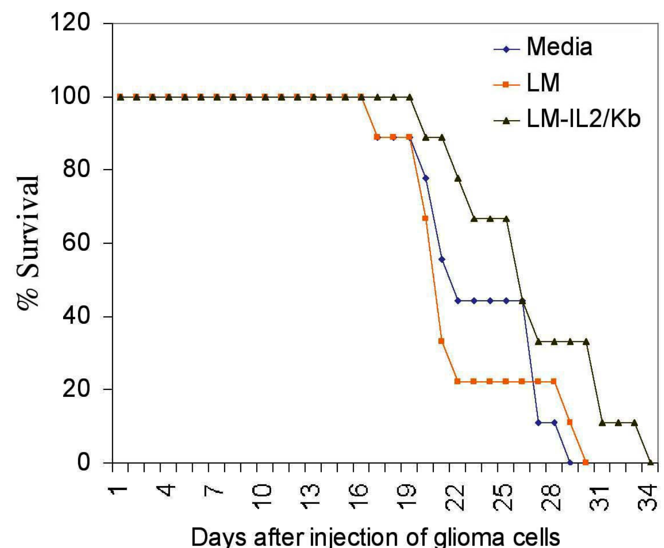

Figure 3. Treatment of an established glioma with IL-2 secreting cells. C57Bl/6 mice (nine animals/group) were injected i.c. through a cannula with 5.0 X 10^4 Gl261 cells followed two days later by the first of three weekly injections of 1.0 X 10^6 LM-IL-$2/K^b$ cells. As controls, animals received an equivalent number of tumor cells followed by treatment with either LM cells or media alone at the same time intervals as described previously. MST (days): media alone, 23.4 ± 4.1; LM, 22.3 ± 4.3; LM-IL-$2/K^b$, 26.7 ± 4.6. *P* values: media alone versus LM-IL-$2/K^b$, $P < 0.05$; LM versus LM-IL-$2/K^b$, $P < 0.025$.

E. Intracerebral versus subcutaneous immunization with allogeneic fibroblasts genetically engineered to secrete interleukin-2 in the treatment of central nervous system tumor

The purpose of this study was to determine the optimal route of delivery of gene therapy for an intracerebral tumor. Systemic delivery of gene therapy is of significant clinical interest. In this study, allogeneic fibroblasts engineered to secrete interleukin-2 were administered either subcutaneously (in the presence or absence of Gl261 cells) or intracerebrally to C57Bl/6 mice with intracerebral (i.c.) glioma. The results indicate a significant prolongation of survival in mice with i.c. glioma treated intracerebrally with LM-IL-2 cells, relative to the survival of mice with i.c. glioma treated subcutaneously with LM-IL-2 cells (either alone or mixed with Gl261 cells) or untreated mice with glioma ($P < 0.05$). The specific release of isotope from ^{51}Cr-labeled glioma cells co-incubated with spleen cells from animals treated either subcutaneously or intracerebrally with LM-IL-2 cells was significantly greater than the release of isotope from glioma cells co-incubated with spleen cells from nonimmunized mice ($P < 0.005$). Direct i.c. administration of fibroblasts genetically engineered to secrete IL-2 was more effective in prolonging survival than peripheral subcutaneous administration in the treatment of mice with i.c. glioma even though both treatments stimulated a strong antiglioma immune response (data not shown).

Similar studies were carried out using an intracerebral melanoma model to determine the possible immunotherapeutic benefits of IL-2 cells in mice with an intracerebral melanoma. In these studies B16F1 cells were stereotactically implanted into the right frontal lobes of C57BL/6 mice. The mice were treated with intracerebral (i.c.) or subcutaneous (s.c.) immunizations of allogeneic fibroblasts genetically engineered to express melanoma associated antigens and secrete IL-2 and/or gamma interferon. For controls, mice were injected i.c. with an equivalent number of B16 cells and treated with non IL-2-secreting RLBA-ZipNeo cells (MAA(+);IL-2(-)). The results indicate that the mice that were injected i.c. with B16 melanoma cells and RLBA-IL-2 cells survived significantly longer ($P<0.005$) than mice injected i.c. with B16 cells alone or with a mixture of B16 and RLBA-ZipNeo cells (**Figure 4**). Similar significant ($P<0.005$) therapeutic responses were observed in mice injected intracerebrally with a mixture of B16 cells and RLBA-IL-2/interferon- double cytokine-secreting cells. There was no increase in survival in the mice immunized subcutaneously with the cytokine secreting cells. Histopathological evaluation of tumors from treated and untreated mice was performed on all animals at the time of cromium release studies (2 weeks) and at the time of death (3-4 weeks). The most extensive lymphocytic infiltration was in mice treated with the IL-2 secreting cells.

