

Paracrine delivery of IL-12 against intracranial 9L gliosarcoma in rats

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Object. Interleukin-12 (IL-12) has potential for the treatment of tumors because it can stimulate an antitumor immune response and possesses antiangiogenic properties. In the study reported here, the authors investigated the therapeutic role of locally delivered IL-12 in a malignant brain tumor model.

Methods. After genetically engineering 9L gliosarcoma cells to express IL-12 (9L-IL12 cells), the authors used these cells as a source of locally delivered cytokine. First, they investigated the behavior of these cells, which were implanted with the aid of stereotactic guidance into the rat brain, by using serial magnetic resonance imaging and histopathological examination. Second, they assessed the antitumor efficacy of proliferating, as well as nonproliferating (irradiated), 9L-IL12 cells by implanting these cells in animals challenged by wild-type 9L gliosarcoma (9Lwt) cells. The IL-12 expression in brain regions injected with 9L-IL12 was confirmed by reverse transcription–polymerase chain reaction. Last, the authors explored whether animals treated with 9L-IL12 cells developed an antitumor immunological memory by rechallenging the survivors with a second injection of 9Lwt cells.

The authors demonstrated that local delivery of IL-12 into the rat brain by genetically engineered cells significantly prolongs survival time in animals challenged intracranially with a malignant glioma.

Conclusions. These findings support continued efforts to refine local delivery systems of IL-12 in an attempt to bring this therapy to clinical trials.

KEY WORDS • cytokine • interleukin-12 • glioma • immunotherapy • brain neoplasm • rat

MALIGNANT brain tumors are the most commonly diagnosed primary intracranial neoplasms, with 13,000 new cases reported each year in the United States.³⁴ Despite aggressive surgical resection and external-beam radiotherapy, the median length of survival in these patients is less than 1 year.³⁴ The addition of systemic chemotherapy may improve length of survival slightly, but its use is associated with significant systemic toxicity.² Thus, the long-term prognosis for patients with glioblastoma multiforme remains poor.¹⁴

This poor prognosis, despite current therapies for malignant gliomas, has stimulated the search for novel therapeutic modalities. Immunotherapy has shown consider-

able promise, especially since genes encoding for several potent stimulators of the immune system have been identified and cloned and have become available for study.

Cytokine-based immunotherapy is designed to trigger an effective antitumor immune response, with the ultimate goals of achieving complete regression of the initial neoplasm and developing long-term immunity against possible tumor regrowth. Among the cytokines that have been studied, IL-12 has been shown to possess potent antitumor properties^{4,5,7,15} because it plays a critical role in mediating inflammatory and immune responses in host defense. Interleukin-12 exerts a variety of biological effects on T cells and NK cells,^{19,40} including induction of IFN γ production,^{3,29} enhancement of proliferation and cytolytic function of T cells and NK cells,^{16,18,31} and promotion of the Th1-type immune response.²⁰ In addition to its stimulatory effects on the immune system, IL-12 is also a potent antiangiogenic factor.^{1,25,42,43}

A number of studies have demonstrated the therapeutic activity of IL-12 in murine tumor models after both local and systemic delivery.^{4,6} In the present study, we investigated the therapeutic role of locally delivered IL-12 in a

Abbreviations used in this paper: CTL = cytotoxic T lymphocyte; IFN γ = interferon- γ ; IL = interleukin; MR = magnetic resonance; mRNA = messenger RNA; = NK = natural killer; PBS = phosphate-buffered saline; RT-PCR = reverse transcription–polymerase chain reaction; TdT = terminal deoxynucleotidyl transferase; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling technique; 9Lwt = 9L wild-type.

malignant brain tumor model. Under normal physiological conditions, IL-12 is secreted at the antigen site by macrophages, B-cells, and microglia.^{4,6} We genetically engineered 9L gliosarcoma cells to express IL-12 (9L-IL12 cells) so that we could deliver this cytokine locally at the site of the tumor and, thus, more closely mimic its natural paracrine bioactivity.

We examined the following four issues: 1) whether tumor cells that were genetically transduced to produce IL-12 lose their tumorigenicity and, if so, is it due to a failure of the tumor cells to replicate, or to tumor regression after an initial period of growth; 2) whether tumor cells expressing IL-12 can stimulate an antitumor immune response against a lethal dose of wild-type tumor cells in the brain (we evaluated this antitumor activity against simultaneously injected wild-type cells and against established tumors [delayed treatment]); 3) whether IL-12-producing tumor cells made replication incompetent through irradiation could induce a similar antitumor response; and 4) whether local delivery of IL-12 against brain tumors could lead to a specific antitumor immunological memory capable of protecting cured animals from a subsequent tumor rechallenge that is fatal to naive control subjects.

Materials and Methods

Experimental Animals

Ten-week-old female Fischer 344 rats, each weighing 180 to 220 g, and 10-week-old male Fischer 344 rats, each weighing 220 to 250 g, were used as indicated in the experiments. These animals were housed in standard animal facilities with three or four rats per cage and given free access to rat chow and Baltimore City water.

Cell Lines

The 9Lwt gliosarcoma cell lines were maintained in tissue culture in Dulbecco's minimum essential medium with 10% fetal bovine serum, streptomycin (80.5 µg/ml), penicillin (base; 80.5 U/ml), and 1% L-glutamine.

