Eradication of Rat Malignant Gliomas by Retroviral-mediated, in Vivo Delivery of the Interleukin 4 Gene

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ABSTRACT

Overexpression of interleukin 4 (IL-4) can impair the tumorigenicity of glioma cells, but direct evidence of its antitumor efficacy after in vivo gene transfer into malignant gliomas has not been provided. To test this, we first injected into the brain of Sprague Dawley rats a 1:1 mixture of C6 rat glioblastoma cells and E86.L4SN20 or E86.L4SN50 retroviral producer cells (RPCs), secreting 20 and 50 ng of IL-4/5 \times 10^7 cells/48 h, respectively. Twenty-seven and 56% of rats receiving injections with these low- or medium-level IL-4 RPCs, respectively, survived tumor injection, whereas control rats died in about 1 month. E86.L4SN50 RPCs coinjected with 9L gliosarcoma cells into syngeneic Fischer 344 rats yielded similar results. A novel IL-4 RPC clone expressing higher levels of IL-4, E86.L4SN50, coinjected with 9L cells increased to 75% the fraction of long-term survivors and induced tumor regression in 50% of rats when injected into established 9L gliosarcomas. Cured rats developed an immunological memory because they rejected a challenge of wild-type 9L cells into the contralateral hemisphere. Magnetic resonance imaging was used to monitor 9L and C6 gliomas and gave direct evidence for tumor rejection in treated rats. Immunohistology showed inflammatory infiltrates in IL-4-treated tumors in which CD8+ T lymphocytes were more abundant, although CD4+ T lymphocytes, B lymphocytes, and macrophages were also present. Overall, these findings suggest that IL-4 gene transfer is a new, promising approach for treating malignant gliomas.

INTRODUCTION

The growth of malignant gliomas takes advantage of an immune-suppressive environment (1). Glioma patients are often characterized by a broad depression of both humoral and cell-mediated immunities, and particularly of T-cell functions (2, 3). These observations encourage the testing of immunotherapy approaches that may up-regulate the immune system and create an immunostimulatory tumor microenvironment.

The identification of the brain as a site of immunological privilege could be somehow contradictory with such approaches. However, the definition of this privilege has been challenged recently by the evidence that tight connections exist between the CNS and the immune system through cervical lymphatics and the blood-brain barrier (4). It has been demonstrated that both the afferent arm (i.e., the presentation of CNS-derived antigens to the immune system and the activation of lymphocytes in the lymphoid organs) and the efferent arm (the presentation of CNS-derived antigens to the immune system and the activation of the immune system through the expression of specific adhesion molecules (4, 5). A redefinition of the CNS immune privilege should now include selective immunoregulatory events that down-regulate cell-mediated immunity and enhance noninflammatory humoral responses (4, 6).

Although infiltrating mononuclear cells can be found in malignant gliomas, their function can be impaired by tumor-derived immuno-suppressive factors like TGF-β (7), IL-10 (8), and Fas ligand (9). Strategies that may overcome immunosuppression should therefore be considered for improving the treatment of gliomas. In this context, we decided to test the therapeutic potential of IL-4 gene transfer. IL-4 is a cytokine with a strong antitumor effect that involves, at the early stages, nonspecific effector cells, such as eosinophils and macrophages, and, later on, T-helper lymphocytes that stimulate a humoral response (10, 11). In mixed tumor transplantation assays, the growth of U-87 glioblastomas was inhibited if J588L plasmacytoma cells engineered to express high levels of IL-4 were coinjected into nude mice (12). The intracranial and s.c. growth of the more malignant C6 glioblastoma could also be limited in nude mice by retroviral and adenoviral-mediated transfer of the IL-4 gene (13, 14). Nevertheless, all IL-4-treated mice injected intracranially were dead by day 55 after tumor injection, likely because of the lack of the late T-cell response. When glioma cells were transplanted in vitro by the IL-4 gene and injected into the brain of immunocompetent rats or mice, ~70% of the animals survived for at least 70 days (15, 16). In these experiments, however, no gene transfer was taking place in vivo, and all tumor cells were overexpressing IL-4 at the moment of the injection, two factors that decrease the significance of these observations in view of the definition of gene therapy strategies for malignant gliomas.