Using a standard ^{51}Cr release assay, the specific release of isotope from labeled B16 cells co-incubated with spleen cells from mice immunized with RLBA-IL-2/interferon- cells either intracerebrally (**Table 3**) or subcutaneously (**Table 4)** was significantly higher than non-immunized mice ($p<.005$). In addition the cellular anti-melanoma response was mediated primarily by NK/LAK and $CD8^+$ cells.

In summary, we find a significantly increased survival time and specific immunocytotoxic responses in mice with CNS melanoma treated intracerebrally with allogeneicfibroblasts modified to secrete IL-2 and IFN- .

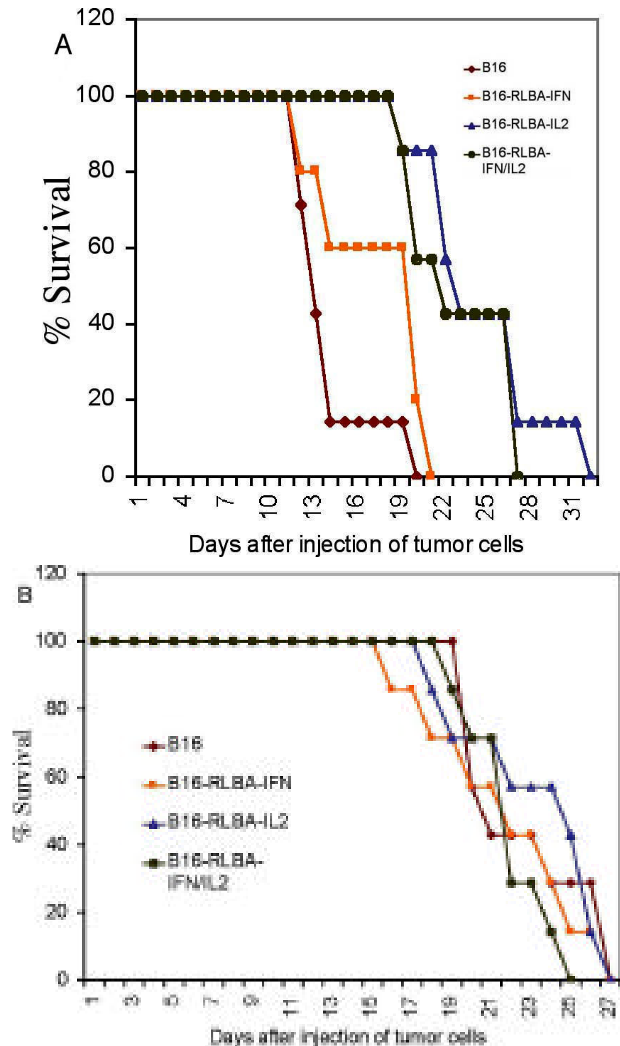

Figure 4. A. Graph showing the survival of mice injected intracerebrally with a mixture of B16F1 melanoma cells and RLBA-IL2 cells. C57Bl/6 mice were injected intracerebrally with a mixture of B16F1 melanoma cells (10^3) and one of the cell types (10^6). Mean survival times in days were as follows: B16 cells alone, 14.0 ± 2.6; B16 + RLBA-IFN- cells, 17.4 ± 3.7; B16 + RLBA-IL-2 cells, 24.6 ± 4.0; B16 +RLBA-IFN- /IL-2 cells, 23.1 ± 3.4. P Values: nonimmunized or RLBA-IFN- versus RLBA-IL-2, $P < 0.005$; nonimmunized or RLBA-IFN- versus RLBA-IFN- /IL-2, $P < 0.005$.**B**. Graph showing the survival of mice injected intracerebrally with B16F1 melanoma cells and subcutaneously with cytokine secreting cells. C57Bl/6 mice were injected intracerebrally with B16F1 cells (10^3) and subcutanously with one of the cell types (10^6 cells). Mean survival time (days): B16 cells alone, 22.7 ± 3.0; B16 + RLBA-IFN- , 21.7 ± 3.6; B16 + RLBA-IL-2, 23.3 ± 3.4; B16 + RLBA-IFN- /IL-2, 22.0 ± 1.9.