The 9Lwt cells were transduced by two cycles of exposure in the presence of 8 µg/ml polybrene to an undiluted supernatant fraction of Am12mIL12N,¹³ which produced the polycistronic retroviral vector coding for both murine IL-12 subunits. At 48 hours after transfection, the cells were diluted and selected in 500 µg/ml of G418. Transduced 9L-IL12 cells were cultured and a single-cell clone producing approximately 500 ng of IL-12 per million cells in 48 hours was isolated by limiting the dilution in selective medium. The IL-12 concentration was determined by performing an enzyme-linked immunosorbent assay. The G418-resistant 9L cells infected with LXS vector alone (9Lneo cells) were used as a control. Cytokinetic studies did not show differences in the proliferation rate between transduced and parental wild-type cells.

To prepare irradiated 9L-IL12 cells immediately before injection, the cells were exposed to 41.34 Gy from a ¹³⁷Cs irradiator discharging 13.78 Gy/minute.

Anesthesia Induction

Before all surgical procedures and MR imaging studies, the animals were anesthetized with an intraperitoneal injection of a stock solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethyl alcohol in normal saline.

Surgical Procedures

The heads of the anesthetized rats were shaved and disinfected with a solution of 70% ethyl alcohol and povidone iodine, after which they were secured in a rodent stereotactic frame. A midline incision was made on the dorsal aspect of the head and the pericra-

nium was swept laterally to expose the bregma. A 1-mm drill hole was made exactly 1 mm anterior and 3 mm left lateral to the bregma. Cells were injected into the white matter at a depth of 4 mm through a 10-µl Hamilton syringe connected to the manipulating arm of the stereotactic device. All injections administered consisted of a total volume of 2 µl over a period of 6 minutes. The 26-gauge needle was retracted over a period of 5 minutes. The skin was closed with surgical clips, and the animals were returned to their cages and given free access to food and water.

On the 5th day after tumor cell inoculation, animals receiving delayed treatment were anesthetized as previously described and subjected to brain MR imaging to confirm the presence of tumor. Following MR imaging, animals were surgically prepared and placed on the stereotactic frame. The skin was reopened and animals were again injected, using the same coordinates and drill hole, with 2 µl of medium containing 4×10^4 9L-IL12 cells. Injections were made as previously described.

Magnetic Resonance Imaging of Tumor

Magnetic resonance imaging was performed using a nuclear spectrometer equipped with actively shielded gradients (internal diameter 15 cm). Rats were anesthetized and placed in a Helmholtz coil tuned to 200 MHz, which was built onto a 4-cm Plexiglas holder. All rats received 1 ml of gadolinium-diethylenetriamine pentaacetic acid (Magnevist) intraperitoneally 20 to 30 minutes before the examination. Spin-echo coronal images were obtained using the following parameters: 1000-msec repeat time, 16-msec echo time, 1-mm slice thickness, and a 64-mm field of view.

Histopathological Examination of Tumors

The brains of the animals that died spontaneously were removed and fixed in 10% formalin. Tissue was embedded in paraffin and 8-µm sections were stained with hematoxylin and eosin according to standard procedures. Brains from animals that were killed at specified times were removed, cut immediately, and embedded in tissue-freezing medium. Specimens were then snap frozen in a slurry of dry ice and isopentane and stored at -75°C. For immunohistochemical studies, paraffin-embedded specimens were sectioned at 4-µm widths, deparaffinized, and subjected to antigen retrieval by limited protein digestion. Slides were then incubated at room temperature with antibodies directed to the antigen TIA-1 (1:500). Antibodies were detected by the avidin-biotin complex method, with diaminobenzidine serving as chromogen.

Detection of Apoptosis

Apoptotic cells were visualized using a commercially available in situ detection kit; however, the manufacturer's recommended staining procedures were slightly modified. Briefly, after routine deparaffinization and rehydration and washing in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl), tissue was digested with proteinase K (20 µg/ml in PBS) for 15 minutes at room temperature and washed. Endogenous peroxidase activity of the sections was blocked by incubation in freshly prepared 3% H₂O₂ for 5 minutes at room temperature followed by washing with PBS. After adding equilibrium buffer for 10 seconds, TdT enzyme was pipetted onto the sections, which were incubated in a humidified chamber for 1 hour at 37°C. The reaction was stopped by adding wash buffer. The slides were subsequently incubated with antidigoxigenin-peroxidase for 30 minutes in a humidified chamber. After washing, the slides were stained with diaminobenzidine substrate and counterstained with methyl green solution. Substitution of TdT with distilled water was used as a negative control.

Interleukin-12 RT-PCR

Expression of mRNA by the transduced cells in vitro and in vivo after intracerebral implantation was assessed by means of RT-PCR. The RNA was isolated using reagent according to the method of Chomczynski and Sacchi.⁹ Briefly, 1 ml of Trizol was added to 50 to 100 mg of tissue or cell pellet and then homogenized. Chloroform was added and the aqueous layer was removed and transferred to a fresh tube. The RNA was precipitated using isopropyl alcohol and

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washed with 75% ethanol. Isolated RNA was redissolved in RNase-free H₂O in preparation for RT-PCR. The RNA (20 µl) was subjected to RT-PCR in the presence of 0.8 µM each of 5' and 3' primers, 25 µl of 2 × reaction mix, and 1 µl of *Taq* polymerase. The RT-PCR was performed in a thermal cycler in a step-down fashion for 35 cycles. Sense and antisense primers used for IL-12 PCR were 5'-GAACTGGCGTTGGAAGCA-3' and 5'-GTTCTTCAAAGGCTTCAT-3', respectively. The expected length of the PCR product was 119 bp. The sense and antisense primers used for β-actin PCR were 5'-ACTCCTACGTGCGCAGAG-3' and 5'-CAGGTC-CAGACGCAGGATGGC-3', respectively. Reaction products were separated using 3% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet lighting.