In initial experiments, we found that coinjection of glioblastoma and RPCs transducing IL-4 into rat brain is followed by survival after 3 months (long-term survival) of a relatively low percentage of rats (23%; Ref. 17). Furthermore, data based on IL-4 transduction in vivo by defective herpes simplex viruses showed 0% survival at the same time point (18). In this report, we show that injection of RPCs producing increasing amounts of IL-4 raises strikingly the fraction of glioma survivors. Our results also suggest that high levels of IL-4 during the initial phases of tumor development are critical for tumor rejection and that CD8+ T-lymphocytes are important mediators of the antitumor function of IL-4.

MATERIALS AND METHODS

In Vivo Experiments. C6 cells were obtained from the American Type Culture Collection and were cultured as suggested by this supplier. 9L cells (a kind gift of Dr. K. Plate, Freiburg University, Department of Neuropathology, Freiburg, Germany) and the mouse T-cell line HT-2 were cultured in RPMI supplemented with 10% FCS.

The RPC line PA317.STK.SBA (here defined as SBA), expressing the HSV-8 gene, was obtained and characterized previously (19). The RPC line \( \Psi L2 \) was also described previously (11). This and other RPC cell lines were named according to the amount of IL-4 that they produced. \( \Psi L2 \) produced 20 ng of IL-4/5 \times 10^7 cells/48 h and were named \( \Psi L2 \) L4SN50. To prepare E86.L4SN RPCs, the murine IL-4 cDNA was cloned in the EcoRI site of the retroviral vector L4SN. 2 These authors contributed equally to this study.
site of a pLXSN plasmid and transfected by lipofection (DOTAP; Boehringer Mannheim) in GP+E86 ecotropic packaging cells (obtained from Dr. A. Bank, Columbia University, New York, NY). After G418 selection (0.5 mg/ml of active drug), several clones were isolated and tested for retroviral titer as described previously (19) and for IL-4 production by an ELISA-based assay (Genzyme). Two clones were used in this study. Clone 6 has a titer of $3 \times 10^5$ cfu/ml, produces $50 \text{ ng of IL-4/5 \times 10^3 cells/48 h}$, and was named E86.L4SN$^{50}$. Clone 31 had a titer of $9 \times 10^7$ cfu/ml and produced $200 \text{ ng of IL-4/5 \times 10^3 cells/48 h}$ and was therefore named E86.L4SN$^{200}$.

For growth rate analysis, $10^3$ C6 or 9L cells were plated in 96-well plates in the presence of different dilutions of conditioned medium from $10^5$ wild-type or IL-4-transduced NIH-3T3 cells (secreting $150 \text{ ng of IL-4/48 h}$). Cell proliferation was measured after 24, 72, and 96 h by WST-1 colorimetric assay (Boehringer Mannheim), according to the manufacturer’s instructions. Statistical analysis was performed by paired t test.

IL-4 receptor expression was evaluated by reverse transcription-PCR. cDNA synthesized using random hexamers (Boehringer Mannheim) was amplified with primers IL-4R-1F 5'-TGTTCTCTCTGACTACCTCGC-3' and IL-4R-1R 5' -TGGAGTGTGAGGTTGTCTGG-3' with 30 cycles of PCR consisting of $30^\circ$ at 94°C, $30^\circ$ at 58°C, and $30^\circ$ at 72°C.

In experiments of intratumoral RPC injection, $4 - 6$ used to seal the burr-hole and avoid the spreading of neoplastic cells outside coordinates for 9L: 1 mm posterior, 2.5 mm left, 6 mm depth). Bone wax was into the left striatum with a Hamilton syringe for 4 min (coordinates for C6, Dawley and 5.5, 6.0, and 6.5 mm for Fischer rats). For tumor challenge animals were placed in a stereotactic frame, and 4 into the right hemisphere with 4

Experiments. In Vivo Experiments. Sprague Dawley or Fischer 344 rats (females, 225–250 g or 160–180 g, respectively; Charles River Italia) were anesthetized before intracerebral implantation using 4% chloral hydrate (1 ml/100 g) after a brief exposure to 1% fluorane or ether. In coinjection experiments, animals were placed in a stereotactic frame, and 4 $\times 10^6$ C6 or 6 $\times 10^6$ 9L cells mixed or not with SBA or $\text{2.L4SN}^{50}$ or E86.L4SN cells (1:1 ratio for most experiments, 10:1 ratio in one case) in 2–3 $\mu$l of PBS were inoculated into the left striatum with a Hamilton syringe for 4 min (coordinates for C6, with respect to bregma: 1 mm anterior, 3 mm left lateral, 5 mm depth; coordinates for 9L: 1 mm posterior, 2.5 mm left, 6 mm depth). Bone wax was used to seal the burr-hole and avoid the spreading of neoplastic cells outside the needle track. In experiments of intratumoral RPC injection, 4–6 $\times 10^6$ glioma cells were implanted as described above. For 4 or 7 days later, $1.6 \times 10^6$ E86.L4SN cells in 8 $\mu$l of PBS were inoculated using the same stereotactic coordinates but at three different depths (4.5, 5.0, and 5.5 mm for Sprague Dawley and 5.5, 6.0, and 6.5 mm for Fischer rats). For tumor challenge experiments, rats treated with IL-4 and surviving over 3 months were injected into the right hemisphere with 4 $\times 10^6$ C6 or 6 $\times 10^6$ 9L cells.

