

Cytokine Gene Therapy of Gliomas: Induction of Reactive CD4⁺ T Cells by Interleukin-4-transfected 9L Gliosarcoma Is Essential for Protective Immunity¹

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ABSTRACT

Tumor cells genetically modified to secrete cytokines stimulate potent immune responses against peripheral and central nervous system tumors; however, variable results on the efficacy of this strategy for therapeutic intervention against established intracranial neoplasia have been reported. We have found that vaccination with rat 9L gliosarcoma cells expressing interleukin 4 (9LmIL4) induced a specific, protective, immune response against rechallenge with parental 9L tumors. In naive rats, sham-transfected 9L (9Lneo) tumors and 9LmIL4 tumors grew at comparable rates for 12–14 days, and then 9LmIL4 tumors regressed. After regression of 9LmIL4 tumors, rats were resistant to rechallenge with parental 9L cells. To investigate the mechanism(s) responsible for 9LmIL4-induced immunity, the phenotype and function of tumor-infiltrating lymphocytes (TILs) in 9Lneo and 9LmIL4 tumors were compared. In flow cytometric analyses, it was determined that CD4⁺ T cells were the predominant cell type in both 9Lneo and 9LmIL4 tumors at day 10. However, at the onset of regression (day 14), 9LmIL4 tumors were infiltrated predominantly by CD8⁺ T cells. To investigate functional aspects of the anti-9L tumor responses, we assessed the capacity of 9LmIL4 TILs to mediate specific lytic function or production of cytokines. In response to parental 9L, TILs isolated from day 14 9LmIL4 tumors were demonstrated to produce substantially greater amounts of IFN- γ than did TILs from 9Lneo tumors. Although freshly isolated TILs from 9LmIL4 or control tumors did not lyse 9L cells in ⁵¹Cr-release cytotoxicity assays, specific cytotoxicity was demonstrable using TILs from day 14 9LmIL4 or splenocytes from 9LmIL4-bearing rats after their restimulation for 5 days with parental 9L tumor cells *in vitro*. Antibody blocking studies demonstrated that cytokine production and lytic activity by TILs, or splenocytes from 9LmIL4-immunized rats, were mediated in a T-cell receptor-dependent fashion. Because interleukin-4 also promotes humoral responses, quantity and isotype of immunoglobulins in sera from 9Lneo or 9LmIL4-immunized rats were compared. The amount of IgG1 antibodies was significantly increased in sera from 9LmIL4-immunized rats compared to sera from 9Lneo-bearing rats. Experiments using sublethally irradiated, naive rats adoptively transferred with splenocytes and/or sera from 9LmIL4-immunized or naive rats demonstrated that immune cells, with or without immune sera, protected recipients from challenge with parental 9L. Immune sera provided no protection when given with lymphocytes from naive rats, and it did not enhance protection against parental 9L when given in conjunction with lymphocytes for 9LmIL4-immunized rats. In additional adoptive transfer experiments, an essential role for CD4⁺ T cells in immunity was observed because their depletion from among splenocytes of 9LmIL4-immunized rats eliminated the protective effect against 9L, whereas depletion of CD8⁺ cells resulted in a more limited effect on protection against 9L. These data suggest that strategies for

inducing systemic, long-term tumor-specific reactivity among CD4⁺ T cells will be critical for the development of immunotherapy of gliomas.

INTRODUCTION

Among children and adults, malignant gliomas are the most common intrinsic brain tumors, and they are the most refractory to conventional therapeutics, including surgery, radiotherapy, or chemotherapy. Limitations of these therapies are well documented and are attributable to multiple factors, such as the highly infiltrative pattern of growth of gliomas, and to the unique features of their anatomical site (recently reviewed in Refs. 1–3). The failure of standard therapies to improve the prognosis of affected patients has focused attention on the development of alternative treatments, particularly immunotherapy. A variety of immunologically based strategies, including passive immunization (4–12), adoptive cellular immunotherapy (13–22), local and systemic delivery of biological response modifiers (23–39), and vaccination with parental and genetically modified tumor cells (29, 30, 32, 34, 36, 37, 39), have been attempted. From these studies, advances in both the understanding of the basic immunobiology of gliomas and in the development of general principles pertinent to therapeutic applications of immune reactivity to CNS⁴ neoplasia have been made. Initially, there were a number of concerns regarding whether the immunologically privileged status of the CNS might be an insurmountable barrier to effective immunity, but it has proven to be a relative rather than absolute barrier (reviewed in Ref. 3). In fact, there is ample evidence for trafficking of immune effector cells into the CNS in a variety of pathological conditions, including neoplasia and autoimmune diseases (reviewed in Ref. 40). As a more general and pertinent concern, there is a poor understanding of whether antigens associated with CNS tumors might be available for induction of systemic responses and whether induction of systemic, specific, antitumor responses might then be induced and manifest in the CNS. Although recent observations have suggested the potential for access of CNS antigens to the periphery (41), there is presently a paucity of candidate glioma antigens for the evaluation of induction of specific immunity. Therefore, most research into these issues has dealt with the induction of systemic immunity to whole tumor cells.

In general, systemic immune reactivity is suppressed in patients bearing gliomas (reviewed in Ref. 42) or in animals bearing experimental gliomas (43, 44). This is mediated by factors produced by gliomas, such as TGF- β 2, IL-10, and prostaglandin E₂ (45–51). There are also reports demonstrating the potential for immunosuppression locally in gliomas by release of these factors as well (45, 50). However, other mechanisms also contribute to immunosuppression within the tumor, such as induction of apoptosis in immune effector cells via

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⁴ The abbreviations used are: CNS, central nervous system; TGF, transforming growth factor; IL, interleukin; CM, complete medium; F344, Fischer 344; TIL, tumor-infiltrating lymphocyte; mAb, monoclonal antibody; TCR, T-cell receptor; PBS/Tw, PBS supplemented with 0.5% Tween-20; PBS/FCS, 25 μ l of PBS supplemented with 1% FCS; FITC, fluorescein isothiocyanate; PE, phycoerythrin; DC, dendritic cells.

Fas:FasL interactions as gliomas express FasL and activated effector cells express Fas (52). Still there are reports indicating that immune responses to gliomas can be induced by immunization with tumor cells, with the caveat that some additional specific, *in vitro* stimulation is often required to make it possible to detect the response (15, 16, 18–21). In this setting, there are indeed reports that adoptive transfer of *ex vivo*-stimulated cytotoxic T cells was able to cure intracerebral tumors in rodents (20, 21, 53, 54). Interestingly, the relevant effector cells in this model can be identified phenotypically as being CD62L^{low} (18, 20, 21). Reports of this nature have engendered numerous attempts to both improve the immunogenicity of gliomas and to reverse the potential immunosuppressive effects of the tumor, which might limit induction of immune responses. Attempts at enhancing immunogenicity of gliomas have included increasing expression of MHC molecules (27, 28), artificially establishing expression of costimulatory molecules (55–57), and artificially establishing expression of cytokines (29, 30, 32, 34, 37, 38). Approaches to reverse immunosuppressive effects of gliomas have involved using antisense technologies to inhibit production of factors such as TGF- β 2 and Schwannoma-derived growth factor (58–60).