Statistical Analysis

The primary statistical outcome for these experiments was time to death measured from time of tumor implantation. Survival distributions were estimated using the product-limit method.²² Differences between survival distributions were assessed in two stages. First, an overall test of heterogeneity among treatment groups was performed using the log-rank test.²⁶ If this test rejected the groups, that is, if treatment differences were present, pairwise comparisons between the treatment groups and the controls were performed using the log-rank statistic. All probability values that are reported are two sided.

Experimental Design

Assessment of In Vivo IL-12 Expression. To assess continued expression of murine IL-12 mRNA after the intracerebral implant of 9L-IL12 cells, two groups of seven rats each underwent left putamen stereotactically guided implantation of a mixture of 10⁴ 9Lwt cells and either 4 × 10⁴ replicating 9L-IL12 or 2 × 10⁶ irradiated 9L-IL12. Three animals from each group were killed on Day 5, three on Day 7, and one on Day 12 postimplantation. An additional group of three animals (negative controls) received only 10⁴ 9Lwt cells and were killed on Day 5 postimplantation. Brains from animals that were killed at scheduled times were removed and each hemisphere was sectioned into 1-mm slices. Each section was snap frozen by using dry ice and isopentane and stored at -75°C. Total RNA was isolated from each section and subjected to RT-PCR by using primers specific for murine IL-12 (subunit p40) and β-actin. Reaction products were separated by agarose gel electrophoresis. The RNA isolated from 9L-IL12 cells served as a positive control. Negative controls included RNA isolated from 9Lwt cells and 9Lneo cells. Additional negative controls included RNA isolated from brains of rats injected with 9Lwt cells or from the contralateral hemisphere of animals injected with 9L-IL12.

Experiment 1. Ten rats were divided into two groups of five. One group (controls) received an intracerebral stereotactically guided injection of 4 × 10⁴ 9Lwt cells. The other group was injected with the same number of 9L gliosarcoma cells genetically engineered to release IL-12 (9L-IL12 cells). To investigate the biological events that occurred after injection, this group of animals was observed using serial brain MR imaging. Animals in both groups were assessed daily for signs of neurological deterioration and their length of survival was recorded. Autopsy was performed in all animals to determine the cause of death.

Experiment 2. In a second experiment, 31 rats were divided into three groups. Animals in the first group (10 rats, controls) received a stereotactically guided implantation of 10⁴ 9Lwt gliosarcoma cells. Animals in the second group (10 rats, simultaneous treatment) was intracranially injected with a mixture of 10⁴ 9Lwt and 4 × 10⁴ 9L-IL12 cells. Animals in the third group (11 rats, delayed treatment) received stereotactically guided injections of 10⁴ 9Lwt cells on Day 0 and were reinjected at the same site with 4 × 10⁴ 9L-IL12 cells on Day 5. Before the delayed injection, these animals underwent brain MR imaging to confirm the presence of a well-established tumor. These images revealed a small, rounded, contrast-enhancing lesion at the site of the injection in all animals. Histological evaluation of the brain of one of these animals killed immediately after the MR imaging session demonstrated a small

area of tumor cells corresponding precisely to the lesion identified on the MR image.

At the beginning of the experiment, two of the animals in the second group were chosen for histological evaluation. These animals were killed on Day 14 and Day 34 postinjection, and their brains were prepared for hematoxylin and eosin staining.

Animals in the second and third groups that survived the initial tumor challenge (four rats) were challenged again by injection of 10⁴ 9Lwt cells into the contralateral hemisphere 112 days after the initial tumor injection. Their length of survival was compared with that of 10 naive control animals.

Experiment 3. In a third experiment 40 animals were divided into five groups. Group 1 animals (eight control rats) received a stereotactically guided injection of 10⁴ 9Lwt cells. Group 2 animals (eight 9Lneo control rats) received an injection of 10⁴ 9Lwt cells and 4 × 10⁶ irradiated 9L gliosarcoma cells transduced with the neomycin resistance gene. This group served as an additional control to ensure that the transduction process itself did not confer any therapeutic benefit. The irradiation rendered the cells replication incompetent. Groups 3, 4, and 5 animals (eight rats in each group) were injected intracranially with 10⁴ 9Lwt cells together with 10⁶, 2 × 10⁶, and 4 × 10⁶ irradiated 9L-IL12 cells, respectively. All animals were assessed daily for signs of neurological deterioration and their length of survival was recorded.

Sources of Supplies and Equipment

The Fischer 344 rats were purchased from Charles River Laboratories (Wilmington, MA) and the 9Lwt gliosarcoma cell lines were kindly provided by Dr. K. Plate, Department of Neuropathology, Freiburg University (Freiburg, Germany).

The Dulbecco's minimum essential medium, fetal bovine serum, streptomycin, penicillin, 1% L-glutamine, and G418 were purchased from Gibco Laboratories (Grand Island, NY); the Trizol reagent, reaction mix, *Taq* polymerase, and SUPERSRIPT One Step RT-PCR system were purchased from Gibco (Gaithersburg, MD). Proteinase K (in PBS) was obtained from Sigma Chemical Co. (St. Louis, MO).

The InterTest-12X mouse IL-12 p70 enzyme-linked immunosorbent assay kit was purchased from Genzyme Corp. (Cambridge, MA). The ¹³⁷Cs Gammacel 1000 irradiator (model no. 62) was obtained from Nordion International (Kanata, Ontario, Canada). David Kopf Instruments (Tujunga, CA) manufactured the rodent stereotactic frame.