To measure the levels of murine IL-4 secreted in vivo, brains from rats injected with 9L cells mixed with SBA or E86.L4SN$^{50}$ cells and sacrificed after 1, 2, and 3 weeks were homogenized on ice in 3 ml of PBS in a glass potter. The suspension was then centrifuged 10 min at 2000 rpm, 4°C, and the supernatants were stored at $-80^\circ$C. IL-4 levels in the supernatants were measured by an ELISA assay (Biosource), and the amounts of total proteins were determined by Lowry method.

Evaluation of Intracerebral Tumor Growth. MRI was performed using a 1.5 T equipment with a 10-cm surface coil. Coronal sequences (Vol 2 mm-T1-FFE, Vol 2 mm T2-TSE) were obtained using the following parameters: the number signal average, matrix, and field of view were, respectively, 4, 256 $\times$ 220, and 220 for T1 sequences and 6, 512 $\times$ 52, and 110 for T2 sequences. Contrast medium (0.3 ml of gadolinium diethylenetriaminepenta-acetic acid) was injected i.p.

For histological analysis, rat brains were either frozen in isopentane or fixed in Carnoy and embedded in Paraplast. In both cases, sections were routinely

stained with H&E. To identify subsets of inflammatory cells, immunohistochemical evaluations were performed on frozen or fixed sections using monoclonal antibodies directed against rat macrophages (antibody ED2; Biosource; 1:250 dilution), CD4 (antibody W3/25; Biosource; 1:100 dilution), CD8 (antibody MRC OX-8; Biosource; 1:100 dilution), B cells (antibody anti-CD45RA, clone OX-33; PharMingen, 1:100 dilution), and NK cells (antibody anti-NK-R-P1, clone 3.2.3; Endogen; 1:100 dilution).

Statistical analysis was performed by the log-rank (Mantel-Cox) test in Kaplan-Meyer nonparametric analysis for survival, using the StatView 4.5 software.

RESULTS

To assess the therapeutic potential of retroviral-mediated transfer of the IL-4 gene, we inserted the murine IL-4 cDNA in plasmid pLXSN and transfected GP+E86 packaging cells with this construct. Several clones were obtained from this transfection, and for the initial experiments, we used clone E86.L4SN$^{50}$. This clone has a titer of $3 \times 10^7$ cfu/ml and secretes $50 \text{ ng IL-4/5 \times 10^3 cells/48 h}$. The titer was evaluated by transduction of NIH-3T3 cells, and transduced cells produced $150 \text{ ng of IL-4/1 \times 10^3 cells/48 h}$.

