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ORIGINAL ARTICLE

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Irradiated tumor cell vaccine for treatment of an established glioma. I. Successful treatment with combined radiotherapy and cellular vaccination

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Abstract Rats bearing a 5-day intracranial (i.c.) syngeneic glioma were treated with a subcutaneous (s.c.) vaccination consisting of irradiated glioma cells or a multimodality approach composed of radiotherapy plus s.c. vaccination. Vaccination of rats harboring a T9 glioma with 5×10^6 irradiated T9.F glioma cells (a clone derived from the T9 glioblastoma cell line) resulted in a marked enhancement of i.c. glioma growth and a significant decrease in survival. Histopathology of the tumors from vaccinated rats revealed a massive glioma composed of healthy tumor tissue lacking any marked inflammation, edema or hemorrhage. Analysis of the tumor-infiltrating mononuclear cells indicated that gliomas from vaccinated rats contained a 10-fold greater lymphoid infiltrate per milligram of tumor as compared to tumors from non-vaccinated rats, suggesting that the vaccination had induced immune cells to localize to the i.c. glioma. Combined treatment consisting of 15 Gy of whole head irradiation of the 5-day glioma followed by vaccination with T9.F cells resulted in a significant increase in survival compared to that of non-treated rats, 45% of which remained tumor-free. Microscopic evaluation in survivors of the tumor implantation site revealed the presence of hemosiderin-laden macrophages and other mononuclear cells, with the absence of tumor cells within the residual lesion. When survivors were challenged s.c. with viable T9.F glioma cells, a delayed-type hypersensitivity (DTH) reaction appeared at the challenge site. T cells purified from these rats proliferated and secreted Th_1-associated cytokines when stimulated with irradiated T9.F glioma cells, and lysed T9.F target cells. In contrast, when these rats were challenged s.c. with the unrelated MadB106 adenocarcinoma, tumor formation was observed. These findings indicate that the treatment of an established i.c. glioma with a cellular vaccination alone may induce enhanced tumor growth; however, when the vaccination is combined with radiation therapy, the results are beneficial in terms of increased survival time or complete remission that is accompanied by the development of tumor-specific cellular immunity.

Keywords Cytotoxic T lymphocyte · Glioma immunity · Glioma vaccine · Radiotherapy

Abbreviations *CTL* cytotoxic $CD8^+$ T lymphocyte · *E:T* effector:target · *DTH* delayed-type hypersensitivity · *FBS* fetal bovine serum · *FACS* fluorescence-activated cell sorting · ^{3}*H-TdR* tritiated thymidine · *i.c.* intracranial · *IL* interleukin · *IFN* interferon · *mAb* monoclonal antibody · *LTS* long-term surviving · *NK* natural killer · *PBS* phosphate-buffered saline · *TIMC* tumor-infiltrating mononuclear cell

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Introduction

It has been estimated that 17,000 new cases of primary malignancies of the central nervous system are diagnosed each year. The most common form of these neoplasms is glioblastoma, which is a malignant grade IV astrocytic neoplasm derived from the glial lineage [9, 19, 40]. Traditional treatments for these tumors include surgery, chemotherapy and radiation. Unfortunately, despite advances in neuro-imaging, neurosurgical and radiological techniques, the median survival for a patient with a glioblastoma is 18 months after primary diagnosis, and the 5-year survival rate is 2% [44].

Glioblastomas invade the normal brain parenchyma and infiltrate along nerve tracts and the Virchow–

Robbins spaces. Although glioblastoma cells rarely metastasize outside the central nervous system, the tumor cells have the ability to migrate within the brain to areas distant from the primary site. Because of the invasive and metastatic propensity of the disease, complete surgical resection is nearly impossible. On the cellular level, a glioblastoma is vastly heterogeneic and exhibits genomic instability, rendering chemotherapy and radiation therapy merely palliative. Newer approaches to the management of this disease will likely involve a multimodality strategy that combines the traditional treatments of cytoreductive surgery or external beam irradiation with a more experimental approach such as anti-angiogenic therapy (for a review, see [25]) or immunotherapy (for a review, see [7, 23]). Over the last decade several immunological approaches have been evaluated in clinical trials, including the intralesional delivery of cytokines [30, 27] and the intra-tumoral implantation of lymphokine-activated killer (LAK) cells alone [24, 28] or in conjunction with interleukin-2 (IL-2) [29, 31, 28]. These attempts yielded limited clinical success, although in a few cases sustained responses were observed.

Analysis of the tumor-infiltrating mononuclear cells (TIMC) of the malignant gliomas has revealed that the cellular infiltrate of these tumors primarily consists of macrophages and cytotoxic T cells (CTL), while B cells are relatively absent [5, 39, 42]. The presence of natural killer (NK) cells in the glioma infiltrate is controversial. In this regard, one study reported that the proportion of NK cells detected in the TIMC was approximately 42% compared to 32% found in the peripheral blood, suggesting an increase in the percentage of NK cells in the glioma [13]. Generally though, it has been found that NK cells are absent or compose only a small fraction of the cellular infiltrate [5, 39, 42]. From the presence of T cells and other lymphoid cells within gliomas, it may be construed that the latter are inherently immunogenic; however, their rapid and invasive growth indicates that the immune cells lack potent tumoricidal function. It is possible that glioma TIMC are overwhelmed by the extensive tumor proliferation, or that the effector functions of the TIMC are inhibited by glioma-derived immunosuppressive factors (for a review see [10, 47]). Nevertheless, the partial success observed in immunotherapy clinical trials demonstrates that it may be possible to stimulate the TIMC and shift the balance towards immunological control of the tumor. We believe that an effective cellular immune response to gliomas is primarily a result of the tumoricidal function of activated CTL, and that a vaccination regimen may augment the effector functions of CTL as well as increase the localization of lymphoid cells within the glioma. Moreover, reduction of the tumor burden by the co-administration of radiotherapy, or by surgical excision of the tumor mass may also favor a cellular anti-tumor response by directly decreasing the number of tumor cells producing immunosuppressive factors.