Magnetic resonance imaging was performed using an Omega 4.7-tesla C.S.I. spectrometer, manufactured by General Electric Medical Systems (Milwaukee, WI), which was equipped with Accustar actively shielded gradients obtained from General Electric (Fremont, CA). Images were obtained using Magnevist contrast medium, available from Berlex Laboratories (Wayne, NJ). Tissue freezing medium, obtained from Triangle Biomedical Sciences (Durham, NC), was used for histopathological examination. Antibodies directed to TIA-1 were obtained from Coulter Immunology (Miami, FL).

Apoptotic cells were visualized using the Apotag in situ detection kit manufactured by Intergen (Purchase, NY). The RT-PCR was performed in a PCR Sprint thermal cycler, which was manufactured by Hybaid (Ashford, Middlesex, UK).

Results

In Vivo Localized IL-12 Expression

The 119-bp IL-12 (subunit p40) reaction product was detected only in the left hemispheric sections corresponding to the region of brain that received both replicating and nonreplicating 9L-IL12 cells (Fig. 1). In both cases, murine IL-12 mRNA expression was detected up to Day 12 after the intracerebral implantation.

Experiment 1: Determination of Tumorigenicity of Intracranially Implanted 9L-IL12 Cells. All animals in the

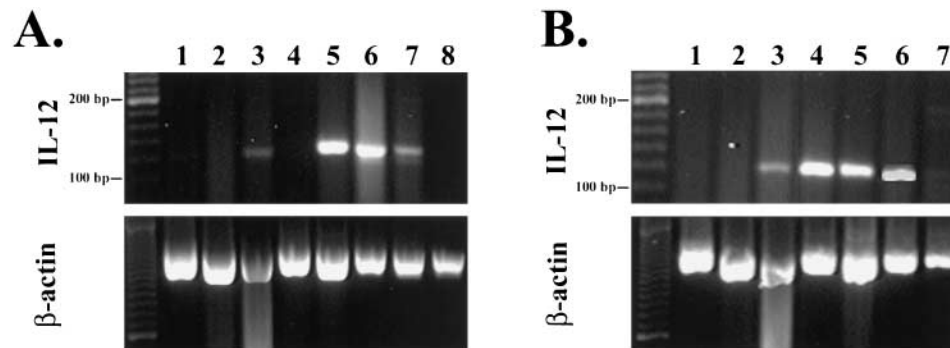


FIG. 1. A: Example of RT-PCR gels showing localized expression *in vivo* of murine IL-12 mRNA in rat brains injected with 4×10^4 replicating 9L-IL12 cells and from rats killed on Day 5 (lane 5), Day 7 (lane 6), and Day 12 (lane 7) postimplantation. Positive controls consisted of RNA isolated from 9L-IL12 cells (lane 3). Negative controls consisted of RNA samples isolated from 9Lneo cells (lane 1) and 9Lwt cells (lane 2). Additional negative controls were RNA samples isolated from the injection site of animals injected with 4×10^4 9Lwt cells (lane 4) and from the contralateral hemisphere of animals injected with 9L-IL12 cells (lane 8). B: Example of RT-PCR gels showing localized expression *in vivo* of murine IL-12 mRNA in rat brains injected with 2×10^6 irradiated 9L-IL12 cells and from rats killed on Day 5 (lane 4), Day 7 (lane 5), and Day 12 (lane 6) postimplantation. Positive controls consisted of RNA samples isolated from 9L-IL12 cells (lane 3). Negative controls consisted of RNA samples isolated from 9Lneo cells (lane 1) and 9Lwt cells (lane 2). Additional negative controls were RNA isolated from the contralateral hemisphere of animals injected with 9L-IL12 cells (lane 7).

control group died, with a median survival time of 21 days (range 18–30 days). Only one of the five animals injected with 9L-IL12 died as a result of tumor growth; this occurred 63 days after the injection (Fig. 2). An MR image obtained in this animal on Day 44 postimplantation demonstrated an enhancing lesion at the level of the left putamen. Postmortem histological examination revealed a large tumor with typical gliosarcoma features, focal regions of necrosis, and the nuclear fragmentation and condensation that is commonly seen in late stages of apoptosis.

The four remaining rats in the 9L-IL12 group survived for more than 2 months. Serial MR imaging was performed at different intervals from the initial injection in

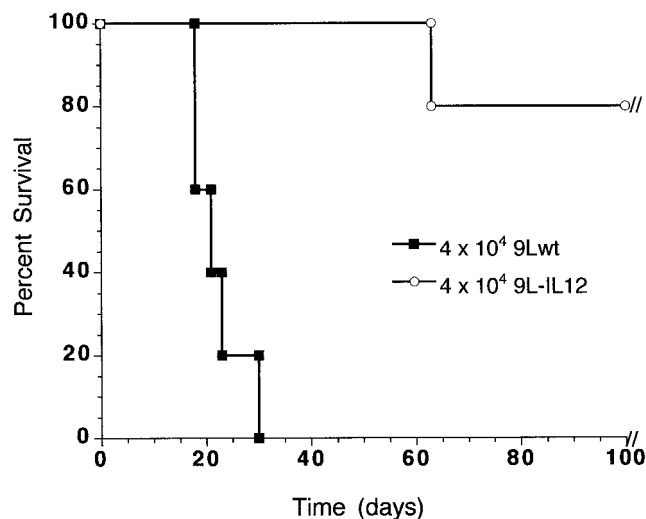


FIG. 2. Kaplan-Meier curves showing length of survival of animals receiving 4×10^4 9L-IL12 cells (five rats) compared with that of animals receiving 4×10^4 9Lwt cells (five rats).

these four animals. In one rat a large lesion developed, which was detected by MR imaging on postimplantation Day 44 as a contrast-enhancing area with a mass effect and midline shift. A repeated MR image obtained in the same animal on Day 79 showed complete involution of the contrast-enhancing lesion with a small, round, residual hypointense area on T_1 -weighted images with no contrast enhancement. There was no longer any mass effect or midline shift (Fig. 3).