One of the most effective ways observed to enhance induction of a systemic, glioma-specific immune response is by immunization with tumor cells that are genetically engineered to secrete cytokines such as granulocyte macrophage colony-stimulating factor, IL-2, IL-4, and IL-12 (30, 61–72). This approach has provided evidence for antitumor efficacy in establishment models, protection models, and in some instances, therapy models (63, 66, 70, 71). Further, in these models, there is ample evidence for the induction of a systemic response, which results in antitumor effects against gliomas established as CNS tumors. Among the cytokines tested in these models, IL-4 has yielded particularly potent antitumor effects, which include direct antiproliferative activity (73, 74), antiangiogenic activity (75), activation of endothelia in the tumor microvasculature, which facilitated increased infiltration by immune cells (76), recruitment of nonadaptive immune effectors, such as eosinophils (75, 77), and induction of specific humoral and cellular immune responses (30, 61, 62, 67–71).

The initial reports regarding IL-4-based immunotherapy of gliomas suggested that innate immune reactivity, in particular, the activity of eosinophils and neutrophils, was responsible for the antitumor effects (30, 62, 75). Support for these conclusions was derived from findings indicating a significant infiltration of gliomas by eosinophils and neutrophils, and further, from findings that IL-4-induced antiglioma effects were observed in athymic, nude animals lacking the capacity to mount T-cell responses (30, 62, 75). However, these findings do not directly support the conclusion that a nonadaptive response is primarily responsible for the potent IL-4-driven antiglioma response in normal animals. In fact, there is ample evidence for the induction of a potent adaptive response to IL-4-transduced gliomas (30, 61, 62, 67–71) and data documenting a significant infiltration of CD8⁺ T cells in regressing gliomas (75, 77). These data suggest that further evaluation of the IL-4-induced immune mechanisms that manifest in the regression of gliomas is warranted.

To establish a more well-defined understanding of immune reactivity induced by IL-4-transduced gliomas, we have recently begun the investigation of specific, systemic immune responses induced by IL-4-transfected 9L (9LmIL4) gliomasarcomas in rats. Using this model, we have studied the outcome of peripheral immunization with 9LmIL4 on both humoral and cellular immunity, and results of some of these studies are detailed here and in particular, contain an analysis of the nature of 9L-reactive T cells.

MATERIALS AND METHODS

Tumor Cells

9L, a F344 rat gliosarcoma cell line, and MADB106, a F344 mammary adenocarcinoma, were maintained as adherent cultures in CM consisting of RPMI 1640 (Cellgro, Herndon, VA) supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 2 mM L-glutamine (Life Technologies, Inc.), 50 μ g of streptomycin (Life Technologies, Inc.), and 50 units/ml penicillin (Life Technologies, Inc.). Neomycin- and mIL4-transfected 9L cell lines were cultured in CM supplemented with 1 mg/ml active G418 (Life Technologies, Inc.).

Isolation of TILs

F344 rats (Taconic Farms, Germantown, NY) were injected s.c. with 2×10^6 tumor cells. After various time intervals, tumors were resected and minced to yield 1–2-mm pieces. To release tumor cells and TILs, the tumor pieces were incubated in a mixture of 30 units/ml hyaluronidase (Sigma, St. Louis, MO), 500 units/ml DNase (Sigma), and 0.01% w/v collagenase (Sigma) in HBSS (Life Technologies, Inc.) at ambient temperature for 45 min with constant stirring. The cell suspension was strained through a sterile grid and washed three times with HBSS. Lymphocytes were separated from the tumor cells by centrifugation on a two-step gradient (75%/100% Ficoll; Uppsala, Sweden) at 1000 rpm for 20 min. The lymphocytes localized on the medium-75% Ficoll interface were harvested and washed twice in CM.

Antibodies

mAbs OX1 (anti-rat CD45), 3.2.3 (anti-rat CD161A), R73.1 (anti-rat TCR- $\alpha\beta$), 1F4 (anti-rat CD3), OX19 (anti-rat CD5), OX8 (anti-rat CD8 α), 341 (anti-rat CD8 β), W3/25 (anti-rat CD4), OX6 (anti-rat MHC class II), OX62 (rat dendritic cell-specific marker), OX42 (anti-rat CD11b/c), OX18 (anti-rat MHC class I), ED1 (anti-rat macrophage) and DB-1 (anti-rat IFN- γ) were used in this study. All antibodies were purchased from PharMingen (San Diego, CA) or from Biosource International (Camarillo, CA), except for mAb 3.2.3, which was produced in our laboratory. FITC- or PE-labeled control mouse IgG1 and unlabeled control mouse Igs were purchased from Serotec (Raleigh, NC).

Detection of Serum Immunoglobulin Subclasses

The concentration of IgG1, IgG2a, IgG2b, and IgM isotypes in pools of normal and immune sera was determined by ELISA assay. Enhanced protein-binding ELISA plates (Nunc) were coated with 2 μ g/ml mouse anti-rat IgG1, IgG2a, IgG2b, or IgM (PharMingen, San Diego, CA) diluted in 0.1 M NaHCO₃ (pH 8.2) for 1 h at 37°C. Plates were washed three times with PBS/Tw. The plates were blocked by incubating with blocking buffer (PBS supplemented with 10% FCS) and washed three times with PBS/Tw. The plates were then incubated with the pools of normal sera, sera from rats given 9LmIL4 (2×10^6 cells s.c.), sera from rats given irradiated 9Lneo (2×10^6 cells s.c.), or standards for rat IgG1, IgG2a, IgG2b, or IgM diluted in blocking buffer for 1 h at ambient temperature. After washing three times with PBS/Tw, plates were incubated with 2 μ g/ml biotinylated mouse anti-rat (light chain and mouse antirat immunoglobulin, γ chain in blocking buffer for 1 h at ambient temperature). Plates were washed six times with PBS/Tw, incubated with avidin-peroxidase in blocking buffer for 30 min at room temperature, and washed six times with PBS/Tw. The ELISA was developed by incubating the plates with substrate buffer containing 0.015% v/v 3-ethylbenzthiazoline-6-sulfonic acid (Sigma) in 0.05 M citric acid (pH 4.35) and 1:1000-diluted 30% H₂O₂. The color-forming reaction was stopped by adding 1% SDS, and the OD405 was read using a Bio-Rad microplate reader (Hercules, CA). Known standard concentrations of immunoglobulin were used to calculate the concentration of the various isotypes in sera. The relative isotype concentration was calculated using the formula: isotype concentration in immune sera/isotype concentration in normal sera $\times 100$.