In the present study, we used experimental rats harboring an established intracranial (i.c.) glioma to investigate whether a subcutaneous (s.c.) tumor cell vaccine could stimulate an anti-tumor response and provide therapeutic benefit when administered alone or combined with radiation therapy. To this end, syngeneic rats bearing a 5-day T9 glioma were vaccinated s.c. with irradiated T9.F glioma cells, a clone derived from the T9 glioblastoma cell line [17, 18]. Subsequently, the TIMC of the i.c. glioma were analyzed and animal survival was assessed. In a second set of experiments, a multimodality approach was adopted to treat the gliomas. In these studies, rats bearing 5-day T9 gliomas were subjected to whole-head irradiation immediately prior to a s.c. vaccination with T9.F glioma cells, and the efficacy of the combined therapy was assessed by measuring survival and the generation of tumor-specific cellular immunity.

Materials and methods

Animals

Inbred female Fischer 344 rats weighing 120 to 140 g were obtained from Harlan Sprague–Dawley (Indianapolis, Ind.). Animals were housed in a climate-controlled AAALAC-approved vivarium, and were provided free access to rat chow and water. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee. Principles of laboratory animal care (NIH publication no. 85–23, revised 1985) were followed, as well as specific national laws where applicable.

Cell lines and culture

The culture of tumor cell lines and their origins have been previously described [17, 18]. Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids (all from Gibco BRL, Grand Island, N.Y.). The T9 glioblastoma tumor was originally induced by *N*-nitrosomethylurea injection [4] and has been reported to be derived from the 9L glioma cell line [3]. The MadB106 mammary adenocarcinoma is a selected pulmonary metastasis produced from a 9, 10 dimethyl-1, 2-benzanthracene-induced tumor [2, 41]. Clone T9.F was isolated from the T9 glioblastoma cell line in a clonogenic assay, and is considered to be immunogenic [17]. All tumors are syngeneic to the Fischer F344 rat.

Vaccination of experimental rats

Monolayers of tumor cells were trypsinized, counted on a hemacytometer and checked for viability by trypan blue exclusion. Cells were washed twice in phosphate-buffered saline (PBS; Sigma, St. Louis, Mo.) and the concentration was adjusted to 5×10^6 cells/ml of D–PBS. Cells were irradiated with 50 Gy from a cesium source administered at a rate of 5 Gy/min. Exposure of T9.F glioma cells to 50 Gy irradiation prevented cellular proliferation, and did not alter the level of expression of major histocompatibility complex class I and intercellular adhesion molecule-1 surface antigens (data not shown). After irradiation, the cell concentration was adjusted to 5×10^7 cells/ml in PBS. The left hind flank of recipient animals was shaved, wiped with 70% ethanol and rats were injected s.c. with 100 µl (5×10^6 cells) of the irradiated cell suspension.

Tumor implantation

The i.c. implantation of T9 glioblastoma cells has been previously described [17]. Briefly, anesthetized animals were placed in a stereotactic apparatus, bregma was located and a shallow depression 4 mm to the right of the sagittal suture and 1 mm posterior to the coronal suture was made. Fifty thousand T9 glioma cells suspended in 5 µl of PBS were injected into the posterior parietal lobe of the brain at a depth of 3.5 mm using a Hamilton syringe and a 26-gauge needle. The needle track was sealed with bone wax and the incision was closed with surgical staples. Long-term surviving rats were challenged s.c. with the T9.F glioma or the MadB106 adenocarcinoma. Tumor cell suspensions were prepared as described for s.c. vaccinations. Rats were injected in the contralateral flank to the original vaccination site with 5×10^6 viable T9.F or MadB106 cells suspended in 100 µl of PBS, and the diameter of the resulting nodule was measured daily with calipers.

Treatment of intracranial T9 gliomas

Rats bearing 5-day T9 gliomas were divided into three groups which received: (a) no treatment; (b) s.c. vaccination only; or (c) a combination of radiation and s.c. vaccination. Rats treated with s.c. vaccination were injected with 5×10^6 T9.F cells. Rats receiving radiotherapy were anesthetized, and the field of radiation exposure was selectively adjusted so that only the cranium of the animals received radiation. Rats received a single dose of 15 Gy delivered from a Picker Zoneguard V4M60 ^{60}Co gamma irradiator (Picker X-ray Manufacturing, Cleveland, Ohio) at a rate of 2 Gy/min. Immediately after the administration of radiation, rats were vaccinated s.c. with 5×10^6 irradiated T9.F cells.

Antibodies

Monoclonal antibodies (mAb) used to phenotype T cells included anti-CD3ϵ (G4.18), anti-CD4 (OX-35), anti-CDα (OX-8), and anti-CD8β (341). Anti-CD161 (10/78) was used to identify NK cells, mAb from the HIS48 hybridoma were used to identify granulocytes, and anti-CD11b/c mAb (OX-42) were used to detect monocytes/macrophages. These mAb were either directly conjugated to a fluorochrome or were biotinylated. Purified anti-CD3 (clone G4.18) was used in the CTL cytotoxicity assay. All mAb were obtained from PharMingen (San Diego, Calif.), except for anti-CD161 from clone 3.2.3 (Pierce Chemicals, Rockford, Ill.) which was used for the magnetic depletion of NK cells.

FACS analysis of leukocytic glioma infiltrate

Tumors were carefully excised, forced through a 70-µm nylon cell strainer and washed once with 5% FBS/PBS. Viable TIMC were enumerated using a hemacytometer and trypan blue exclusion. Cell surface staining was performed using standard methodology, and mAb were used according to the supplier's recommendations. One million TIMC were stained in a 96-well, V-bottomed microplate in a volume of 50 µl of 5% FBS/PBS containing a cocktail of three different mAb for 30 min on ice. Cells were then washed twice with 5% FBS/PBS and incubated on ice for 30 min with a streptavidin–peridinin chlorophyll protein conjugate (PharMingen). Cells were then washed twice, fixed with 1% paraformaldehyde and stored in the dark at 4°C. Three-color FACS analysis was performed using a Coulter Epics XL-MCL flow cytometer.

In vitro immune assays

Spleens from naive or long-term survivor (LTS) rats were aseptically removed and forced through a 70-µm nylon cell strainer. The cell suspension was then layered onto a Ficoll-Hypaque density gradient (Histopaque-1077, Sigma) and centrifuged for 30 min at 700 *g*. Mononuclear cells localized at the Ficoll interface were collected, washed twice with PBS and resuspended in RPMI-1640 (Gibco BRL) with 10% FBS, 0.05 mM 2-mercaptoethanol (Sigma) and an antibiotic/antimycotic mixture (Gibco BRL). Cells were then incubated at 37°C for 1 h in a tissue culture dish to remove macrophages/monocytes by plastic adherence, followed by removal of B cells by magnetic cell sorting using mouse anti-rat kappa microbeads in conjunction with a MACS MS column (both from Miltenyi Biotech, Auburn, Calif.). Positively selected B cells from naive rats were irradiated (10 Gy) and were used as accessory cells. The enriched T cell population was enumerated, and viable T cells were used in proliferation, cytokine secretion and cytotoxicity assays using T9.F or MadB106 as irradiated (50 Gy) stimulator cells or viable target cells. FACS analysis indicated that the composition of the enriched T cell populations was generally 40% $CD3^+CD8b^+$; 30% $CD3^+CD4^+$ and 30%$CD161^+$(NKR-P1 A); other cell types were negligible in this population.