We compared the efficacy of E86.L4SN$^{50}$ cells with that of a previously tested, less efficient IL-4 RPC, producing $5 \times 10^5$ cfu/ml and secreting 20 ng of IL-4/5 $\times 10^3$ cells/48 h ($\text{2.L4SN}^{50}$ cells). Coinjection of C6 and $\text{2.L4SN}^{50}$ cells in the brain of Sprague Dawley rats (1:1 ratio; $4 \times 10^5$ cells for each cell type) increased rat survival significantly ($P = 0.0034$ versus controls, log-rank test; Fig. 1), and long-term survivors (i.e., animals surviving >90 days) were 27%. The survival of rats inoculated with C6+E86.L4SN$^{50}$ cells, however, was higher, and 56% of these rats remained alive more than 3 months ($P = 0.0003$ versus controls; Fig. 1). Raising to 10:1 the ratio of C6 to E86.L4SN$^{50}$ infected cells did not decrease the fraction of long-term survivors (data not shown). Furthermore, the injection of $1.6 \times 10^6$ E86.L4SN$^{50}$ cells into established C6 glioblastomas (i.e., tumors formed by the inoculation of $4 \times 10^4$ C6 cells 1 week before) was followed by tumor rejection in 40% of the animals ($P = 0.0207$ versus controls;Fig. 1). A fraction of these animals was examined by MRI and had radio-
logical evidence for complete tumor regression. One example is shown in Fig. 2A. MRI allowed a close follow-up of tumor growth in the presence or absence of IL-4 RPC. In general, tumor growth was detected during the first 3–4 weeks after tumor graft and was followed by the death of the animals in untreated controls or by tumor regression in many IL-4-treated rats. To characterize the immune effecter cells involved in tumor rejection, some animals were sacrificed during the regression phase, usually 40–50 days after the injection of C6 and E86.L4SN50 RPC. Fig. 2B shows an example of MRI sequences taken 22 and 50 days after injection of C6 and IL-4 RPCs. The large, bilobulated lesion enhanced by gadolinium in the MRI scan taken at day 22 is decreased at day 50, suggesting the presence of an antitumor reaction. This animal was sacrificed 2 days later, and the histological analysis showed large inflammatory infiltrates in the vicinity of residual neoplastic cells (Fig. 3C). Similar observations were made in other rats sacrificed 6–7 weeks after tumor injection (Fig. 3, A, B, and D). The immunohistochemical analysis demonstrated that these infiltrates were mostly constituted by CD4+ and CD8+ T-lymphocytes, B-lymphocytes, and macrophages (not shown).

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C6 cells are allogeneic to Sprague Dawley, thus favoring spontaneous tumor rejection (20). In fact, 6 of 33 controls injected with C6 tumors were tumor free at the moment of the sacrifice, a result consistent with those from other groups (21, 22). To evaluate the effects of IL-4 gene transfer in a syngeneic model, we performed an additional series of experiments using 9L gliosarcoma cells and Fischer 344 inbred rats. With this system, the amount of spontaneous rejections of brain tumors was considerably lower (1 of 55 controls tested, 1.8%), although 9L cells are considerably immunogenic (23). We injected inbred 344 Fisher rats with 6 × 10⁴ 9L gliosarcoma mixed or not with equal amounts of E86.L4SN50 cells or of PA317.STK.SBA (SBA; Ref. 19), a control RPC used to test nonspecific immunogenic effects of retroviral transduction. PA317.STK.SBA RPC transduce the “suicide” HSV-tk gene and were used in the absence of ganciclovir. Five of 12 rats inoculated with 9L+E86.L4SN50 RPCs were long-term survivors, whereas 11 of 12 rats injected by 9L cells and 13 of 13 injected by 9L and PA317.STK.SBA cells died by day 46 (Fig. 4A; 9L versus 9L+SBA was not significant, P = 0.4643; 9L+ E86.L4SN50 versus 9L, P = 0.0007; 9L+ E86.L4SN50 versus 9L+SBA, P = 0.0227).

To test further the effects of IL-4 concentration on tumor growth, Fischer 344 rats were injected with 9L cells mixed with E86.L4SN200, a new RPC clone with titer of the same order of magnitude as E86.L4SN50 but producing four times the amount of IL-4. Three months after coinjection of 9L and E86.L4SN200 cells, 75% of the rats survived (Fig. 4A; 9L+E86.L4SN200 versus 9L, P = 0.0001; E86.L4SN200 versus 9L+SBA, P < 0.0001; E86.L4SN50 versus E86.L4SN200 was not significant, P = 0.1126). This confirms, together with data obtained in C6 tumors (see Fig. 1), the correlation between the fraction of rats cured by the treatment, the amount of IL-4 due to the production by packaging cells, and the efficiency of transduction of the IL-4 gene. Although a direct evaluation of the concentration of IL-4 in the tumor microenvironment was not performed, the amount of IL-4 levels in brain homogenates of rats injected with 9L admixed with SBA cells or E86.L4SN200 cells was measured 1, 2, and 3 weeks after cell grafting. These preliminary data showed that IL-4 levels in brains of rats injected with 9L and E86.L4SN200 cells were 20, 25, and 7 times higher than in controls (average values of two rats for each time point). On the contrary, no relevant variation of IL-4 was found in 9L/SBA controls at any time point (5.8 ± 3.4 pg of IL-4/mg of protein, n = 5).