Immunostaining and Flow Cytometry

Cells were incubated with 0.5 μ g of FITC- or PE-labeled or unlabeled mAbs diluted in PBS/FCS for 30 min at 4°C. The cells were washed twice with PBS/FCS. The cells incubated with unlabeled mAbs were resuspended

and incubated with an excess of FITC (Harlan, Indianapolis, IN)- or PE (Caltag, Burlingame, CA)-labeled goat antimouse IgG for 30 min at 4°C. The cells were washed twice, resuspended in PBS/FCS, and fixed in 0.5% paraformaldehyde (Sigma) in PBS and analyzed in a FACScan or FACScan Plus cytometer (Becton & Dickinson, Mountain View, CA). A total of 5000 vital cells/sample was analyzed. Further FACScan data analyses were performed using REPROMAN software (True Facts Software, Seattle, WA).

Specific Reactivity against Parental 9L by 9L- and 9LmIL4-immunized Rats

Immunization. F344 rats were immunized by three s.c. injections (days 0, 7, and 14) with 2×10^6 irradiated (8000 rads) 9L, irradiated 9Lneo, 9LmIL4 in PBS, or 0.5 ml of PBS. At day 28, spleens were resected, and single cell suspensions of the splenocytes were cultured at 1×10^6 cells/ml with 3.3×10^4 /ml irradiated (8000 rads) control 9L cells in the presence of 50 units/ml human IL-2 (Chiron, Emeryville, CA), $50 \mu\text{M}$ 2-mercaptoethanol (Sigma), and $50 \mu\text{M}$ mono-methyl-L-arginine (Cyclops, Salt Lake City, UT) in 24-well plates (Corning, Corning, NY).

Cytokine Production. For the determination of IFN γ production, supernatants were harvested after 2–5 days of culture. The supernatants were tested for IFN- γ concentration using a rat IFN- γ -specific ELISA kit (Biosource International). Relative concentrations were determined based upon establishing the concentration of IFN- γ as a value of 1.0 in supernatants of cultures receiving no stimulation and dividing this into the values obtained for IFN- γ concentrations in supernatants of cultures stimulated with either parental 9L or MADB106.

Cytolytic Function. Cytolytic activity was assessed after 2–5 days of culture. Effector cells were tested in a standard 4-h ^{51}Cr -release assay as described. Target cells (1×10^6) were labeled with $100 \mu\text{Ci Na}^{51}\text{CrO}_4$ (Amersham, Arlington Heights, IL) for 1 h at 37°C. The target cells were washed twice in CM and resuspended at 5000 cells/well in a 96-well round-bottomed plate (Corning). Effector cells were added to the target cells at various effector to target (E:T) ratios. The plate was centrifuged (800 rpm, 2 min) and incubated for 4 h at 37°C. After the incubation, the plate was centrifuged again (800 rpm, 5 min.), and $50 \mu\text{l}$ of supernatant were harvested and added to wells of a LumaPlate-96 (Packard Instrument Company, Downers Grove, IL). The plate was dried overnight at room temperature, and the amount of ^{51}Cr present was measured in a Micro Plate Scintillation Counter (Packard Instrument Company). The percentage lysis was calculated using the formula: % lysis = $(ER - SR)/(MR - SR \times 100)$. Experimental release (ER) was the release of target cells in presence of effector cells. Maximal release (MR) of ^{51}Cr -labeled cells was defined as the release obtained by the addition of 1% Triton X-100 to target cells, and spontaneous release (SR) was determined by incubating target cells without effector cells. The SR was always <20% of the MR.

Adoptive Transfer. F344 rats were immunized s.c. with 2×10^6 9Lneo (irradiated), 2×10^6 9LmIL4 cells, or no tumor cells in 0.5 ml of PBS. Thirty days after the immunization, naive rats were sublethally irradiated (850 rads). Three days later, sera and spleens were harvested from the

immunized rats. The splenocytes were depleted from B cells and macrophages/monocytes using nylon wool columns (3×10^8 splenocytes/0.5 g nylon wool, 1 h at 37°C). CD4⁺, CD8 α ⁺, or CD161A⁺ subpopulations were depleted from nylon wool nonadherent splenocytes obtained from 9LmIL4-immunized rats by incubating the cells with the specific mAb for 15 min at 4°C, followed by one wash and incubation of the mAb-coated cells with Biomag beads coated with goat antimouse IgG (Perseptive Biosystems, Framingham, MA) according to the manufacturer's protocol. Nondepleted (5×10^7) or CD4, CD8 α , or CD161A-depleted splenocytes were injected i.v. into irradiated rats. After 24 h, all rats were challenged in the right flank with 2×10^6 parental 9L cells. Some rats were given an i.p. administration of 4 ml of a pool of normal sera or sera from 9LmIL4-immunized rats on the day of the tumor challenge and 3 days after tumor challenge. The tumors were measured twice weekly, beginning at day 7 after the tumor challenge.

RESULTS

Characterization of Immune Cell Infiltration of 9L Tumors and Assessment of Specific Anti-9L Responses by TILs. To assess local antitumor responses, the phenotypes of immune cells infiltrating s.c. 9LmIL4 and parental 9L tumors were determined at 7, 12, 14, and 16 days after tumor implantation. These time points are favorable because tumors of sufficient size to allow these analyses have been established by day 7. Days 12–14 represent the time frame of onset of rejection of 9LmIL4, but continued growth of 9Lneo and day 16 represents a time point at which substantial regression of 9LmIL4 has occurred. In these studies, the tumors were physically disaggregated and digested with collagenase, DNase, and hyaluronidase. The tumor cells and TILs were separated using a 75%/100% two-step ficoll gradient. The phenotypes of the TILs were analyzed by flow cytometry using mAbs recognizing prototypical markers expressed by NK cells, T-cell subsets, macrophages, and DCs, as well as lymphoid activation markers, such as MHC class II and CD25 (Table 1).