Table 3

The effect of CD8⁺ or NK/LAK mAbs on the cytotoxic activities of spleen cells from mice injected i.c. with a mixture of B16F1 melanoma and cytokine(s)-secreting cells

Cell-types injected[a]	Treatment	% Cytolysis[b]
B16F1	--	1.3 ± 0.5
	anti Lyt 2.2	-2.0 ± 1.0
	anti asialo GM1	-1.7 ± 0.6
B16F1 + RLBA-IFN-γ	--	6.6 ± 3.8
	anti Lyt 2.2	2.4 ± 4.6
	anti asialo GM1	4.8 ± 5.8
B16F1 + RLBA-IL-2	--	24.8 ± 3.3[c]
	anti Lyt 2.2	16.0 ± 3.2
	anti asialo GM1	6.5 ± 3.8
B16F1 + RLBA-IFN-γ/IL-2	--	26.0 ± 9.4[c]
	anti Lyt 2.2	10.9 ± 9.0
	anti asialo GM1	7.1 ± 2.0

[a]C57BL/6 mice received a single i.c. injection of a mixture of 10^3 melanoma cells together with one of the modified fibroblast cell-types (10^6 cells). Two weeks afterward, mononuclear cells from the spleens of the injected mice (Ficoll-Hypaque) were used for the ^{51}Cr-release assay. All values represent the mean ± SD of triplicate determinations.

[b]Toward ^{51}Cr-labeled B16F1 cells; E: T ratio = 100 : 1.

[c]$P < 0.005$ relative to ^{51}Cr-release for spleen cells from mice injected i.c. with B16F1 cells alone.

Table 4

The effect of anti CD8⁺ or NK/LAK mAbs on the cytotoxic activities toward B16F1 cells of spleen cells from C57BL/6 mice injected i.c. with B16F1 cells and s.c. with the cytokine(s)-secreting cells

Cell-types injected[a]	Treatment	% Cytolysis[b]
B16F1	--	4.0 ± 3.6
	anti Lyt 2.2	2.9 ± 0.3
	anti asialo GM1	-2.7 ± 3.4
B16F1 + RLBA-IFN-γ	--	17.1 ± 3.3
	anti Lyt 2.2	6.9 ± 1.5
	anti asialo GM1	25.7 ± 2.7
B16F1 + RLBA-IL-2	--	43.0 ± 1.5[c]
	anti Lyt 2.2	24.0 ± 4.3
	anti asialo GM1	11.3 ± 5.2
B16F1 + RLBA-IFN-γ/IL-2	--	54.3 ± 5.9
	anti Lyt 2.2	22.5 ± 1.5
	anti asialo GM1	17.4 ± 6.4

[a]C57BL/6 mice received a single i.c. injection of (10^3) B16F1 melanoma cells and a s.c. injection of one of the modified fibroblast cell-types (10^7 cells). Two weeks afterward, mononuclear cells from the spleens of the injected mice (Ficoll-Hypaque) were used for the ^{51}Cr-release assays. All values represent the mean ± SD of triplicate determinations.

[b]E : T ratio = 100 : 1.

[c]$P < 0.005$ relative to ^{51}Cr-release from B16F1 cells co-incubated with spleen cells from mice injected i.c. with B16F1 cells alone.
[d]$P < 0.0005$ relative to ^{51}Cr-release from B16F1 cells co-incubated with spleen cells from mice injected i.c. with B16F1 cells alone, and $P < 0.005$ versus ^{51}Cr-release from B16F1 cells co-incubated with spleen cells from mice injected i.c. with RLBA-IL-2 cells.

There was no increase in survival in animals treated subcutaneously, despite a significant systemic immunocytotoxic response.

F. Survival of C3H/He mice when injected i.c. with a mixture of intracerebral breast carcinoma and IL-2 secreting allogeneic fibroblasts

On the basis of previous experiments, 10^6 cytokine secreting cells were chosen as the treatment dose. Confirmation of IL-2 secretion by the LM-IL-2/K^b cells was detected by an enzyme-linked immunoadsorbent assay. Next C3H/He mice (eight mice/group) were injected i.c. with a mixture of 10^6 IL-2 secreting fibroblasts and 10^4 SB-5b breast carcinoma cells. LM fibroblasts which are syngeneic with C3H/He mice were modified to express H-2K^b class-I allogeneic MHC determinants (LM-K^b or LM-IL-2/K^b) to provide a potent immune adjuvant. The results indicated that the mean survival time of mice injected with the mixture of breast carcinoma cells and the LM-IL-2/K^b cells was significantly longer than mice injected i.c. with an equivalent number of breast carcinoma cells alone ($P < 0.01$), or mice injected i.c. with breast cancer cells and non-cytokine secreting LM-K^b fibroblasts ($P < 0.05$) (**Figure 5**). Thus, the presence of IL-2 secreting fibroblasts in the tumor bed prolonged survival in mice with intracerebral breast carcinoma.