Fig. 2. MRI of rat C6 glioblastomas transduced in vivo by the IL-4 gene (T1-w. i. on a coronal plane after injection of gadolinium). A, on the left, a deep enhancing lesion (arrowhead) is recognizable 48 days after surgery. On the right, 120 days after surgery the lesion disappeared. Signal alterations and contrast enhancement are not visible anymore. The hypointensity in the left hemisphere (small arrow) is related to the needle track, reactive gliosis, and organization processes. B, on the left, a large tumor with a deep necrotic portion (two arrowheads) is present 22 days after surgery. On the right, 50 days after surgery the tumor regression is visible, and only a small deep nodule of contrast enhancement is still recognizable (small arrow).
The effects of the inoculation of RPCs transducing IL-4 into established gliomas was also investigated in the 9L system. Using the same stereotactic coordinates, Fischer 344 rats were injected with E86.L4SN200 or SBA cells (as a control) 4 days after the brain injection of 9L cells. This experiment confirmed that the animals injected by IL-4 RPCs survived significantly longer than the controls (Fig. 4B) and that a relevant fraction of them (50% in this experiment) can survive tumor injection.

In several animals, the packaging cells injected into the established gliomas had been incubated with BrdUrd 2 days before surgery. Immunohistochemistry with anti-BrdUrd antibodies was performed at different time points (1, 5, 11, 14, 18, and 45 days after surgery), and the results indicated that packaging cells were present, usually at the boundary of the tumor, until 18 days after surgery. This observation is in agreement with results reported by Ram et al. (24).

To evaluate whether IL-4 long-term survivors had developed an immunological memory that could reject a challenge with wild-type tumors, $6 \times 10^4$ 9L cells were injected into the contralateral hemisphere of seven rats 3 months after the first injection of tumor cells. All of these animals survived the challenge. In a similar experiment on 10 long-term survivors to C6 glioblastomas, 8 animals survived the C6 challenge in the right hemisphere. Surprisingly, one of the two succumbing animals died because of a relapse of the first tumor in the left hemisphere.

Brains injected with 9L tumors were also analyzed by MRI and histological techniques. Of controls and treated rats dying spontaneously, all showed a very large malignant glioma compressing the remaining part of the brain. In treated animals sacrificed at different time points, but not in controls, inflammatory infiltrates, mostly constituted by lymphocytes, were detected. In general, these infiltrates appeared earlier (i.e., during the first 4 weeks after injection) and were larger in rat brains injected with E86.L4SN200 cells than in brains injected by $\Psi$2.L4SN20 and E86.L4SN50 cells. Furthermore, these, but not the infiltrates associated with $\Psi$2.L4SN20 and E86.L4SN50 cells, showed that CD8+ T-lymphocytes were more numerous than CD4+ and B lymphocytes. NK cells and eosinophils were almost
with the same number of PA317.STK.SBA (SBA) RPCs (5P5 versus 9L P5).

versus 9L P5. The control tumor is clearly larger, suggesting comparison in Fig. 6.

A MRI picture of this tumor, 3 days before the histological analysis, is shown in Fig. 6. The control tumor in Fig. 6A. The control tumor is clearly larger, suggesting that inflammatory cells give a major contribution to tumor shrinkage.

That tumor rejection is mostly due to the action of specific and nonspecific inflammatory cells is also confirmed by in vitro experiments performed to test whether IL-4 may affect per se the proliferation of glioma cells. IL-4 receptors are expressed by astrocytes and by astrocytic tumors and may mediate negative growth signaling by IL-4 (27, 28). This negative effect on proliferation, however, seems only present in a subgroup of glioblastomas (28, 29). Immunohistochemistry and Western blotting with polyclonal antibodies failed to identify the α chain of the IL-4R in 9L and C6 cells (rat spleen and mouse HT-2 cells were the positive controls). Reverse transcription-PCR, however, gave a positive result (not shown), suggesting that the expression level of this receptor is rather low in both of these glioma cell lines. We subsequently evaluated whether the interaction of IL-4 with these receptors had some effect on proliferation. C6 and 9L cells were overlayed with different dilutions of the medium of NIH-3T3 cells transduced by the IL-4 gene and producing 150 ng of IL-4/1 × 10⁷ cells/48 h. As a control, we used the medium of untransduced NIH-3T3 cells. Under these conditions, IL-4 did not affect the proliferation of the tumor cells, and similar results were obtained by adding recombinant murine IL-4 to the culture medium in concentrations ranging from 10 pg to 100 ng/ml (data not shown).