A small subpopulation of NK cells (CD161A^{bright}/TCR⁻; 1–8%) was consistently present in both 9Lneo and 9LmIL4 tumors at all time points. At early stages of tumor development, a large infiltrate of ED1⁺/CD11b/c⁺ macrophages was seen in both tumor types. In evaluating T-cell infiltration of 9Lneo tumors, it was determined that T cells were present in tumors over a range of 30–59% of total lymphocytes. T-cell populations in 9LmIL4 were present over a comparable range (*i.e.*, 39–67% of total) at time points up to 14 days. Because 9LmIL4 tumors regress at around days 12–14, we also investigated the total T-cell compartment at day 16 and determined it to be 91% of lymphocytes. In 9Lneo tumors, CD4⁺ cells were found to comprise 33% of the total at day 7 and to increase to 61% at day 14. Correlating with these findings, it was determined that CD8⁺ cells

Table 1 Infiltration of immune cells in 9Lneo and 9LmIL4 tumors^a

Cells	Markers	9Lneo			9LmIL4			
		Day 7	Day 12	Day 14	Day 7	Day 12	Day 14	Day 16
$\alpha\beta$ T cell		30 ^b	59	52	39	54	67	91
	$\alpha\beta$ /CD4	33 ^c	58	61	44	64	38	22
	$\alpha\beta$ /CD8	58	42	38	43	41	68	85
	$\alpha\beta$ /CD161 ^{dim}	22	15	16	11	13	11	16
	$\alpha\beta$ /MHC cl.2	43	46	47	50	n.d.	71	69
NK cell	$\alpha\beta$ /CD25	n.d. ^e	11	23	n.d.	25	30	9
	CD161 ^{bright}	8	4	3	2	4	2	1
	CD11b/c	59	30	24	65	37	27	6
Myeloid cells	OX62 ⁺	10	30	23	17	25	25	19
	OX62 ⁺ /MHCcl.II ⁺	75 ^d	64	69	98	85	83	84

^a A representative experiment among three is shown.

^b Percentage of positive cells.

^c Percentage of positive cells of total $\alpha\beta$ T cells.

^d Percentage of positive cells of total OX62⁺ DC cells.

^e n.d., not done.

comprised 58% of the total at day 7, but decreased to 38% by day 14. In 9LmIL4 tumors, the inverse of this was observed because CD8⁺ cells were present at 43% at day 7 and increased to 68% by day 14. By day 16, TILs were 91% T cells, and ~85% of these cells were CD8⁺. A greater number of T cells in 9LmIL4 tumors expressed activation markers such as MHC class II than those in parental 9L. Interestingly, all OX62⁺ DC cells present in 9LmIL4 tumors expressed MHC class II, suggesting that these cells were highly activated, mature antigen-presenting cells.

As a means of evaluating immune reactivity, the capacity for specific induction of cytokine production was analyzed in TILs from 9Lneo and 9LmIL4 tumors. TILs were harvested from 9LmIL4 on days 7, 9, 14, and 16 and cultured for 48 h in the presence of parental 9L or MADB106, a syngeneic mammary adenocarcinoma. TILs from 9Lneo tumors did not produce IFN- γ upon stimulation with 9L (Fig. 1A). However, TILs from 9LmIL4 tumors, harvested on days 14 and 16, were found to produce substantial amounts of IFN- γ (377 pg/ml at day 14, 430 pg/ml at day 16; Fig. 1B). IFN- γ production was 9L-specific because stimulation with MADB106 did not result in IFN- γ production. To study whether the IFN- γ production was TCR-mediated, mAbs directed against MHC class I, CD4, CD3, and CD8 were evaluated for their effect on IFN- γ production (Table 2). IFN- γ production was inhibited by anti-MHC class I, anti-CD4, and anti-CD8 β mAbs. The anti-CD3 mAb did not inhibit, and in fact, it slightly stimulated IFN- γ production (Table 2). This is most likely attributable

Table 2. Specific production of IFN- γ by TILs of class I-presented antigen from 9LmIL4 tumor requires TCR and T-cell co-receptor expression^a

mAbs directed against	Day 14		Day 16	
	IFN- γ concentration (pg/ml) ^b	% inhibition ^c	IFN- γ concentration (pg/ml)	% inhibition
Control immunoglobulin	377		430	
MHC class I	66	82	75	82
CD3	418	-11	n.t. ^d	
CD4	190	49	n.t.	
CD8 α	104	72	n.t.	

^a A representative experiment from among three is shown.

^b IFN- γ production by TILs cultured in presence of parental 9L cells with or without mAbs minus IFN- γ production by TILs without parental 9L cells.

^c The percentage inhibition was calculated by dividing the IFN- γ production in presence of the mAb by IFN- γ production by TILs with control mAb.

^d n.t., not tested.

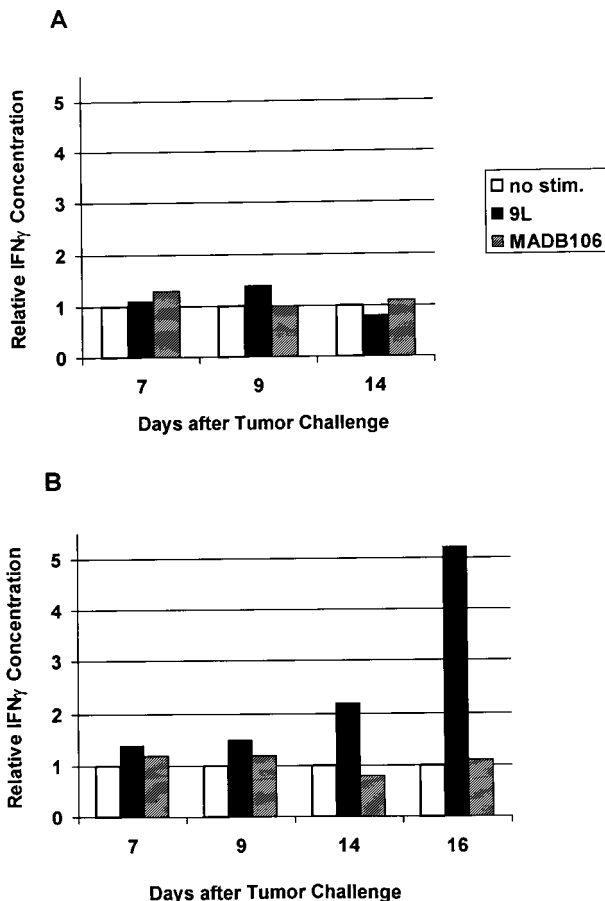


Fig. 1. IFN- γ production by TILs from 9LmIL4 or 9Lneo tumors. TILs were harvested at various time points (days 7, 9, 14, or 16) from 9Lneo (A) or 9LmIL4 (B) tumors and cultured *in vitro* with parental 9L cells or MADB106 cells or without stimulator cells. Supernatants were harvested after 48 h, and IFN- γ concentration was determined by ELISA. The data are shown as relative IFN- γ concentration calculated as described in "Materials and Methods." A representative experiment from among three is shown.