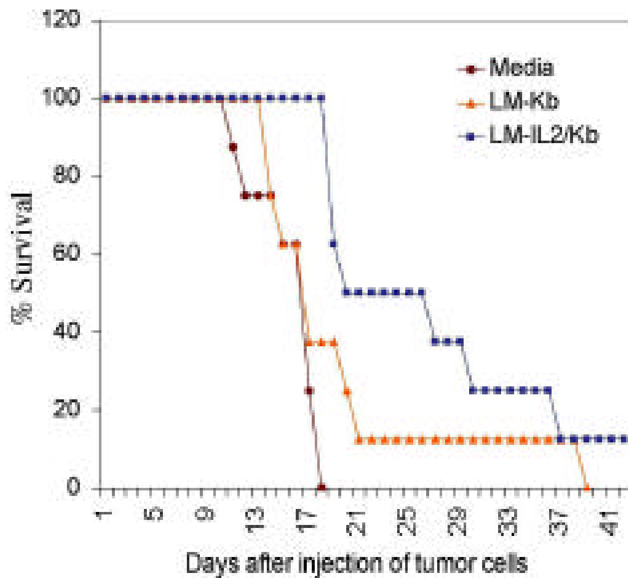

Figure 5. Treatment of C3H/He mice with intracerebral SB-5b breast carcinoma with LM-IL-2/K^b cells. C3H/He mice (eight animals/group) were injected with a mixture of 1.0 X 10^6 LM-IL-2/K^b cells and 1.0 X 10^4 SB-5b cells or, as controls, with an equivalent number of SB-5b cells and either 1.0 X 10^6 LM-K^b cells or media alone. MST (days): media alone, 15.6 ± 2.7; LM-K^b, 19.6 ± 8.2; LM-IL-2/K^b, 27.8 ± 11.5. *P* values: media alone versus LM-IL-2/K^b, $P < 0.01$; LM-K^b versus LM-IL-2/K^b, $P < 0.05$

G. Pretreatment of mice with allogeneic cytokine secreting cells prior to i.c. injection of tumor cells

We found previously that the survival of C57Bl/6 mice injected with Gl261 glioma cells mixed with allogeneic IL-2 secreting fibroblasts is significantly prolonged in comparision to various control groups. In previous studies, we also found that allogeneic LM-IL-2 fibroblasts modified to express H-2K^b determinants (syngeneic in C57Bl/6 mice) to form semiallogeneic LM-IL-2/K^b cells are more effective than IL-2-secreting fibroblasts that express allogeneic determinants alone in treating mice with Gl261 glioma. In order to investigate the mechanism involved in using these genetically engineered cells for treatment of an intracerebral tumor, cannulas were placed into the right frontal lobe of C57Bl/6 mice. The animals were subsequently injected two times at weekly intervals with LM-IL-2/K^b cells through the cannulas prior to injection of glioma cells. The tumor cells were mixed with the vaccine and introduced through the cannulas one week following the second injection. The results demonstrate a significant delay in the development of glioma ($P < 0.005$) in the animals treated with either non-secreting cells or IL-2-secreting syngeneic/allogeneic fibroblasts (**Figure 6**).