The proliferation of T cells was assessed by the incorporation of ^{3}H-thymidine (^{3}H-TdR; Amersham Pharmacia Biotech, Piscataway, N.J.) in a 96-well, flat-bottomed tissue culture plate. T cells were cultured at a concentration of 2×10^5 cells/well in medium containing human recombinant IL-2 (50 IU/ml; Chiron, Emoryville, Calif.) in the presence of 4×10^3 stimulator cells and 5×10^4 accessory B cells in a final volume of 200 µl/well for four days. During the last 15 h of culture, cells were pulsed with 1 µCi of ^{3}H-TdR. The plates were then stored at –20°C. Incorporation of ^{3}H-TdR was analyzed using a 96-well plate harvester and a beta-plate reader (Packard, Meridien, Conn.). Data were expressed as the mean cpm of triplicate wells ± SE.

Using a 12-well tissue culture plate, 2×10^6 T cells were cultured with 4×10^4 stimulator cells and 5×10^5 accessory B cells per well in 1 ml of the medium. After four days the culture medium was removed, clarified by centrifugation and the cell-free supernatants were analyzed by enzyme-linked immunosorbent assays (ELISA) for the presence of IL-2, IL-4 (both from PharMingen) and interferon-gamma (IFN-γ; BioSource Int., Camarillo, Calif.). Cultures were set up in duplicate and the concentration of cytokines was expressed as pg/ml ± SE.

T cells were cultured with T9.F stimulator cells and accessory B cells as described above in 6-well tissue culture plates, and after four days NK cells were removed by magnetic cell sorting using mAb anti-CD161 from murine hybridoma 3.2.3 coupled with goat anti-mouse IgG microbeads (Miltenyi Biotech) and a MACS MS column. The purified T cells were then used in a 5-h cytotoxicity assay with chromium-51 labeled T9.F glioma or MadB106 adenocarcinoma cells at various effector:target (E:T) ratios. Briefly, 1×10^6 viable target cells were incubated for 2 h in the presence of 100 µCi of $Na^{51}CrO_4$ (Amersham), washed twice with PBS and resuspended in 10 ml of the above-mentioned RPMI-1640 medium without the addition of IL-2. Five thousand target cells were added to each well of a 96-well V-bottomed microplate. T cells suspended in the same medium were added to the wells in triplicate to achieve E:T ratios of 100:1, 50:1 and 25:1 in a final volume of 200 µl/well. To block cytotoxicity, T cells (5×10^6/ml) were incubated for 30 min on ice with anti-CD3 mAb (10 µg/ml) and then used directly in cytotoxicity assays. No T cells were added to spontaneous release or maximum release control wells, which contained target cells only in a total volume of 200 µl/well. Microplates were briefly centrifuged to initiate cell contacts and incubated at 37°C. At the end of 5 h, 20 µl of 10% sodium dodecyl sulfate (SDS) was added to the control maximum release wells, microplates were centrifuged to pellet cells and 100 µl of the supernatant from control wells and experimental wells, for experimental release, was removed for analysis. The percentage of cell lysis was calculated using the formula: % cytotoxicity = [(experimental release)/(maximum release–spontaneous release)]×100. Results were expressed as cpm of the mean of triplicate wells ± SE.

Histology

Animals were killed with sodium pentobarbital, then their brains were carefully removed and fixed in a 10% neutral buffered

formaldehyde solution. After fixation, tissue samples were embedded in paraffin. Serial coronal sections (7 μm) were mounted on glass slides and stained with hematoxylin and eosin for microscopic analysis and photography.

Statistics

Statistical analysis was performed using the Student's paired *t*-test, and differences with a *P*-value of less than 0.05 were considered significant. In the case of survival plots, results were analyzed using the Lifetest procedure (SAS Institute, Cary, N.C.) and significance was determined using the log-rank method.

Results

Intracranial T9 glioblastoma model

Intracranial implantation of 5×10^4 T9 glioblastoma cells unfailingly produced a lethal glioma when implanted into syngeneic rats, and the animals succumbed to tumor progression in approximately 28 days. Five days after the injection of 5×10^4 T9 glioblastoma cells, an established tumor was clearly identifiable at the implantation site, and exhibited the morphological characteristics typical of an anaplastic astrocytoma (Fig. 1A–C). At low power, a 5-day glioma could be observed as a dense, cellular mass with no evidence of necrosis or hemorrhage. The architecture of the tumor consisted of areas of short, interwoven cellular bundles that infiltrated into the surrounding neuropil. The tissue of the glioblastoma was predominately composed of euchromatic, fusiform neoplastic cells with large nuclei. Numerous mitotic figures with an occasional abnormal tri-mitotic figure were present and, at the advancing edge of the tumor, the parenchyma was edematous and blood vessels were routinely cuffed by glioma cells.

Subcutaneous vaccination of rats with established T9 glioblastomas

We investigated whether a s.c. vaccination with T9.F cells could activate anti-tumor effector cells which would be effective in the brain against a pre-existing T9 glioma. Rats were implanted i.c. with 5×10^4 T9 glioblastoma cells, and five days later were vaccinated with 5×10^6-irradiated T9.F glioma cells. The combined results of two independent experiments are shown as a survival plot in Fig. 2, and demonstrate that animals with a pre-existing, i.c. glioma that received a vaccination had a significantly reduced mean survival of 17.9 days ± 3.2 SD compared to the non-vaccinated control rats which had a mean survival time of 27.9 days ± 3.7 SD ($P<0.0001$). These unexpected results were highly reproducible. Fifteen days after T9 glioma implantation, the majority of the vaccinated rats were moribund, whereas the non-vaccinated glioma-bearing rats exhibited none of the neurological deficits or behavioral changes normally associated with a progressive i.c. tumor.