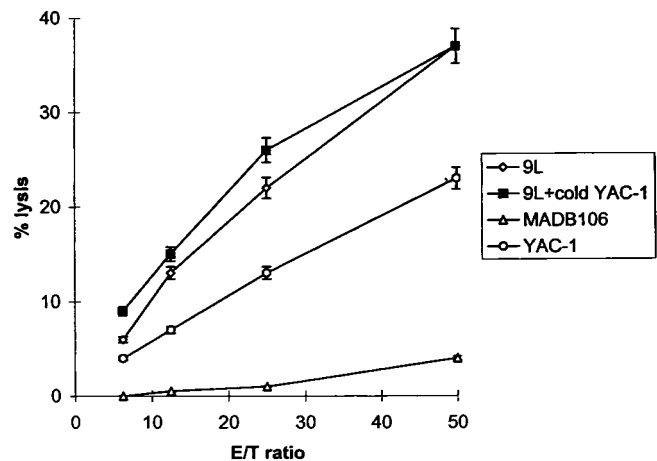


Fig. 2. Cytolytic activity by splenocytes from rats immunized with 9LmIL4. Splenocytes from 9LmIL4-immunized rats were restimulated *in vitro* with 9Lneo for 5 days and used as effector cells in a ⁵¹Cr-release assay against parental 9L in the absence or presence of cold YAC-1, against MADB106, and against YAC-1 target cells. A representative experiment from among three is shown.

to stimulation of the T cells by anti-CD3 as has been previously described by others (78). Additionally, because a great percentage of CD8⁺ T cells representative of cytolytic T cells was observed in 9LmIL4 tumors at the time of tumor regression, the cytotoxic activity of freshly isolated TILs was assessed in ⁵¹Cr-release assays, and these cells were not able to lyse parental 9L (data not shown). Similarly, freshly isolated TILs from these tumors were not able to mediate specific induction of apoptosis of 9L (data not shown).

Immunization with 9LmIL4 Induces Specific, Systemic Responses to Parental 9L Cells. We have previously demonstrated that rats that had rejected s.c. 9LmIL4 tumors were protected against intracranial rechallenge with parental 9L, whereas rats immunized with irradiated 9Lneo were not protected against subsequent rechallenge (70). These data indicate that a potent systemic immune response to 9LmIL4 was induced and that this response could mediate antitumor effects against intracranial tumors. These data also indicate that comparable immunity was not induced by 9Lneo or parental 9L. In the data presented here, we provide an assessment of the response induced by comparing 9L-specific reactivity among splenocytes from 9LmIL4- and 9Lneo-immunized rats. Splenocytes from 9LmIL4- or 9Lneo-immunized rats were restimulated *in vitro* with irradiated (8000 rads) parental 9L for 5 days, after which they were used as effector cells in ⁵¹Cr-release assays to determine their capacity to mediate specific induction of necrotic cell death. After restimulation, splenocytes from 9LmIL4-immunized rats were found to lyse 9L, but not MADB106 target cells (Fig. 2). While the restimulated spleno-

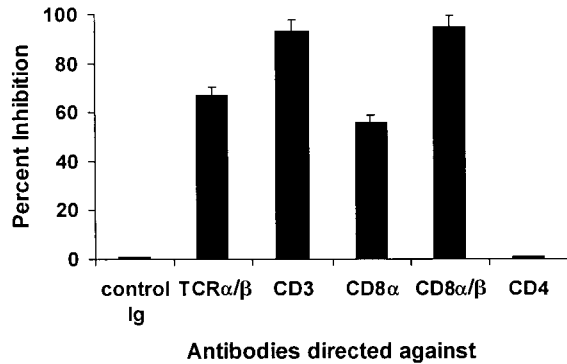


Fig. 3. Inhibition of cytolytic activity mediated by splenocytes from rats immunized with 9LmIL4 using anti-MHC class I, anti-TCR- α/β , anti-CD3, and anti-CD8 α and/or - β . Splenocytes from 9LmIL4-immunized rats were restimulated *in vitro* with 9Lneo for 5 days and used as effector cells in a ⁵¹Cr-release assay with parental 9L as target cells and in the presence of the mAbs indicated (4 μ g/ml). A representative experiment from among three is shown.

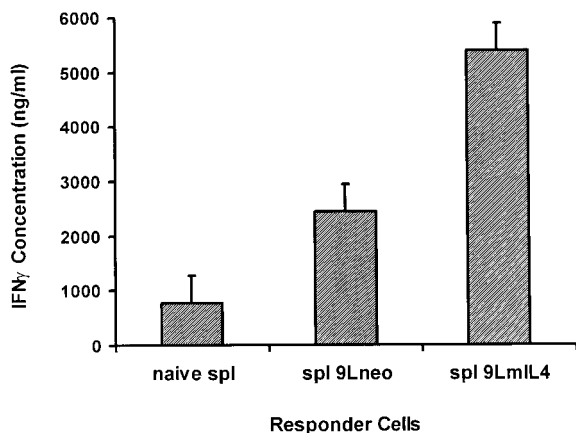


Fig. 4. IFN- γ production by splenocytes from rats immunized with 9LmIL4 or 9Lneo. Splenocytes from naive rats, 9Lneo-immunized rats, or 9LmIL4-immunized rats were cultured *in vitro* in the presence of parental 9L days. Supernatants were harvested and tested for IFN- γ concentration by ELISA. A representative experiment from among three is shown.

cytes also lysed YAC-1, the addition of nonlabeled YAC-1 to ⁵¹Cr-labeled 9L resulted in comparable lysis levels to those in the absence of cold YAC-1. These data indicate that the lysis of 9L was not NK cell-mediated. To more directly assess the involvement of T cells in mediating these responses to 9L, we determined that mAbs directed against CD3, TCR- α/β , and a combination of anti-CD8 α and anti-CD8 β mAbs inhibited the lysis of 9L. These data suggest that CD8⁺ T cells were responsible for the lysis of 9L (Fig. 3).

As an additional means of determining specific responses to 9L, supernatants from *in vitro* restimulated splenocytes from naive 9Lneo- and 9LmIL4-immunized rats were tested for cytokine production. The determination of IFN- γ concentrations revealed that splenocytes from 9LmIL4-immunized rats produced substantial amounts of IFN- γ , and this was significantly greater than IFN- γ produced by splenocytes from 9Lneo-immunized rats or nonimmunized rats (Fig. 4). Stimulation of 9LmIL4 splenocytes with MADB106 resulted in the production of minor amounts of IFN- γ (Table 3). Blocking studies showed that mAbs directed against CD3, MHC class I, CD4, or CD8 inhibited the IFN- γ production by 9LmIL4 splenocytes upon stimulation with 9L (Table 3). Depletion of CD4⁺ or CD8⁺ T cells from splenocytes from 9LmIL4-immunized rats resulted in smaller amounts of IFN- γ produced by the bulk cultures (data not shown). These data suggest that the IFN- γ production by splenocytes from 9LmIL4-immunized rats was 9L-specific and mediated by both CD4⁺ and CD8⁺ T cells.