**DISCUSSION**

Glioblastomas are very aggressive tumors characterized by the infiltrative capacity, the high proliferation rate, and the strong stimulation of neoangiogenesis. Progress in neurosurgery, radiotherapy, and chemotherapy did not modify significantly their prognosis (30). Gene therapy approaches based on transfer of “suicide” genes have been encouraging at the preclinical level, and clinical trials have been started (31, 32). Other gene therapy approaches have been based on the transfer of wild-type copies of tumor suppressor genes inactivated during the malignant progression of glioblastomas (33).

All of these strategies are hampered by the difficulty of targeting the majority of neoplastic cells and, particularly, the infiltrating cells, because in vivo transduction with defective viral vectors relies mostly on contiguity between viral producer cells or viral particles and the tumor cells. The search for new, nondefective vectors that may transfer genetic information more efficiently, maintaining the selectivity for neoplastic cells, is very active and includes the creation of new retroviral vectors with engineered envelope proteins or of replicase-competent attenuated derivatives of herpes simplex virus (34, 35).

Gene therapy protocols based on the possibility of raising a strong immune response against the tumor have the potential to generate a therapeutic response affecting all glioblastoma cells without the need for a generalized transduction of neoplastic cells. In this perspective, we have developed a series of experiments to test the effects of IL-4 gene transfer on rat brain malignant gliomas.

We have first demonstrated that the coinjection of glioma cells with IL-4 RPCs causes a significant prolongation of survival with respect to controls and the virtual cure of a sizeable fraction of treated animals. Our previous results indicated that 23% of rats with C6 tumors coinjected with E86.L4SN²⁰⁰ cells, whose viral titer and IL-4 production are, respectively, 5 × 10⁷ ctu/ml and 20 ng/5 × 10⁵ cells/48 h, survived >3 months (17), a finding now confirmed on a larger number of animals (27% on 18 rats; Fig. 1). Coinjection of 9L or C6 cells with E86.L4SN²⁰⁰ cells, characterized by a viral titer of 3 × 10⁷ ctu/ml and by a release of 50 ng of IL-4/5 × 10⁵ cells/48 h, brought to 42–56% the percentage of long-term survivors. The injection of 9L and E86.L4SN²⁰⁰ cells (similar titer as E86.L4SN²⁰⁰ but 200 ng of IL-4/5 × 10⁴ cells/48 h) raised to 75% such percentage.

These findings indicate a direct correlation between amounts of IL-4 at the tumor site and rat survival. In particular, the different

undetectable. This is an intriguing finding, because the presence of eosinophilic granulocytes was strongly associated with antitumor action of IL-4 (25). In IL-4-treated gliomas, eosinophils were detected in some cases (12, 13, 15) but not in others (Ref. 18 and this work). This may reflect variations in the amount of IL-4 (high levels may be initially necessary to recruit a large number of eosinophils) and/or variations in the presence of key factors in the brain, such as IL-5 or eotaxin, necessary to attract these cells. Recent data, however, suggest that the growth suppression of IL-4-secreting tumors is not dependent on the presence of the eosinophils (26).

An example is shown in Fig. 5, where the inflammatory cells were detected 23 days after injection of 9L and E86.L4SN²⁰⁰ cells. The MRI picture of this tumor, 3 days before the histological analysis, is shown in Fig. 6. The MRI of a control tumor at day 19 is shown for comparison in Fig. 6A. The control tumor is clearly larger, suggesting that inflammatory cells give a major contribution to tumor shrinkage.

Fig. 4. Survival analysis of Fischer 344 rats with 9L brain tumors in the presence of IL-4 RPCs. A, survival of rats injected with 6 × 10⁵ 9L either alone (C; n = 12) or mixed with the same number of PA317.STK.SBA (SBA) RPCs (D; n = 12) or E86.L4SN²⁰⁰ RPCs (O; n = 12). Statistical analysis (log-rank test): 9L versus 9L + SBA was not significant (P = 0.4643); 9L versus 9L + E86.L4SN²⁰⁰, P = 0.0087; 9L versus 9L + E86.L4SN²⁰⁰, P = 0.0001; SBA versus E86.L4SN²⁰⁰, P = 0.0227; SBA versus E86.L4SN²⁰⁰, P < 0.0001; E86.L4SN²⁰⁰ versus E86.L4SN²⁰⁰ was not significant (P = 0.1126). B, survival of rats injected with 1.6 × 10⁶ PA317.STK.SBA (C; n = 6) or E86.L4SN²⁰⁰ (D; n = 6) RPCs into 4-day-old 9L gliomas (P = 0.008).