In two of the remaining rats, MR images obtained on postimplantation Day 101 revealed a large contrast-enhancing area at the site of the injection. One of these animals died soon after the MR imaging procedure as a result of an overdose of an anesthetic agent, and the brain was harvested for histopathological examination (Fig. 4). Staining with hematoxylin and eosin showed a large tumor situated in the left putamen, with wide areas of necrosis, cells showing nuclear fragmentation, and mononuclear inflammatory infiltrate that was predominantly lymphocytic (Fig. 5A and C). An *in situ* TUNEL assay demonstrated intense nuclear staining of apoptotic cells (Fig. 5B). A subset of lymphocytes also showed immunoreactivity for TIA-1, an antigen expressed on cytolytic granules of NK cells and CTLs (Fig. 5D). The other animal underwent successive MR imaging sessions, results of which revealed regression of the lesion and its mass effect (Fig. 6).

The final rat in the 9L-IL12 group also underwent serial MR imaging sessions starting 44 days after tumor injection. Results from these sessions did not reveal any obvious tumor—only a thin linear hypointensity that likely represented the track of the injection needle.

Experiment 2: Determination of Antitumor Effect of Implanted 9L-IL12 Cells. All animals injected with only 9Lwt cells (control group) died, with a median survival time of 25 days (range 23–37 days). Rats injected with a mixture of 9Lwt and 9L-IL12 cells (simultaneous treatment) had a significantly prolonged survival (median 53.5

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days, range 25–112 days; log-rank test, $p = 0.0003$), with 25% long-term survivors. Rats bearing established tumors, which were treated with an intratumoral injection of 9L-IL12 cells (delayed treatment), also had significant prolongation of survival time (median 51.1 days, range 23–112 days; log-rank test, $p = 0.0018$) compared with controls, with 20% long-term survivors. There was no significant difference between animals treated with simultaneous injection of 9L-IL12 cells and animals treated with 9L-IL12 cells 5 days after tumor implantation (Fig. 7). Moreover, none of the treated animals showed signs of toxicity.

Animals surviving their initial tumor implantation were again challenged by injection of tumor cells into the contralateral hemisphere on Day 112. Half of these animals rejected this second challenge, which proved to be fatal in all naive control animals.

Experiment 3: Determination of Antitumor Effects of Implanted Irradiated 9L-IL12 Cells. All animals in the control group and the irradiated 9Lneo group died, with a median survival of 23 days (ranges 19–31 and 21–27 days, respectively). Animals receiving irradiated 9L-IL12 cells showed a statistically significant increase in survival time. Animals receiving 10^6 irradiated 9L-IL12 had a median survival time of 36 days (range 20–40 days; log-rank test, $p = 0.0155$). Those receiving 2×10^6 irradiated 9L-IL12 cells had a median survival time of 32 days (range 31–35 days; log-rank test, $p = 0.0004$). Those receiving 4×10^6 irradiated 9L-IL12 cells had a median length of survival of 37 days (range 31–40 days; log-rank test, $p = 0.0002$) (Fig. 8). None of the treated animals showed any signs of central nervous system or systemic toxicity.

Discussion

The identification and cloning of genes encoding cytokines has enabled us to learn about these immune response modifiers, and this has opened the door to their use for cancer therapy. Aimed at triggering an effective immune response against tumor, cytokine-based immunotherapy is also exciting because it can potentially induce a long-lasting antitumor immunological memory. Given the impressive degree of immune suppression seen in many patients with malignant glioma, strategies aimed at augmenting the immune response may be especially valuable in these individuals.³⁵ Over the past decade, several cytokines, including IL-2, IL-4, tumor necrosis factor- α , granulocyte/macrophage colony-stimulating factor, and IFN γ have been investigated for their potential use in cancer therapy. At present, IL-2 is being used in human clinical trials for the treatment of a variety of cancers, including gliomas.²⁷ The expression of various cytokine receptors by human glioma cells supports the notion that these immune response modifiers may play a promising role in the therapy of glial neoplasms.³⁸

Recently, a newly identified cytokine^{23,37,44} called IL-12 has been characterized. Not only does it appear to be an important element in the immune system, but also a potentially powerful antitumor cytokine.^{4,5,7,15} Treatment with IL-12 has been shown to be efficacious in a variety of experimentally induced tumor models.^{4,5,29,30,33,46} By enhancing the cytolytic activity of both NK and CTLs,^{16,23} induc-

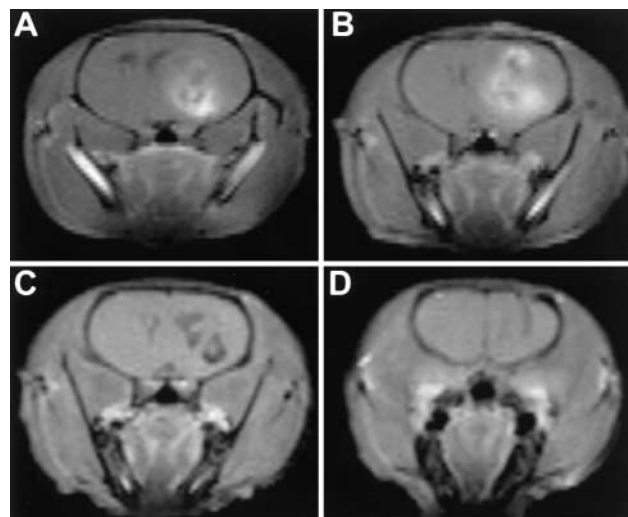


FIG. 3. A and B: Gadolinium-enhanced T₁-weighted coronal MR images of a rat brain obtained 44 days after implantation of 4×10^4 9L-IL12 cells. Note the large enhancing mass that is causing significant midline shift. C and D: Gadolinium-enhanced T₁-weighted coronal MR images obtained 79 days after implantation of 4×10^4 9L-IL12 cells into the same rat brain. Note that the mass has regressed. There is no longer any contrast enhancement and the midline shift has resolved.