Immunization with 9LmIL4 Results in an Enhanced Production of IgG1 Isotype Antibody. IL-4 is known to affect both cellular and humoral immunity (reviewed in Refs. 79 and 80). To assess whether IL-4 was inducing a substantial humoral response in 9LmIL4-immunized rats, sera from 9LmIL4- and 9Lneo-immunized rats were harvested, and the concentration of various immunoglobulin isotypes was determined by ELISA (Fig. 5). In rats immunized with 9LmIL4, an increased concentration of the IgG1 isotype was found. We did not, however, observe an increase in IgG1 in the rats immunized with 9Lneo. The concentration of the other isotypes was not changed in the sera from 9LmIL4-immunized rats, relative to 9Lneo-immunized rats. These data indicate that immunization with 9LmIL4 enhanced the humoral immune response in terms of increased production of IgG1 antibodies.

Adoptive Transfer of Immune Effector Cells, but Not Antibody, Results in 9L Immunity. Because immunization with 9LmIL4 enhanced both cellular and humoral immune responses, adoptive transfer experiments were performed to investigate which arm of the immune response was mediating the 9L regression. To provide a source of immune cells and sera, 4 weeks after rats were immunized with 9LmIL4, sera and spleens were harvested. Sera and/or nylon wool nonadherent splenocytes were transferred to sublethally irradiated recipient rats. One day after transfer, recipient rats received s.c. parental 9L tumors. The rate of tumor development was assessed on days 7, 12, 17, 21, and 25 by calculating the tumor area. Substantive tumor growth was observed in the groups to which nonimmunized splenocytes and/or sera from 9LmIL4-immunized rats were transferred (Fig. 6). The transfer of splenocytes from 9LmIL4-immunized rats eradicated tumor growth in the recipient rats with or without immune sera, indicating that the cellular arm of the immune response was responsible for the 9L-specific immunity.

To determine whether immunization with 9LmIL4 or 9Lneo was

Table 3 IFN- γ production by splenocytes from 9LmIL4-immunized rats is tumor-specific and MHC-restricted^a

Restimulation with	IFN- γ (pg/ml)	% inhibition ^b
None	980	
9L	4326	
9L + anti-MHC class I mAb (OX18)	2304	47
9L + anti-CD3 mAb (1F4)	1869	69
9L + anti-CD4 mAb (W3/25)	1067	75
9L + anti-CD8 α mAb (OX8)	1363	69
MADB106	1332	

^a Splenocytes were restimulated with irradiated parental 9L or irradiated MADB106 for 5 days.

^b The percentage inhibition was calculated by dividing the IFN- γ production in the presence of the mAb by IFN- γ production in the presence of control mAb.

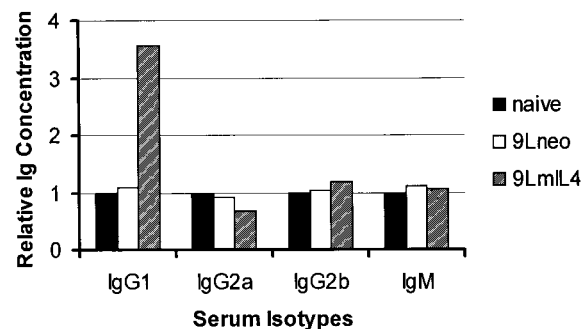


Fig. 5. Analyses of antibody isotype concentrations in sera after 9LmIL4 immunization. Rats were immunized once with 9LmIL4 or 9Lneo, and the sera were harvested at day 28. Concentrations of serum IgG1, IgG2a, IgG2b, and IgM were determined by ELISA and are represented as relative concentrations based upon comparisons of values of these isotypes in sera from naive rats. A representative experiment from among three is shown.

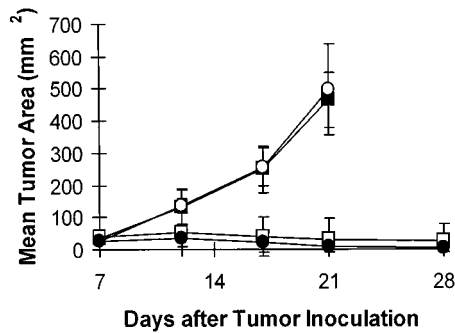


Fig. 6. Adoptive transfer of sera and/or splenocytes from 9LmIL4-immunized rats. Four weeks after a single injection of PBS or 9LmIL4, sera and splenocytes were harvested and transferred to sublethally irradiated naive recipient rats. Recipient rats were rechallenged with parental 9L cells after 1 day. ■, sera and splenocytes from PBS-injected rats; □, sera from PBS-injected rats and splenocytes from 9LmIL4-immunized rats; ○, sera from 9LmIL4-immunized rats and splenocytes from PBS-injected rats; ●, sera and splenocytes from 9LmIL4-immunized rats.

capable of inducing an immune response against parental 9L *in vivo* and to determine which cells were responsible for the anti-9L tumor response after immunization, rats were immunized with 9Lneo or 9LmIL4, or were given PBS. After 4 weeks, splenocytes from the various groups were harvested and transferred to sublethally irradiated recipient rats. As illustrated in Fig. 7A, 9L tumors grew rapidly in rats that received splenocytes from PBS-treated rats. Adoptive transfer of splenocytes from 9Lneo-immunized rats significantly inhibited 9L tumor growth from day 17 ($P = 0.004$), and one of five rats rejected the tumor. These data indicated that 9L was weakly immunogenic. Complete regression of parental 9L was observed in rats receiving an adoptive transfer of bulk populations of splenocytes derived from 9LmIL4-immunized rats. In similar experiments, splenocytes from 9LmIL4-immunized rats were harvested 4 weeks after immunization with 9LmIL4, and CD161A⁺, CD4⁺, or CD8 α ⁺ cells were depleted from among nylon wool nonadherent splenocytes by immunomagnetic bead depletion (Fig. 7B). The selectively depleted splenocytes were then transferred to sublethally irradiated recipient rats. Complete regression of 9L was observed in rats receiving an adoptive transfer of nondepleted and CD161A⁺ cell-depleted splenocytes derived from 9LmIL4-immunized rats. In rats receiving CD4⁺ cell-depleted splenocytes derived from 9LmIL4-immunized rats, the growth of parental 9L was comparable to the growth of 9L in rats receiving splenocytes from nonimmunized rats ($P = 0.1$), with the exception of

one rat, which slowly rejected the tumor. Transfer of the CD8⁺-depleted splenocytes from 9LmIL4-immunized rats resulted in tumor regression in three of five rats. However, the kinetics of regression were significantly slower compared to the regression of parental 9L after 9LmIL4 immunization ($P = 0.001$). These results suggested that immunization with 9Lneo induced weak 9L immunity, which had a limited capacity to inhibit parental 9L tumor growth, in contrast to immunization with 9LmIL4, which induced a potent, protective immune response to parental 9L. In addition, we found that CD4⁺ T cells were necessary for anti-9L tumor responses induced by 9LmIL4 immunization and that CD8⁺ T cells were also reactive, but had a lesser capacity to modulate tumor regression.