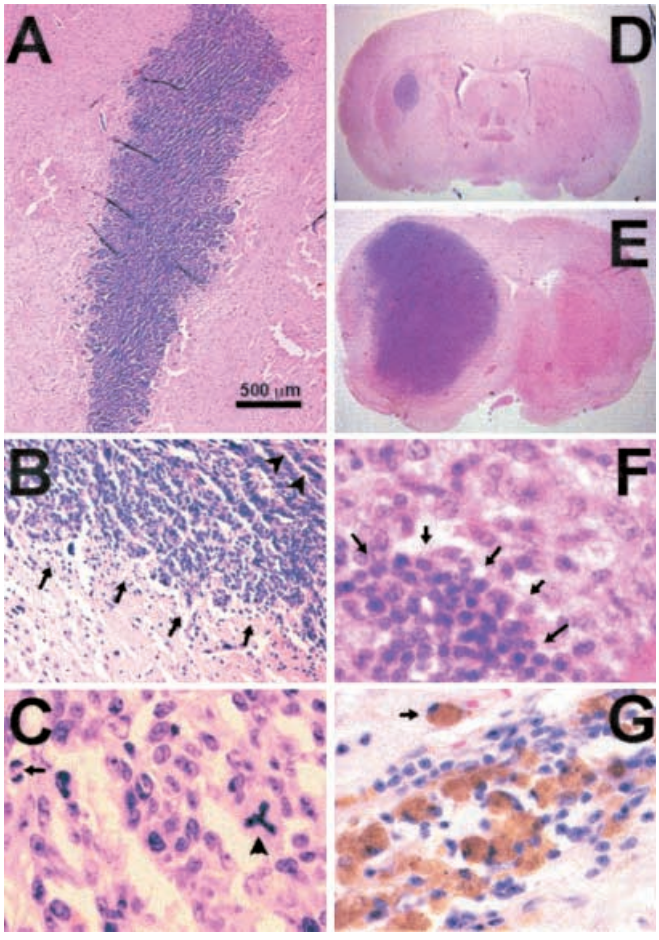

Fig. 1A–G. Histology of the T9 glioma stained with hematoxylin and eosin. **A** Coronal section bisecting the tumor implantation site showing relative size 5 days post-injection (40×). **B** Advancing edge of the 5-day glioma showing secondary tumor structure of short interwoven bundles (➤), tumor infiltration into the parenchyma (➨) with accompanying edema (200×). **C** Higher magnification of the preceding section of the infiltrative border showing the presence of a glioma cell in anaphase (➨) and an abnormal tri-mitotic figure (➤); 1,000×. **D** Coronal brain section of a non-vaccinated control rat showing the relative size of the glioma 17 days post-implantation as compared to **E**, a similar cross-section from a vaccinated moribund rat 17 days post-implantation. **F** Higher magnification of a glioma from a moribund vaccinated rat revealing a cluster of mononuclear cells (➨) within the glioma; 1,000×. **G** Evaluation of the implantation site of a rat treated with combined radiation and vaccination 2 months after glioma implantation showing the presence of hemosiderin-laden macrophages (➨) and mononuclear cells in the residual lesion; 1,000×

The histopathology of the brains from glioma-bearing rats is shown in Fig. 1D–F. Fifteen days after the i.c. tumor implant, vaccinated rats had developed a massive glioma with well-defined margins. The tumor mass was centered around the injection site in the caudate nucleus and disseminated throughout the basal ganglia. In contrast, non-vaccinated control rats harbored a significantly smaller glioma which localized to the caudate putamen. Generally, gliomas from moribund vaccinated rats had an approximate tumor volume that was three times greater than that of control rats killed at the same

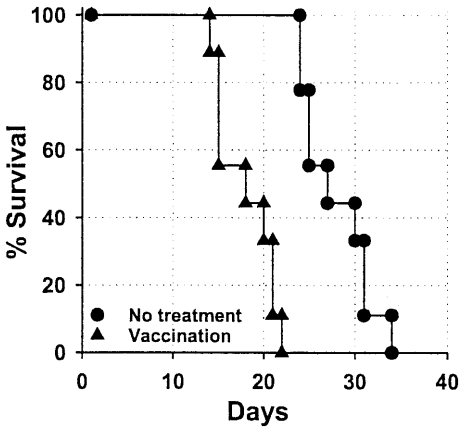

Fig. 2. Vaccination of glioma-bearing rats results in decreased survival. Rats bearing a 5-day T9 glioma were vaccinated s.c. with 5×10^6 irradiated T9.F cells (p; $n=9$) or were sham-treated (●; $n=9$). Vaccinated rats showed a significant reduction of survival ($P<0.0001$). The combined results of two independent experiments are shown

time point, as estimated from coronal sections that bisected the implantation site and by debulked tumor weights (Table 1). More extensive microscopic analysis disclosed that T9 gliomas from vaccinated rats were composed of healthy tumor tissue which was devoid of inflammation and associated edema, suggesting that experimental animals died of excessive tumor burden and not from complications derived from an immune response elicited by the vaccination. Interestingly, small islands of clustered mononuclear lymphoid cells were identifiable within the gliomas of vaccinated rats (Fig. 1F) that were not present in the tumors of control animals, suggesting that the vaccination procedure may have induced the infiltration of lymphocytes into the i.c. glioma.

Table 1. Density of TIMC in i.c. gliomas

Group	Days[a]	Tumor weight (mg)	No. of TIMC ($\times10^5$)	TIMC density[b] ($\times10^3$)
1[c]	15	53.0	29	55
2	15	37.0	18	48
3	15	41.0	24	60
Mean ± SD	–	43.7 ± 8.3	27 ± 5.5	54 ± 6.0
1[d]	29	131	60	46
2	30	157	116	74
Mean ± SD	–	144.0 ± 18.4	88 ± 40	60 ± 40
1[e]	14	206.0	197	96
2	13	90.0	95	106
3	13	112.0	120	107
4	14	167.0	224	134
5	15	136.0	210	154
6	15	156.0	250	160
Mean ± SD	–	144.5 ± 41.3	183 ± 61	126 ± 2.7

[a]Number of days after tumor implantation and TIMC analysis
[b]Density is defined as the number TIMC per milligram of tumor
[c]Non-vaccinated control group. Rats did not manifest neurological or behavior deficits associated with excessive glioma burden at the time of tumor excision
[d]Moribund control group. Gliomas were analyzed from moribund non-vaccinated rats
[e]Vaccinated group. Rats bearing i.c. gliomas were vaccinated s.c. and tumors were excised for analysis when rats became moribund