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Fig. 5. Histology and immunohistochemistry of one rat brain injected with 9L and IL-4 RPC cells. Low power view (A: H&E, ×45) showing a small lesion along the site of tumor injection (arrowheads) with an admixture of neoplastic cells, macrophages, and inflammatory infiltrates (B: H&E, ×200). Details on high magnification (×700) of different patterns of response to antibodies directed against various subsets of lymphocytes (C, CD4; D, CD45; E, CD8) and directed to macrophages (F).
survival rates obtained with RPCs of similar titer but producing different amounts of IL-4 suggest that if more IL-4 is available at the tumor site, the threshold needed to trigger the immune response is reached more rapidly. To be clinically efficacious, the treatment needs an early activation of the immune response before the formation of a large tumor. Thus, IL-4 that is present early during tumor development (measurements on brain homogenates demonstrated high levels during the first 2 weeks after injection of E86.L4SN200) could stimulate the presentation of tumor antigens by macrophages or B lymphocytes (these cell types were found in inflammatory infiltrates of IL-4-treated tumors) and elicit an antitumor response, mostly based on CD8+ T-lymphocytes. Furthermore, transduced tumor cells, which in vitro proliferate like wild-type cells (see “Results”), may contribute to maintain high levels of IL-4.

These two features of retroviral-mediated gene transfer, the temporary survival of RPC producing high levels of IL-4 before the formation of a large glioma and the stable integration of the transduced gene into actively dividing tumor cells, may underlie the high survival rates obtained in some of our experiments. Their lack, on the contrary, may explain why IL-4 transduction of GL261 gliomas by herpes simplex-based defective constructs could only increase slightly the survival of C57BL/6 mice (18).

It should be acknowledged that in our experiments we have matched mouse IL-4 with rat IL-4Rs. Human/mouse IL-4 species specificity has been demonstrated previously (36, 37). Rat/mouse IL-4 and IL-4R have higher similarity than human/mouse IL-4 and IL-4R (58% versus 39%, respectively, for IL-4; 79% versus 52%, respectively, for IL-4R). Furthermore, previous reports have demonstrated that mouse IL-4 can counteract graft rejection in the rat (38) and have an antitumor effect on rat C6 gliomas (15). These observations, in combination with our results, suggest that the species specificity of rat and mouse IL-4 is not as tight as for human IL-4. It is possible, however, that the use of species-specific IL-4 may contribute further to the antitumor efficacy of this cytokine.

In view of future clinical applications of this gene therapy approach, three other points need to be mentioned: (a) signs of toxicity that could be linked to IL-4 were never detected, and rats’ deaths were always due to a large brain tumor and not to inflammatory edema of the brain. Preliminary experiments based on injection into the brain of Sprague Dawley rats of ST14A neural progenitor cells derived from the same rat strain (39) and that we transduced to overexpress IL-4 confirmed the absence of brain or systemic signs of toxicity.5 This seems rather relevant in light of recent findings indicating that intracranial injection of cells overexpressing IL-2, IFN-γ or granulocyte/macrophage-colony-stimulating factor does not increase tumor survival and can cause severe CNS toxicity (40–42); (b) established 9L gliomas injected with IL-4 RPCs were cured in 50% of the rats. This experimental setting mimics quite closely a clinical situation in which, after tumor debulking, RPCs could be injected at the boundaries of the tumor cavity; and (c) IL-4 has a relevant inhibitory effect on angiogenesis (43). This can be an important component of its antitumor effect (44) that may have contributed significantly to our results. The investigation of the mechanisms of the antiangiogenic effect of IL-4 on malignant gliomas and the precise identification of immune cells that in different districts of the immune system are responsible for tumor rejection (tumor-infiltrating lymphocytes and tumor-specific T lymphocytes in the spleen and in cervical lymph

5 S. Benedetti and E. Cattaneo, unpublished observations.
IL-4 GENE THERAPY FOR GLIOMAS


44. Colombo, B. M., Benedetti, S., Ottolenghi, S., Poli, G., Pollo, B., Mora, M., and Finocchiaro G. The “bystander effect”: association of U-87 cell death with ganci
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Sara Benedetti, Maria Grazia Bruzzone, Bianca Pollo, et al.


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