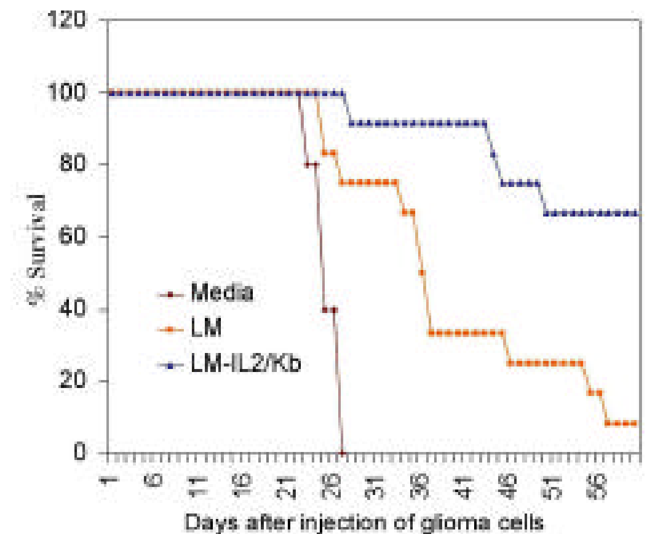

Figure 6. Pre-treatment with allogeneic fibroblasts prevents the development of a glioma. C57Bl/6 mice (twelve animals/group) were injected with 1.0 X 10^6 LM-IL-2/K^b cells through a cannula on two occasions separated by one week. One week following the second injection the animals were injected a third time with a mixture of 1.0 X 10^6 LM-IL-2/K^b cells and 5.0 X 10^4 Gl261 cells. As controls, animals were injected through the cannula with either 1.0 X 10^6 LM cells or media at the same time points along with an equivalent number of Gl261 cells at the time of the third injection. MST (days): media alone, 25.4 ± 1.6; LM, 39.6 ± 12.2; LM-IL-2/K^b, 53.9 ± 10.3. *P* values: media alone versus LM, $P < 0.005$; media alone versus LM-IL-2/K^b, $P < 0.0005$; LM versus LM-IL-2/K^b, $P < 0.005$.

Six animals in the IL-2 treated group that survived for over three months were then re-challenged with an intracerebral injection into the same site as the previous injections of 5 X 10^4 Gl261 glioma cells alone to determine if a long-term resistance toward glioma had been established in these animals.

The results demonstrated a significant prolongation of survival (P < 0.01) for those animals that had been previously injected with a mixture of tumor and LM-IL-2/K^b cells in comparison to the naïve animals injected with glioma cells alone (**Figure 7**). There were four long-term survivors (> 90 days) of the six total animals in the group previously treated with LM-IL2/K^b cells after receiving a second tumor challenge. These results suggest that a long-term immunity was established at the injection site in the animals that underwent multiple intracerebral injections of LM-IL-2/K^b cells prior to tumor injection. Whether or not a more generalized systemic immunity against glioma was established in these animals has not been determined.

IV. Discussion

The efficacy of active tumor immunotherapy with cytokine-transduced syngeneic or allogeneic fibroblasts has been reviewed in this paper. Intracerebral injections with IL-2 transduced allogeneic fibroblasts generated systemic anti-tumor immunity capable of eradicating brain tumors. In particular we constructed a cellular vaccine

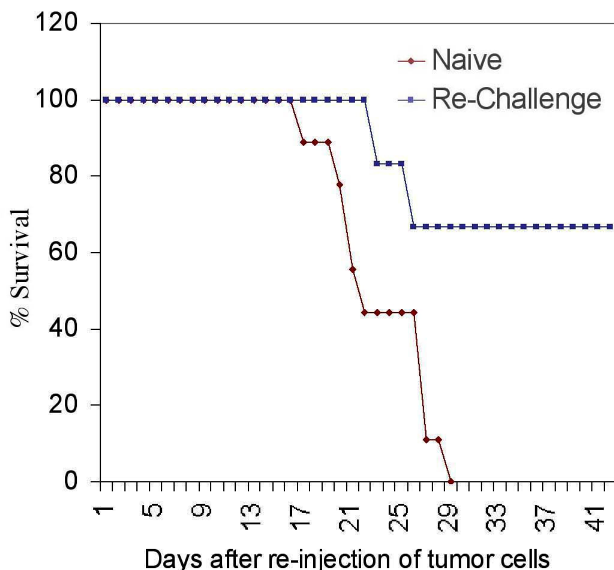

Figure 7. Long-term immunity in mice with glioma that survived prior treatment with IL-2 secreting allogeneic fibroblasts. Six C57Bl/6 mice surviving 90 days after prior injection of Gl261 cells and LM-IL-2/K^b fibroblasts were injected through the same right frontal burr hole a second time with 5.0 X 10^4 Gl261 cells alone. As a control, eight naïve C57Bl/6 mice were injected intracerebrally with an equivalent number of Gl261 cells alone. MST for the untreated naïve animals injected with tumor cells was 23.4 ± 4.1 days, and 36.2 ± 7.2 for the animals that had previously been vaccinated with LM-IL-2/K^b cells and re-challenged with tumor cells. The four animals that were still alive at the conclusion of this experiment all of which had previously been treated with LM-IL2/K^b cells survived for longer than 90 days without evidence of any neurologic deficit. $P < 0.01$ for the difference in survival of mice in the two groups.