Analysis of the glioma-infiltrating leukocytes from control rats and vaccinated rats was performed to study the effects of the s.c. vaccinations on the localization of immune cells to the i.c. glioma. Data shown in Table 1 unequivocally demonstrate that the s.c. vaccination with irradiated T9.F glioma cells induced a large number of immune cells to localize within the glioma. This increase of TIMC was quantitatively assessed using the density of the glioma-infiltrating leukocytes, i.e. the number of TIMC per milligram of glioma. In this regard, the density of TIMC in the gliomas of vaccinated rats was more than twice that of non-vaccinated rats bearing i.c. T9 gliomas for the same length of time. To control for glioma size and moribund status in these density studies, approximately 30 days post-implantation rats bearing terminal gliomas were killed when they became moribund, and the TIMC was analyzed. As shown in Table 1, these gliomas were the same size as that of gliomas removed from moribund vaccinated rats. However, the TIMC density was similar to that of the non-vaccinated rats analyzed at day 15, i.e. it was approximately half the TIMC density found in the vaccinated rats.

Next, we used FACS analysis to distinguish the lymphoid subsets present in the TIMC of the i.c. gliomas, and compared the density of each specific population within the gliomas of vaccinated rats to that of control rats. Leukocyte populations identified included $CD4^+$ T cells ($CD3^+$, $CD4^+$); $CD8^+$ T cells ($CD3^+$, $CD8\alpha^+$); granulocytes ($HIS48^{bright}$); NK cells ($CD3^-$, $CD161^{dim+bright}$) and monocytes/macrophages ($HIS48^-$, $CD11b/c^+$). The results are shown in Fig. 3, and demonstrate that the TIMC of vaccinated rats displayed a significantly higher density of each lymphoid

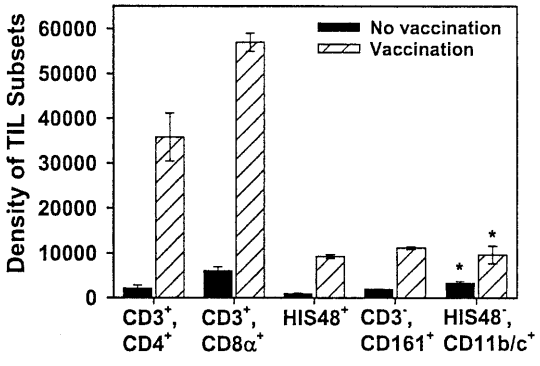

Fig. 3. Compositional analysis of leukocytes infiltrating T9 gliomas in vaccinated or control rats. Leukocytes isolated from 15-day T9 gliomas from control (*solid bar*; $n=3$) or vaccinated (*hatched bar*; $n=2$) rats shown in Table 1 were analyzed by FACS using a panel of mAb recognizing markers expressed on different leukocyte populations. The number of TIL staining positive for a specific phenotypic marker per milligram of tumor weight, i.e. density, is shown. Granulocytes are represented by $HIS48^+$ TIMC, $CD3^-$, $CD161^{+(dim+bright)}$. TIMC are identified as NK cells, and monocytes/macrophages are phenotyped as $HIS48^-$, $CD11b/c^+$ (*$P=0.23$). Mean values are plotted ± SE

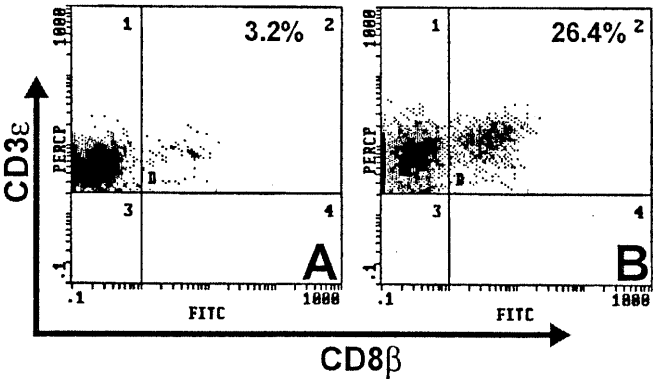

Fig. 4A, B. FACS analysis of CTL from 15-day gliomas. Tumor-infiltrating mononuclear cells were isolated, stained with phenotypic T cell markers, and gating was based upon the $CD3\epsilon^+$, $CD4^-$ population. **A** Glioma-derived CTL from non-vaccinated rats show a severe reduction in the expression of the CD8β co-receptor as compared to **B**, glioma-derived CTL from vaccinated rats. Histograms are representative of at least three independent experiments

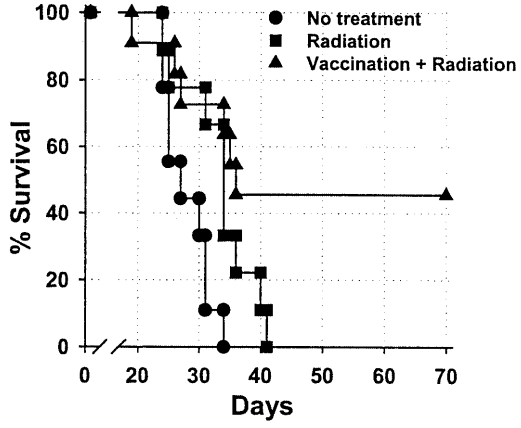

Fig. 5. Successful treatment of the T9 glioma with combined radiation and vaccination. Rats bearing a 5-day T9 glioma treated with 15 Gy of whole-head irradiation (■; $n=9$) had a significant extension of survival ($P=0.015$) compared to rats receiving no treatment (●; $n=9$). Rats that received combined radiation and vaccination had an even greater extension of survival compared to controls ($P=0.004$), and 45% of the rats remained tumor-free (p; $n=11$). Note, as shown in Fig. 2, glioma-bearing rats that received a vaccination only had a mean survival time of approximately 18 days. The combined results of two independent experiments are shown

subset analyzed, except for the monocyte/macrophage population ($P=0.23$). Most impressive was the increased density of the T cell populations, particularly in the CTL subset of the vaccinated rats. In addition, we further characterized the glioma-infiltrating CTL in terms of the level of expression of the CD8 α- and β-chains. Reduced expression of critical T cell receptor-associated membrane proteins such as the CD8 heterodimer [1], the β-chain of the CD8 co-receptor [38] and the T cell receptor-ζ-chain [48] have been associated with degenerative changes and apoptosis. Analysis of the TIMC CTL from control and vaccinated rats revealed that the β-chain of the CD8 co-receptor was dramatically down-modulated in the gliomas of control rats compared to the CTL from the glioma of vaccinated rats (Fig. 4). In this regard, only 3.2% of the non-vaccinated glioma-derived CTL expressed the CD8 β-chain, whereas 26.4% of the CTL TIMC from vaccinated rats expressed the β-chain of the CD8 heterodimer. This difference in phenotype suggested that the TIMC CTL of vaccinated rats did not undergo extensive degenerative changes, as seen in the CTL from gliomas in the control rats.