DISCUSSION

Cytokine gene therapy for the induction of specific therapeutic immunity to gliomas has proven to have significant potential because several reports indicate positive benefits in both preclinical models and clinical trials. However, these reports also indicate that improvements in the efficacy of the induction or maintenance of the immune responses induced will be necessary before maximal effects can be achieved. Because the specific immune mechanism(s) responsible for the observed antitumor effects have not been clearly elucidated, it will be necessary and beneficial to gain a greater understanding of the nature of the antiglioma activity and how it may be most efficiently induced and used.

In the present study, we investigated the immune mechanisms responsible for specific antitumor effects mediated against parental tumor after immunization of rats with IL-4-transduced 9L tumor cells, *i.e.*, 9LmIL4. In our studies (70), as well as those of others (30, 61, 62, 67–71, 79–81), it is clear that IL-4 boosts immune reactivity to gliomas. Given the pleiotropic effects of IL-4 (reviewed in Ref. 79), it could be hypothesized that a number of factors, both quantitative and/or qualitative, could be responsible for the enhanced immunity observed. First, because it is known that IL-4 can activate expression of elevated levels of adhesion molecules on endothelial cells, it is possible that the enhanced immune response observed could be due simply to an increased number of immune effector cells in the tumor. In our studies, we have determined that there is a marked increase in the number of immune cells infiltrating 9L gliomas expressing IL-4 compared to sham-transduced 9L tumors (data not shown), although it has not yet been determined whether this phenomenon is based upon

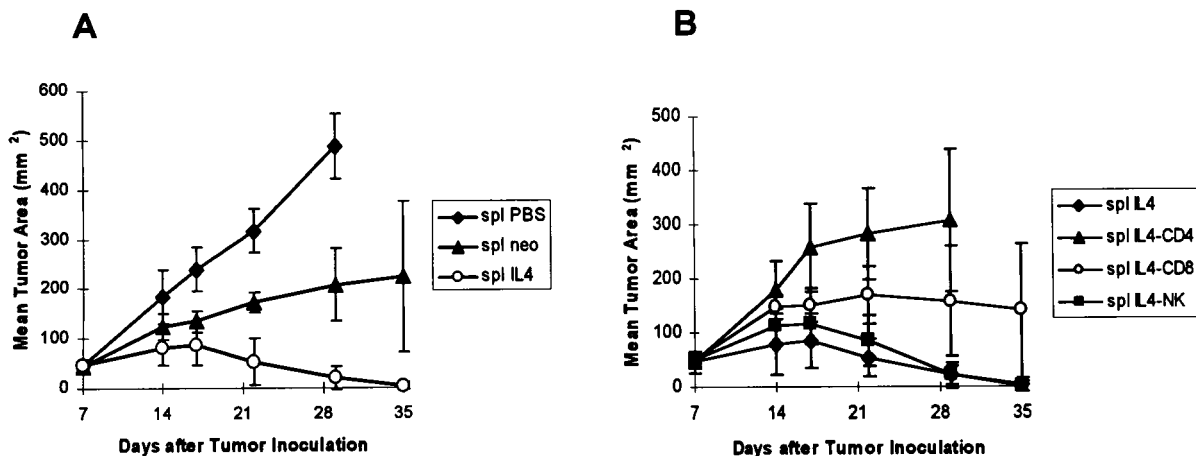


Fig. 7. Adoptive transfer of splenocytes from 9L-immunized rats and comparison of a role for CD4⁺, CD8⁺, and CD161⁺ cells in immunity. A, 4 weeks after a single injection of 9Lneo, 9LmIL4, or PBS, splenocytes were harvested and transferred (5×10^7) to sublethally irradiated recipient rats, which were then challenged 1 day later with 2×10^6 parental 9L tumor cells. B, 4 weeks after a single injection of 9Lneo, 9LmIL4, or PBS, splenocytes were harvested and depleted of CD4⁺, CD8⁺, or CD161A⁺ cells; then, 5×10^7 cells of each population were given to sublethally irradiated recipient rats. Recipient rats were then challenged with 2×10^6 parental 9L tumor cells.

increased expression of adhesion molecules either directly on tumor cells or on endothelial cells in the neovasculature of the 9L tumor.

Earlier reports investigating the cellular infiltrate in tumors having high local concentrations of IL4, based upon delivery of exogenous cytokine or on gene transfer into syngeneic fibroblasts or tumor cells themselves, have suggested an elevated presence of eosinophils and neutrophils early after tumor implantation (27, 39, 62, 75, 81). Our studies suggest that there was not a substantial infiltration of eosinophils or neutrophils in 9L tumors, although we only analyzed this at later time points in tumor growth, *e.g.*, days 14–18 (data not shown). Early in 9Lneo and 9LmIL4 tumor development, we also observed a large percentage of ED1⁺/CD11b/c⁺ macrophages and OX62⁺ DCs in the tumors (Table 1). In the 9LmIL4 tumors, all DCs expressed abundant levels of MHC class II at day 7, indicating that these cells were mature DCs and thus should be highly capable of effective antigen presentation. Interestingly, IL-4 has been described as one of the cytokines necessary for maturation and activation of rat DCs *in vitro* (82), and a previous report indicated the presence of large numbers of DCs in IL-4-producing tumors (83). However, in this report, the DCs were in an immature and nonactivated state (83). In contrast, our results suggest that the local microenvironment in 9LmIL4 activated the tumor-infiltrating DCs and likely resulted in a greater potential for antigen presentation by the DCs and induction of immunity against 9L as demonstrated by the specific induction of a cytolytic T-cell response. The disparity in these results could be hypothesized to be based upon differences in the local microenvironment of the tumors attributable to differences in production of immunosuppressive factors such as TGF- β 2 and represents a very interesting area of investigation for future evaluation of the use of IL-4 gene therapy.

There is evidence for increased numbers of CD8⁺ cells in IL-4-expressing gliomas after tumor implantation (75, 77). As illustrated in Table 1, we in fact also observed qualitative differences in the cellular infiltrate in 9LmIL4 compared to 9Lneo. Interestingly, there was a greater number of CD4⁺ cells relative to CD8⁺ cells in 9LmIL4 and 9Lneo at day 7. However, in 9LmIL4 there was a shift to substantially more CD8⁺ cells than CD4⁺ cells at a time corresponding to tumor regression (days 12–14). Our data on the timing of tumor regression, on the capacity of 9LmIL4 to grow at a comparable rate to 9Lneo in nude rats (data not shown), and on the shift in cell populations to predominantly CD8⁺ cells at the time of regression strongly support the importance of a T-cell-mediated adaptive response driven by IL4.