ing the production of IFN γ and favoring the Th1 response over Th2,^{8,23} IL-12 appears to be particularly well suited to counteract the actions of immunosuppressive agents such as transforming growth factor- β , prostaglandin E-2, IL-10, and insulin-like growth factor-I, which are produced or induced by gliomas. Despite a number of studies demonstrating the antitumor activity of IL-12 in systemic murine tumor models, no observations have been reported regarding the possible role of this cytokine in orthotopic glioma models. Jean, et al.,²¹ have demonstrated the effectiveness of continuous systemic infusion of IL-12 in the presence of irradiated 9L gliosarcoma cells by inducing regression of 9L tumors implanted subcutaneously. In the present study, we investigated the effect of locally delivered IL-12 against this same tumor cell line orthotopically implanted in the rat brain.

Interleukin-12 has been shown to possess antitumor activity following either systemic or local administration.^{4,6} Under physiological circumstances, however, IL-12 is secreted by professional antigen-presenting cells (such as macrophages, B cells, and microglia) local to the site of antigen.⁴⁰ Systemic administration of IL-12 at pharmacological doses, therefore, is likely to produce unusually high IL-12 concentrations at sites distant from a tumor and relatively lower levels in tissues at the tumor site. This distribution problem following systemic administration is exacerbated in the case of brain tumors because of the poor penetration of proteins through the blood-brain barrier. It should also be noted that adverse side effects have been reported with systemic administration of IL-12 in mice, primates, and humans.^{10,17,36,39} In contrast, interstitial cytokine delivery can provide high concentrations of IL-12 at the site of a tumor, while greatly reducing systemic toxicity. Thus, although IL-12 has shown potent antitumor ac-

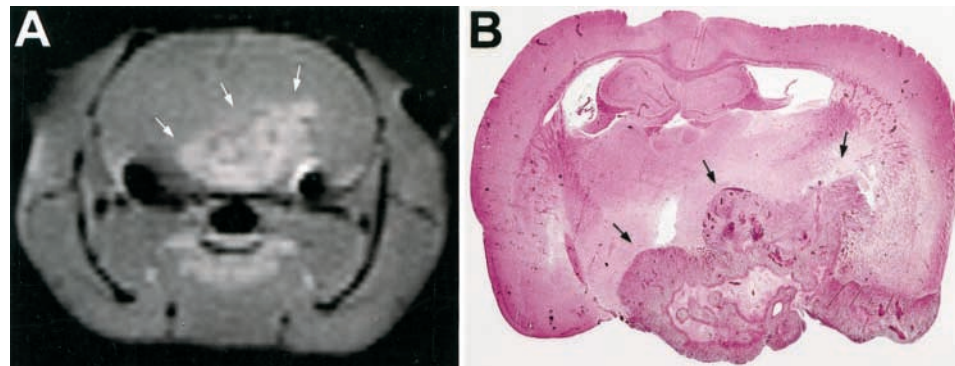


FIG. 4. A: Gadolinium-enhanced T₁-weighted coronal MR image of a rat brain obtained 101 days after implantation of 4×10^4 9L-IL12 cells. Note the large enhancing mass in the left putamen. B: Histopathological specimen (coronal section) obtained from the same animal. H & E, original magnification $\times 2$. Note the precise correlation between the radiological image and the pathological specimen (indicated by arrows).

tivity after both systemic and local administration, in an attempt to mimic its natural paracrine bioactivity more closely, we have developed a way to deliver IL-12 locally within the brain by implanting genetically transduced tumor cells engineered to secrete IL-12.

We first evaluated whether tumor cells genetically modified to express IL-12 maintain their oncogenic potential when implanted into the brain. When cells engineered to release a cytokine are implanted in an animal, the cytokine-triggered antitumor action may occur as a succession