Combined treatment of a pre-existing i.c. T9 glioblastoma with radiation and s.c. vaccination with T9.F glioma cells

Subcutaneous vaccination with T9.F cells dramatically increased the localization of T cells as well as other immune cells to the i.c. glioma; however, vaccination also resulted in enhanced i.c. T9 glioma growth. We theorized that partial destruction of the i.c. tumor by the administration of radiation prior to vaccination might benefit an anti-tumor response by: (a) changing the glioma-to-TIMC ratio to one that is more favorable for an immune response; (b) reducing the concentration of glioma-derived immunosuppressive factors because of radiation-induced cell killing; and (c) inducing a "danger signal" as glioma cells undergo γ-irradiation-induced cell death, which may in turn elicit local phagocytes to scavenge tumor debris and present tumor antigens. In a pilot titration experiment using the 5-day T9 glioblastoma model, we ascertained that 15 Gy of whole-head irradiation extended the survival of T9 glioma-bearing rats by approximately seven days, but the rats ultimately succumbed to their tumor burden (data not shown).

Given these findings, we conducted multimodality treatment survival studies, in which we implanted 5×10^4 T9 glioblastoma cells, then five days later administered 15 Gy whole-head irradiation. Immediately after radiotherapy, rats were vaccinated s.c. with 5×10^6 T9.F glioma cells. The combined results of two independent survival experiments are shown in Fig. 5, and demonstrated that rats receiving combined therapy had a significant extension of survival in comparison to untreated glioma-bearing rats ($P=0.004$). Furthermore, 45% (5/11) of the rats that received combined treatment remained tumor-free for over 70 days, and were considered to be cured of their disease. Histological evaluation of the tumor implantation site from successfully treated rats revealed the marked presence of hemosiderin-laden macrophages and other mononuclear cells within the residual lesion and adjacent neuropil (Fig. 1G). Tumor cells were not detectable in the lesion or in the surrounding parenchyma.

Successful treatment of i.c. T9 glioblastomas results in long-term cell-mediated immunity

To determine whether the surviving rats that received multimodality therapy developed immunity to T9.F

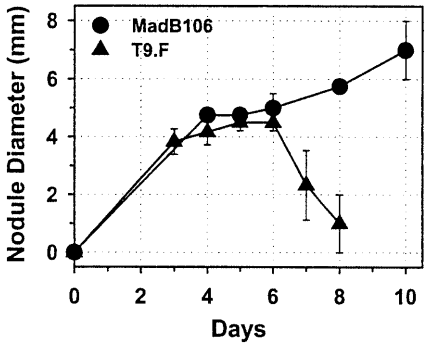

Fig. 6. Rats successfully treated for their glioma rejected a T9.F glioma challenge, but not that of a MadB106 adenocarcinoma. Rats were injected s.c. with 5×10^6 MadB106 cells (●; $n=3$) or with an equal number of T9.F cells (p; $n=3$), and the diameter of the MadB106 tumor nodule or the T9.F-invoked induration was measured daily. Mean values are plotted ± SE

gliomas, these animals were challenged s.c. in the flank contralateral to the vaccination site with 5×10^6 viable T9.F glioma cells or MadB106 adenocarcinoma cells approximately 80 days after primary treatment. Injection of this tumor inoculum is completely tumorigenic. As shown in Fig. 6, LTS rats implanted with the adenocarcinoma developed a progressive tumor at the injection site which did not regress. Conversely, 48 h after being challenged with the T9.F glioma, rats exhibited a delayed-type hypersensitivity (DTH) reaction at the site of injection, as indicated by induration and erythema, which started to subside after approximately 1 week. The appearance of a DTH response elicited by the T9.F glioma challenge and not by the MadB106 adenocarcinoma challenge suggested the presence of a tumor-specific cell-mediated memory response.

To explore the cellular memory response in greater depth, the effector functions of T cells isolated from the spleens of LTS rats were evaluated in vitro by several immune assays using syngeneic T9.F glioma and MadB106 adenocarcinoma cells. Purified T cells from naive and LTS rats were assessed for their proliferative potential when stimulated with T9.F and MadB106 cells. The results, shown in Fig. 7A, indicate that naive T cells responded poorly to both tumors, while T cells from LTS rats proliferated vigorously when stimulated with T9.F glioma cells. T cells from LTS rats also exhibited a moderate response to MadB106 adenocarcinoma cells. Similar results were observed when we evaluated the cytokine secretion potential by T cells from LTS rats in response to stimulation with the two tumors. In this regard, T cells obtained from LTS rats secreted Th_1-associated cytokines (IFN-γ and IL-2) when co-cultured with T9.F glioma cells, but secreted significantly lower levels when stimulated with MadB106 adenocarcinoma cells (Fig. 7B). Naive T cells secreted nominal amounts of IL-2 and IFN-γ in response to either tumor, and IL-4 was not detected in the co-culture medium from T cells derived from naive or LTS rats when stimulated with