with enhanced anti-tumor effectiveness by transducing LM cells, a mouse fibroblast cell-line expressing defined MHC-determinants (H-2^k), with a modified retroviral vector that specified the gene for IL-2. C57BL/6 mice (H-2^b) injected intracerebrally (i.c.) with a mixture of Gl261 glioma cells and LM cells (H-2^k) modified for IL-2 secretion (LM-IL-2) survived significantly longer than mice in various other treatment groups. The anti-tumor immune responses in the tumor-bearing mice were mediated predominantly by $CD8^+$ and NK/LAK cells. Of special interest, mice injected i.c. with the cytokine-secreting allogeneic cells alone exhibited no neurologic deficit and there were no adverse effects on survival. The injection of cytokine-secreting allogeneic cells into the microenvironment of an intracerebral tumor is hypothesized to induce an anti-tumor immune response capable of prolonging survival.

The toxic effects of cytokines in the CNS may limit the quantity that can be administered (Robinson et al, 1987; Birchfield et al, 1992; Kim et al, 1994). Neurologic effects have been seen in animals injected intracranially with syngeneic cytokine-secreting cells. The co-implantation into the rat brain of syngeneic (RG-2) glioma cells and RG-2 cells modified by retroviral transduction to secrete IL-2 or IFN- resulted in short-term cell mediated anti-glioma responses. However the survival of the tumor bearing rats was not prolonged, and the animals died from secondary effects including severe cerebral edema (Tjuvajev et al, 1995). The toxicity of a cellular-based cytokine gene therapy for tumors is likely to depend in part on the survival of the genetically modified cells in the CNS. We investigated the survival of an allogeneic IL-2 secreting vaccine in the CNS by two different means: PCR and bioassay (Griffitt et al, 1998). We found that the survival of allogeneic cells in the CNS was less than 28 days. The cells like other allografts were rejected. The cells were well tolerated, and the animals did not demonstrate any significant neurologic or systemic toxicity. This suggests that cytokine-secreting allogeneic cells may serve as a useful vehicle for the safe delivery of cytokines into brain tumors, and supports the possibility and safety of using a monthly retreatment schedule in a clinical protocol. Most of the systemic toxicities of IL-2 therapy should be avoided by the introduction of the gene for IL-2 directly into the tumor mass, resulting in primarily local concentrations of the cytokine. This form of treatment is particularly attractive in the treatment of primary gliomas, since these tumors usually only recur locally and are rarely metastatic.

More recently, the use of a small intracerebral cannula enables one to inject the treatment cells directly into the tumor bed on numerous occasions (Lichtor et al, 2002). This allows us to investigate both protective vaccine strategies using pretreatment via the cannula prior to tumor injection as well as the effect of the vaccine on the treatment of an established tumor. One of the major concerns related to the immunologic treatment of brain tumors is the effect of the blood brain barrier on the development of a host immune response in the CNS. Studies using IL-4 secreting plasmacytoma cells implanted into the brains of nude mice along with human glioma

cells demonstrated a dramatic eosinophilic infiltrate in regions of necrotic tumor, suggesting that an immune response can take place against a tumor of the central nervous system *in situ*. The response, however, was non T-cell dependent (Yu et al, 1993). We found that a specific and significant systemic immunocytotoxic response (by ^{51}cromium release assay) was present in animals with glioma treated with allogeneic IL-2 secreting fibroblasts (Glick et al, 1995; Lichtor et al, 1995). Thus the secretion of IL-2 by the cellular immunogen, or an immunogenic derivative of the cells, may have altered the blood brain barrier (BBB) enabling the immunogen to reach the spleen and lymph nodes in the periphery (Watts et al, 1989; Zhang et al, 1992).

Although preclinical studies with cytokine gene therapy appear promising (Sampson et al, 1997; Yu et al, 1997; Natsume et al, 1999; Giezeman-Smits et al, 2000, Okada et al, 2001; Lichtor et al, 2002), clinical trials for brain tumors have been limited. These trials have involved immunization with tumor cells modified with the IL-2 gene (Sobol et al, 1995), the IL-4 gene (Okada et al, 2000) or TGF- 2 antisense gene (Fakhrai et al, 2000). In summary, our studies suggest that Immuno-Gene therapy using IL-2 secreting fibroblasts as a cellular vaccine can be useful as a new therapeutic approach in treatment of a primary or metastatic intracerebral tumor especially when the tumor burden is small or at the time of tumor resection. The use of cytokine secreting tumor vaccines as a protective treatment introduced following tumor resection hopefully will play an important role in delaying tumor recurrence. We believe that this is where immunotherapy is most promising.

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