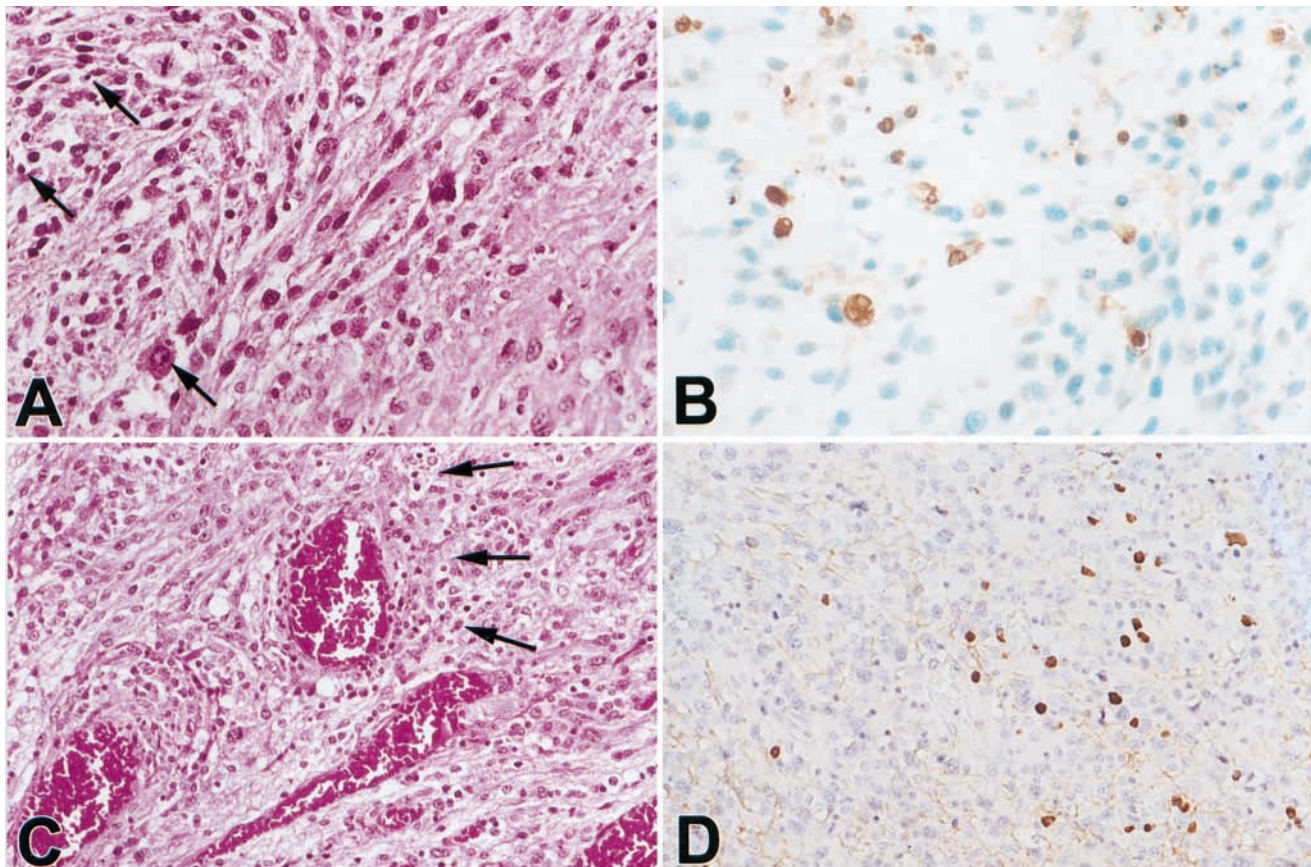


FIG. 5. Photomicrographs of the lesion noted in Fig. 4. A: Nuclear condensation and fragmentation (arrows) consistent with apoptosis. H & E, original magnification $\times 400$. B: Results of TUNEL assay with methyl green nuclear counterstaining demonstrating intense nuclear staining of apoptotic tumor cells. Original magnification $\times 400$. C: Lymphocytic infiltrate (arrows). H & E, original magnification $\times 250$. D: Immunohistochemical staining for TIA-1, an antigen expressed on cytolytic granules of NK cells and CTLs, revealing the presence of these cell types. Original magnification $\times 250$.

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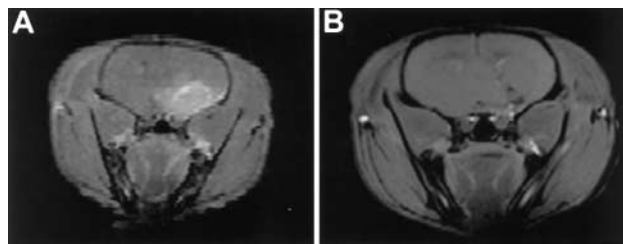


FIG. 6. A: Gadolinium-enhanced T_1 -weighted coronal MR image of a rat brain obtained 101 days after implantation of 4×10^4 9L-IL12 cells. Note the large enhancing mass in the left putamen. B: Subsequent gadolinium-enhanced T_1 -weighted coronal MR image of the same rat's brain showing complete regression of the tumor.

of phases.¹¹ First, there is an initial phase of tumor growth. Then the increasing production of cytokine leads to an inflammatory reaction based on the recruitment of large numbers of leukocytes. This is followed by a debulking phase. A final phase signals the end of the reaction with reparative phenomena. By monitoring our animals with MR images obtained after the intracranial injection of 9L-IL12 cells, we found a similar succession of phases, including initial growth followed by shrinkage and disappearance of the tumor. These observations demonstrate that, when implanted into the brain, 9L cells engineered to release IL-12 lose the ability possessed by their wild-type counterparts to kill the animals bearing them. This phenomenon appears to be the consequence of an inflammatory reaction against the tumor, which is likely induced by the increasing production of cytokine from an increasing number of proliferating cells, rather than the result of a simple inability of the transduced cells to replicate. It has

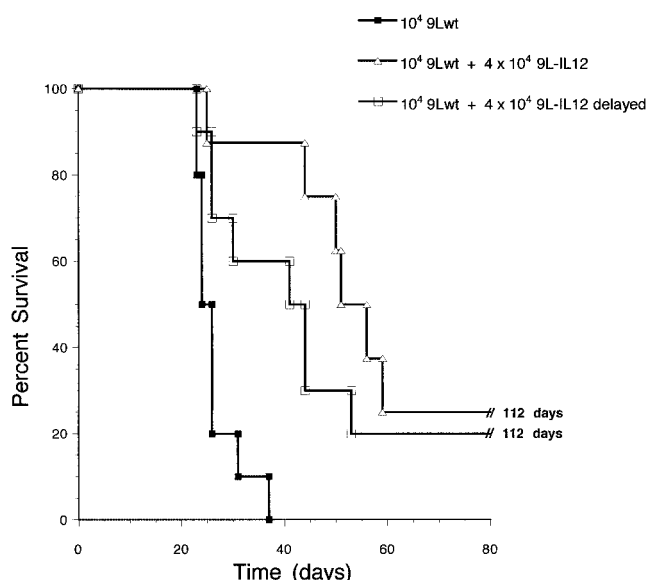


FIG. 7. Kaplan-Meier curves showing survival times of animals receiving 10^4 9Lwt cells simultaneously with 4×10^4 9L-IL12 cells (open triangles, eight rats) or 10^4 9Lwt cells and a delayed injection of 4×10^4 9L-IL12 cells (open squares, 10 rats), compared with controls receiving only 10^4 9Lwt cells (solid squares, 10 rats).