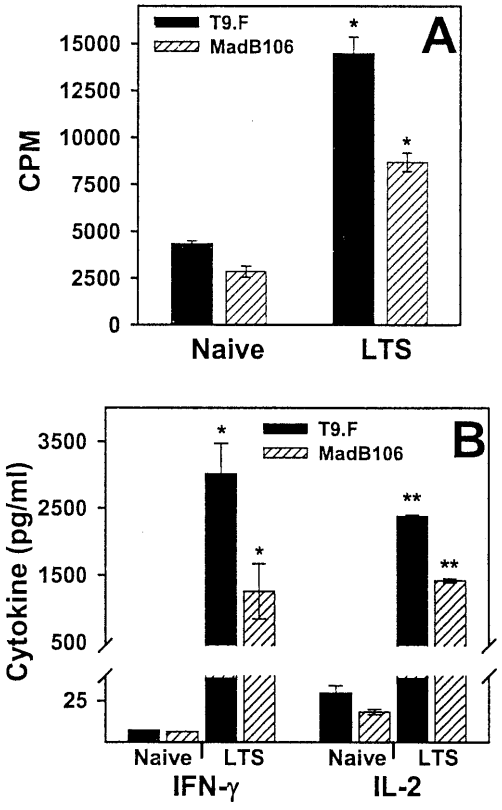

Fig. 7A, B. Purified T cells from LTS rats were reactive towards T9.F and MadB106 cells. T cells were purified from rats (as shown in Fig. 5) challenged with T9.F cells or from naive rats, and were co-cultured with T9.F or MadB106 stimulator cells; the effector function of the T cells in response to the stimulator cells was assessed in terms of **A)** proliferation (*$P=0.01$), and **B)** the production of IL-2 and IFN-γ (*$P=0.02$ and **$P=0.04$ respectively). Interleukin-4 was not detected in the co-culture supernatants. One of three independent experiments yielding similar results is shown. Mean values are plotted ± SE

T9.F or MadB106 cells. T cells derived from naive or LTS rats were also used in a cytolytic assay to evaluate their ability to lyse T9.F or MadB106 target cells. The results of a representative 5-h cytotoxicity assay are shown in Fig. 8, and demonstrate that T cells from LTS rats were capable of killing T9.F target cells at several E:T ratios, whereas naive T cells exhibited marginal cytolytic activity at high E:T ratios. Addition of soluble anti-CD3 mAb to the cytotoxicity reaction significantly decreased the lysis of T9.F target cells by T cells derived from LTS rats. As observed in the proliferation and cytokine secretion assays, T cells from LTS rats were also reactive towards MadB106 cells. To this end, T cells were capable of lysing MadB106 cells, particularly at a high E:T ratio; however, the killing of MadB106 cells was greatly reduced as the E:T ratio declined. The noted killing of the MadB106 cells could not be attributed to the NK sensitivity of the MadB106 tumor [2, 46] because the effector cell population was depleted of NK cells by magnetic cell sorting immediately prior to the initiation of the cytotoxic assays. The observation that T cells from LTS rats were partially reactive towards the

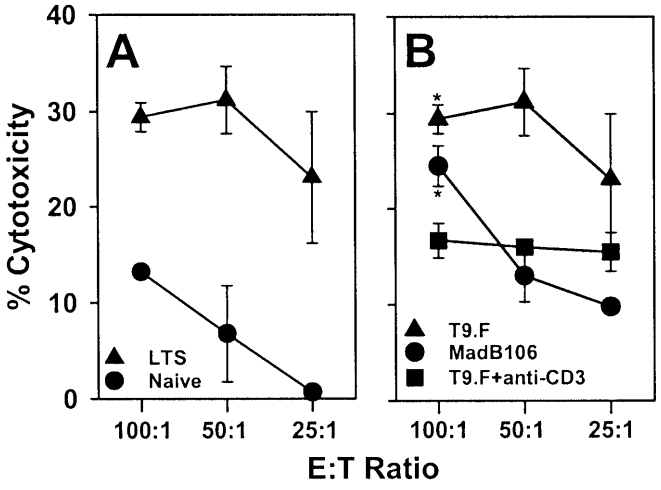

Fig. 8A, B. T cells from LTS rats are cytotoxic toward T9.F glioma cells. T cells were purified from rats (as shown in Fig. 5) challenged with T9.F cells or from naive rats, and were co-cultured with T9.F stimulator cells for four days, after which they were used in cytotoxicity assays. **A** T cells from naive rats were marginally cytotoxic towards T9.F target cells and T cells from LTS rats could lyse T9.F targets. **B** Lysis of T9.F target cells by T cells from LTS rats was blocked by anti-CD3 mAb and LTS-derived T cells were partially cytotoxic towards MadB106 target cells at high E:T ratios (*$P = 0.03$). One of two independent experiments yielding similar results is shown. Mean values are plotted ± SE

unrelated MadB106 adenocarcinoma in the immune assays is not altogether surprising, given the highly activated status of the T cells from in vivo and in vitro priming with tumor antigens.

Discussion

Other brain tumor researchers, as well as the members of our own laboratory, have shown in experimental glioma models that tumor cells genetically altered to express immune-potentiating cytokines can elicit an immunologically-based anti-tumor response with resultant cellular, tumor-specific immunity [15, 16, 18, 49]. However, there are few reports on the treatment of established orthotopic gliomas with cytokine-secreting tumor cells administered in vaccine form. In this regard, Okada et al. were able to treat rats bearing a 3-day i.c. 9L glioma with a s.c. vaccination of IL-4-secreting 9L cells [35], and in the GL261 murine glioma model Herrlinger et al. reported partial success in the treatment of a 3-day glioma with a s.c. vaccination of granulocyte/monocyte-colony stimulating factor secreting glioma cells [20]. Our own attempts to treat rats harboring 3-, 5- or 7-day i.c. T9 gliomas by vaccination with cytokine-secreting glioma cells yielded unexpected results (M.R. Graf, unpublished data) which prompted us to re-evaluate our glioma model and vaccination strategy by using a rudimentary tumor cell vaccine. The results demonstrated that the s.c. injection of sublethally irradiated non-genetically modified glioma cells had severe consequences on the survival of experimental rats harboring a pre-existing i.c. glioma.

The aims of this study were four-fold: (a) to define a clinically relevant treatment model for an established glioblastoma; (b) to determine whether s.c. vaccinations with irradiated glioma cells could induce the localization of lymphoid cells to the i.c. glioblastoma; (c) to assess the therapeutic efficacy of the increased cellular infiltrate of the tumor; and (d) to ascertain whether a multimodality form of treatment, i.e. radiotherapy combined with s.c. vaccination, would be more effective in the treatment of the established glioma than the individual therapeutic components. To this end, we demonstrated that the i.c. implantation of 5×10^4 T9 glioblastoma cells results in an established glioma which presents histological characteristics of an anaplastic astrocytoma five days after injection; s.c. vaccinations induced a significant increase of the cellular infiltrate within the gliomas, although curative effects were lacking; and radiotherapy combined with s.c. vaccination resulted in a successful approach for the treatment of established gliomas.