As an indication of the immune mechanisms involved in the specific antitumor activity mediated against 9L, we investigated the capacity for production of cytokines by TILs derived from 9Lneo and 9LmIL4 and splenocytes from 9Lneo- and 9LmIL4-immunized rats. In these experiments, it was determined that freshly isolated TILs from 9LmIL4 or 9Lneo tumors could not be demonstrated to produce cytokines such as IFN- γ , tumor necrosis factor α , or IL-4 (data not shown). However, after an *in vitro* restimulation with parental 9L, TILs from 9LmIL4 were determined to produce substantially more IFN- γ than TILs from 9Lneo-immunized rats (Fig. 1). Similarly, splenocytes from 9LmIL4-immunized rats were found to be capable of producing substantially more IFN- γ than splenocytes from 9Lneo-immunized rats (Fig. 4), but not detectable amounts of tumor necrosis factor α or IL-4 (data not shown). Interestingly, in these studies, IFN- γ was found to be produced by both CD4⁺ and CD8⁺ T cells. The facts that 9LmIL4 promotes the induction of specific T cells, which are capable of production of cytokines representative of a Th₁-type response and promotes both CD4⁺ and CD8⁺ cells capable of IFN- γ production, are striking features of the 9L model. In contrast to our results indicating that IL4 gene therapy results in the induction of Th₁-type reactivity, Pericle and colleagues (81) have reported that transducing the TS/A mammary adenocarcinoma with IL-4

results in specific antitumor immunity, but that it is a Th₂-type response characterized by Th₂ isotype switching and a decreased capacity for IFN- γ production by lymphocytes from regional lymph nodes. Further, in their model, the adoptive transfer of immune sera provided protection against challenge with parental TS/A. However, Schuler and colleagues (84) have reported that IL-4 knockout mice are incapable of mounting a specific antitumor response against either a mammary or colon carcinoma and that IL-4 genes delivered at the time of induction of a response to these tumors resulted in the development of a Th₁-type response (84). These data strongly support a role for IL-4 in the development of an antitumor response and even more interestingly, indicate that IL-4 can be requisite for the development of specific IFN- γ -producing T cells. The disparity in results in these models may be attributable to strain differences in mounting responses, as evidenced quite prominently in analyses of antiparasite immunity in which Th₁- or Th₂-type responses are mounted depending upon the strain of mice challenged (reviewed in Ref. 85). However, it must also be considered that the differences observed in terms of antitumor responses could be attributed to a differential production of immunomodulating factors by the various tumors. We, and others have observed that 9L tumors produce substantial amounts of TGF- β 2 and that this cytokine is important for the growth and development of this tumor (86). Because it has been reported that combinations of IL-4 and TGF- β can influence the outcome of an immune response and specifically that this combination of cytokines promotes a Th₁-type response (87), it seems likely that 9LmIL4 could promote Th₁-type responses as supported by our data on cytokine production by 9L-specific effector cells. Whether this phenomenon will be generalizable to any tumor-producing TGF- β or whether there are in fact other tumor-derived factors that influence this will also be an important area of investigation for evaluating the use of IL-4 cytokine gene therapy of tumors.

To further investigate the mechanisms of antitumor activity induced by 9LmIL4, we analyzed the inherent and inducible cytotoxicity to 9L in naive and 9LmIL4- or 9Lneo-immunized rats. In these experiments, we determined that freshly isolated TILs from either 9Lneo or 9LmIL4 were not capable of mediating lysis of parental 9L in standard ⁵¹Cr-release assays (data not shown). To determine whether cytolytic cells with specific reactivity for 9L were in fact induced, we isolated TILs from day 14 9Lneo and 9LmIL4 tumors, restimulated them *in vitro* for 5 days, and then carried out assays indicative of induction of either necrotic or apoptotic cell death against parental 9L. We determined that both specific necrotic and apoptotic (data not shown) cell death could be observed among T cells from 9LmIL4-immunized rats, but not from 9Lneo-immunized rats. Further, the reactivity was mediated by prototypical T cells via TCR: class I interactions as antibodies to these determinants, and to CD8, blocked specific reactivity.

The effects of IL-4 on the immune response are pleiotropic, and this cytokine can enhance both humoral and cellular responses (reviewed in Ref. 79). The data in the previous sections clearly support the fact that 9LmIL4 induced a potent cellular response against parental 9L tumors. We have also demonstrated that immunization with 9LmIL4 results in an enhanced humoral response to 9L tumor-associated antigens (data not shown), and this involves an enhanced production of IgG1 isotype antibodies (Fig. 5), which is similar to results reported in other models evaluating IL-4 regulation of humoral responses (79). To evaluate the immune mechanisms responsible for antitumor effects induced by immunization with 9LmIL4, we carried out adoptive transfer studies in which preimmune sera with splenocytes from naive or 9LmIL4-immunized rats or immune sera with splenocytes from naive or 9LmIL4-immunized rats were given to sublethally irradiated naive rats challenged with parental 9L. These experiments showed that the only effective means of transferring 9L-specific immunity involved the delivery of immune effector cells, and immune sera had

no effect on tumor growth. Interestingly, we determined that the elimination of CD4⁺ cells from among immune effectors totally eliminated the ability of adoptively transferred cells to cause the elimination of tumors. Although depletion of CD8⁺ cells from immune splenocytes resulted in slower 9L tumor growth, only 60% of the rats eventually eliminated the tumor. These findings were somewhat surprising because all other aspects of the response, including specific production of IFN- γ by immune effector cells, specific production of IFN- γ by both CD4⁺ and CD8⁺ cells, elevated levels of CD8⁺ cells in the tumor corresponding to onset of regression, and specific cytolytic activity of CD8⁺ cells against parental 9L, all suggested that CD8⁺ cells would likely be more capable of mediating the antitumor effects in adoptive transfer experiments. It can be hypothesized that adoptively transferred CD4⁺ cells activate endogenous CD8⁺ effector cells in recipient rats, but these animals were irradiated before transferring immune cells and would therefore have no function or substantially reduced function among endogenous CD8⁺ cells, suggesting either a direct antitumor effect of the CD4⁺ cells or recruitment and activation of other radioresistant effector cells. Interestingly, we have generated a 9L-specific T-cell line, which expresses CD4 and which is capable of mediating cytolytic activity against parental 9L (data not shown). These data support the hypothesis that there is direct antitumor activity of the adoptively transferred CD4⁺ cells *in vivo*, and evaluation of this possibility will be carried out in subsequent analyses.

The data presented here can be interpreted in a number of ways, but a hypothesis which incorporates our findings is that the lack of induction of effective antitumor reactivity against parental 9L is primarily attributable to a paucity of helper cell function, likely as a consequence of suppressive factors in the tumors. Local secretion of IL-4 in 9LmIL4 overcomes the immunosuppression in the tumor microenvironment and allows the establishment of a glioma-specific T-cell response. Alternatively, IL-4 works in conjunction with tumor-derived TGF- β 2 to promote the production of cytokines indicative of a Th₁ response, which is more efficacious in mediating antitumor effects against the glioma.

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