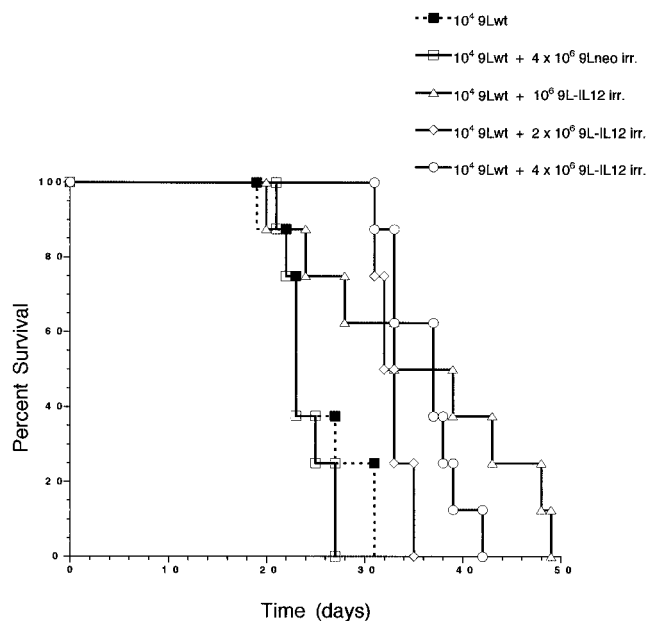


FIG. 8. Kaplan-Meier curves showing survival times of animals receiving 10^4 9Lwt cells alone (solid squares, eight rats) or in combination with 4×10^6 irradiated 9Lneo cells (open squares, eight rats), 10^6 irradiated 9L-IL12 cells (open triangles, eight rats), 2×10^6 irradiated 9L-IL12 cells (open diamonds, eight rats), or 4×10^6 irradiated 9L-IL12 cells (open circles, eight rats).

been reported that inflammatory cells infiltrating the tumor and subsequent T-cell recruitment are responsible for the debulking phase, the efficacy of which depends on the amount and type of cytokine released, the tumor histological type, and the host tissue.^{12,28} Our histopathological findings suggest a role for NK cells and $CD8^+$ lymphocytes that is consistent with previous reports (see Fig. 5B and C).^{5,33}

We also considered whether the intracranial injection of 9L cells engineered to release IL-12 could improve the length of survival in animals injected with wild-type tumor cells. We examined this issue by using both replicating and nonreplicating 9L-IL12 cells because nonproliferating cells would be more suitable for translation into clinical settings. Our results showed a significant increase in the length of survival in animals that received either replicating or nonreplicating 9L-IL12 cells simultaneously with the wild-type tumor challenge. Previous studies have indicated that the amount of IL-12 expressed locally at the tumor site correlates with the degree of tumor regression.^{13,24} These observations could explain the better results obtained in animals treated with replicating cells, compared with groups receiving irradiated—replication-incompetent—cells. It is reasonable, indeed, to hypothesize that proliferating cells provide a prolonged and escalating production of cytokine.

The ability of any therapy to affect the growth of established tumors is crucial. It has been previously demonstrated that IL-12-based treatment can be effective in a variety of established tumor models.^{5,15,30,45} Our findings corroborate these observations, demonstrating that locally delivered IL-12 can be effective in treating animals bearing established intracranial 9L gliosarcoma. Note that pro-

longation of survival in our animals was similar whether the IL-12 treatment was simultaneous with tumor implantation or delayed by 5 days. Other authors have reported a more striking antitumor effect when IL-12 was used against established tumors than when used against incipient tumors,^{32,41} and this effect has been correlated with the number of infiltrating T and NK cells in the region of the tumor.

The final issue addressed in this study is that of long-term antitumor immunological memory. Long-term survivors from each of the experiments were rechallenged with wild-type tumor cells. Half of these animals survived the rechallenge, suggesting that they had developed a specific immunological memory against the tumor. It is difficult to explain why 50% of the animals did not survive the rechallenge. Interestingly, the animals that did not reject the tumor were those that received 9L-IL12 cells simultaneously with the initial tumor challenge. This finding would support the hypothesis that a more rapid disappearance of the incipient tumor, due in this case to an early administration of the cytokine to a relatively small number of tumor cells, could result in an insufficient loading of antigen-presenting cells and a consequent lack of memory induction.¹¹

Conclusions

In our study we examined the therapeutic efficacy of IL-12 against an orthotopically implanted brain tumor. Systemic toxicity and poor penetration across the blood-brain barrier have placed significant limitations on the systemic delivery of cytokines for brain tumor therapy. Therefore, we used tumor cells transduced with genes encoding for IL-12 as a vehicle for local cytokine delivery. In our experiments, IL-12 was shown to have potent antitumor activity and to induce immunological memory against intracranial 9L gliosarcoma. Further experiments to elucidate the mechanisms of IL-12 activity and continued refinement of the local delivery system may bring IL-12 closer to clinical trials against malignant brain tumors.

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