In our vaccination paradigm, we used a clone (T9.F) which was derived from the T9 glioblastoma cell line for s.c. vaccinations. Although T9.F glioma cells are more tumorigenic than the parental T9 glioblastoma cell line, T9.F cells retain their immunogenic nature in that s.c. vaccination with irradiated T9.F cells can protect an experimental rat from a subsequent i.c. challenge with the T9 glioblastoma [17]. The increased glioma progression and decreased survival induced by the vaccination of glioma-bearing rats were unexpected findings, but have been extensively reproduced. Necropsy of the experimental rats revealed that the vaccination had induced the i.c. gliomas to grow more rapidly than those in control rats, and that the former group had indeed succumbed to a massive i.c. glioma burden over a time period that was approximately half that of the non-vaccinated rats. Preliminary data suggest that vaccination-enhanced glioma growth may not be generalized to all glioma cell lines. In this regard, rats bearing an intracranial 5-day T9.F glioma do not exhibit decreased survival after s.c. vaccination with T9.F cells, as reported with animals harboring a T9 glioma. It is possible that the enhanced glioma growth and decreased survival observed in rats bearing a T9 glioma after vaccination may be related to the secretion of certain factors by the T9 glioma which are not secreted by the T9.F glioma. We are actively investigating this aspect, and our findings will be presented in a subsequent report.

A comparison of debulked glioma weights indicated that gliomas from moribund vaccinated rats were approximately three times the weight of those from control rats which had harbored their tumors for the same length of time. The difference in glioma weights was ~100 mg, and cannot be accounted for by the elevated number of infiltrating leukocytes because the weight of 2×10^7 splenic leukocytes in a loose pellet is ~10 mg. Histological evaluation of the gliomas showed that the tumor tissue was not edematous or hemor-

rhagic. Assessment of the glioma infiltrate from vaccinated and control rats revealed that the s.c. vaccination resulted in a greater localization of lymphoid cells within the glioma, as demonstrated by the density of leukocytes per milligram tumor. These results suggested that the vaccination was effective in that it augmented the T cell infiltrate, particularly the number of CTL. The number of NK cells in the gliomas of control and vaccinated rats did not appear to be substantial compared to other lymphoid cell populations, a finding which differs with a previous report on rat glioma cellular infiltrate [8].

Initially, we hypothesized the glioma-infiltrating T cells might be undergoing activation-induced cell death from excessive antigen stimulation provided by the vaccination as well as tumor antigens from the i.c. glioma. However this does not appear to be the case, because vaccination with 100-fold less T9.F cells had the same adverse effect on survival and glioma growth (data not shown). Furthermore these CTL did not display degenerative changes, as indicated by the expression of CD8β, contrary to the CTL from non-vaccinated rats. In fact, a concurrent study by Prins et al. (submitted) has demonstrated that the glioma -infiltrating CTL from the vaccinated rats are functionally competent and more responsive to T9.F glioma antigens than glioma-derived CTL from non-vaccinated rats; however, their CTL effector functions may be suppressed by the presence of myeloid progenitor cells in the glioma-bearing vaccinated rats, which could be a possible mechanism by which the vaccination of tumor-bearing rats results in enhanced glioma growth.

Irrespective of the effects of vaccination on glioma progression, we considered that the vaccination effort harbored promise because it resulted in a significant increase of T cells infiltrating the i.c. gliomas. We hypothesized that a reduction of tumor burden immediately preceding vaccination might provide for a more efficient T cell response by shifting the glioma-to-immune cell ratio. In addition, this form of combined treatment would more closely resemble a clinical situation. Because surgical tumor debulking was impractical, we opted to initially treat glioma-bearing rats with 15 Gy of whole-head irradiation, a dose which extends survival for approximately one week, immediately followed by a s.c. vaccination with irradiated T9.F cells. Following this multimodality regimen, almost half of the rats were successfully treated and remained tumor-free. Microscopic analysis of the tumor implantation site of these animals showed an absence of glioma cells at the lesion and surrounding parenchyma, suggesting that the i.c. glioma had been completely eradicated.

Challenge of LTS rats with viable T9.F or MadB106 cells demonstrated that the animals were protected by this treatment from the glioma but not from the adenocarcinoma. In this regard, injection of MadB106 adenocarcinoma cells produced a progressive tumor; however, the injection of T9.F glioma cells did not result in tumor formation, but induced a rapid DTH response which subsided after 10 days. These results suggested that the LTS rats had developed glioma-specific cellular immunity. However, in subsequent in vitro assays, T cells purified from LTS rats were partially reactive towards MadB106 cells, i.e. they proliferated and secreted cytokines in response to MadB106 cells, and were able to lyse MadB106 target cells. At first, these findings appear to be inconsistent with the in vivo challenge data; however, they can be attributed to non-specific in vitro stimulation of the LTS rat T cells which have been extensively primed in vivo by antigens from the vaccination, i.c. glioma and the subsequent s.c. T9.F tumor challenge. The observed unrestricted activity can be accounted for by the presence of serum antigens on the cultured MadB106 cells; weak, non-specific major histocompatibility complex class I interactions between the T cells and MadB106 cells; and the formation of complexes between the lymphocyte function-associated antigen-1 molecules on the T cells and intercellular adhesion molecules-1 on the MadB106 cells [11, 22]. These presumed interactions most likely provide sufficient stimuli to trigger the T cells to proliferative, produce cytokines and lyse MadB106 cells, although to a significantly lesser degree than T cells reacting in a glioma-specific fashion towards T9.F cells.

We have shown that s.c. injection of irradiated tumor cells is an effective method to induce the localization of T cells to an i.c. glioma in tumor-bearing rats. However, the increased cellular infiltrate did not correlate with a curative effect, but rather appeared to augment tumor progression. To our knowledge, this is the first report of a tumor cell vaccine inducing the progressive growth of a pre-existing i.c. tumor. The results of combined radiation therapy and cellular vaccination were extremely promising in that the decreased survival associated with the vaccinations was not observed, that almost half of the treated rats were cured of their gliomas and that these animals developed glioma-specific cellular immunity. We believe that the successful treatment of human glioblastoma patients will involve the formation of a cellular immune response, that it may be possible to activate tumor-specific T cells with a tumor cell vaccine, and that the administration of glioma vaccines should be used in conjunction with radiation therapy and surgical tumor resection. Furthermore, these results raise new concerns that should be considered in the development and administration of cellular vaccines to glioma patients.

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