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Authors

Graf, MR
Jadus, MR
Hiserodt, JC
[et al.](#)

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Development of Systemic Immunity to Glioblastoma Multiforme Using Tumor Cells Genetically Engineered to Express the Membrane-Associated Isoform of Macrophage Colony-Stimulating Factor¹

Martin R. Graf,^{2*} Martin R. Jadus,^{†‡} John C. Hiserodt,[†] H. Terry Wepsic,^{†‡} and Gale A. Granger*

We investigated the ability of Fischer rat T9 glioblastoma cells transduced with cDNA genes for the secreted (s) or membrane-associated (m) isoform of M-CSF to elicit an antitumor response when implanted into syngeneic animals. Intracranial (i.c.) implantation of 1×10^5 T9 cells expressing mM-CSF (T9/mM-CSF) resulted in 80% tumor rejection. Electron microscopy of the T9/mM-CSF tumor site, 2–4 days postimplantation, showed marked infiltration by macrophages, many of which were in physical contact with the T9/mM-CSF cells. Animals that rejected T9/mM-CSF cells were resistant to i.c. rechallenge with T9 cells, but not syngeneic MadB106 breast adenocarcinoma cells, suggesting that T9-specific immunity can be generated within the brain via the endogenous APCs. Intracranial injection of parental T9, vector control (T9/LXSN), or T9 cells secreting M-CSF (T9/sM-CSF) was 100% fatal. Subcutaneous injection of 1×10^7 T9/sM-CSF, T9/LXSN, or parental T9 cells resulted in progressive tumors. In contrast, T9/mM-CSF cells injected s.c. were destroyed in 7–10 days and animals developed systemic immunity to parental T9 cells. Passive transfer of CD3⁺ T cells from the spleens of immune rats into naive recipients transferred T9 glioma-specific immunity. In vitro, splenocytes from T9/mM-CSF-immunized rats specifically proliferated in response to various syngeneic glioma stimulator cells. However, only marginal T cell-mediated cytotoxicity was observed by these splenocytes in a CTL assay against T9 target cells, regardless of restimulation with T9 cells. Subcutaneous immunization with viable T9/mM-CSF cells was effective in eradicating i.c. T9 tumors. *The Journal of Immunology*, 1999, 163: 5544–5551.

Each year, ~15,000 cases of high grade gliomas are diagnosed in the United States, and these numbers are rising in both pediatric and adult populations (1). The prognosis of patients with these tumors is poor, and traditional therapies, such as surgical tumor resection followed by external beam radiation and/or chemotherapy, have done little to alter the fatal outcome of this disease. Even with multimodality therapies, the mean life expectancy of patients with glioblastoma multiforme is only 1 yr from the time of initial diagnosis and only several months after its progression (2). Hence, new strategies must be investigated for its treatment. Novel approaches using immunotherapy are currently being explored for the treatment of brain cancer as well as other solid tumors. In this regard, tumor cells that have been genetically engineered to secrete immune enhancing cytokines have been shown to elicit strong antitumor responses in animal models of many different solid tumors (see Refs. 3–10 for review).

Reports of antitumor responses elicited by cytokine-secreting glioma cells are fewer, and the results are not as extensive as

studies with other types of tumors. Ram et al. (11) demonstrated that IL-2 secreted from transduced 9L rat glioma cells resulted in reduced s.c. tumor formation, but was completely lethal and conferred no survival advantage when implanted in the brain. Tjuvajev et al. (12) reported similar results with RG-2 rat glioma cells also genetically altered to secrete IL-2 or IFN- γ . In this regard, both the IL-2- and the IFN- γ -secreting RG-2 cells exhibited attenuated s.c. tumor growth, although no s.c. tumor regression was observed, and no increase in survival was noted when the IL-2- or IFN- γ -secreting RG-2 cells were implanted intracranially (i.c.)³ (3). In contrast, Fakhrai et al. (13) reported that 9L glioma cells genetically modified to secrete IL-2 can stimulate an effective i.c. antitumor response, but only when combined with TGF- β antisense therapy to abrogate TGF- β production by the IL-2-secreting 9L glioma cells. Furthermore, another group compared the efficacy of IL-2, IL-3, IL-4, IL-6, TNF- α , IFN- γ , or GM-CSF secretion to induce an i.c. antitumor response in a spontaneously arising murine astrocytoma model (14). Of the cytokines evaluated in this study, only astrocytoma cells genetically altered to secrete IL-2, IL-4, or TNF- α elicited an effective antitumor response that resulted in long-term survival (14).

Studies utilizing tumor cells altered to secrete cytokines have revealed that certain cytokines that have the ability to activate APCs, such as macrophages and dendritic cells, induce a more efficient antitumor response that, in some cases, may result in the establishment of long-lasting tumor-specific immunity. Tumor cells engineered to secrete IL-4 (15–17) or GM-CSF (18) have

Departments of *Molecular Biology and Biochemistry and [†]Pathology, University of California, Irvine, CA 92697; and [‡]The Veterans Affairs Medical Center, Long Beach, CA 90822

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² Address correspondence and reprint requests to Dr. Martin R. Graf at the current address: Department of Anatomy, Virginia Commonwealth University, P.O. Box 709, 1101 East Marshall Street, Richmond, VA 23298. E-mail address: mgraf@hsc.vcu.edu

³ Abbreviations used in this paper: i.c., intracranial; s, secreted; CM, complete medium; m, membrane-associated; MLTR, mixed lymphocyte-tumor reaction.

been shown to be efficient at inducing tumor immunity and have been effective in destroying small established parental tumors or noncytokine-secreting bystander tumor cells in a few tumor models (17). In these studies, it appears that for dendritic cells to become efficient APCs for tumor-specific Ags, the cytokine-secreting tumor cells must be lethally irradiated, whereas injection of the viable cytokine-secreting tumor cells may not be rejected and result in progressive tumor growth (18). Other cytokine genes such as IL-2, IL-7, and IFN- γ , when transfected into tumor cells, induced tumor rejection, but long-lasting antitumor immunity was not generated (18). In a different study, mice were vaccinated with a mixture of irradiated leukemia cells genetically modified to express GM-CSF or IL-4. It was reported that bone marrow mononuclear cells harvested from these vaccinated animals contained an increased number of dendritic cells and that transfer of the bone marrow cells into tumor-bearing mice was associated with their prolonged survival (19). Moreover, spleen cells from the recipients of the bone marrow cells that contained an increased number of dendritic cells showed specific cytotoxicity against parental leukemic cells, suggesting that dendritic cells from the vaccinated donor mice activated specific T cells in the tumor-bearing recipients (19).

Transduction with M-CSF has also been used to improve antitumor responses, but has produced mixed results. Dorsch et al. (20) have demonstrated that tumors resulting from plasmacytoma cells engineered to secrete M-CSF have a marked increase of infiltrating macrophages; however, no suppression of tumor growth was observed. In contrast, Kimura (21) showed that L1210 leukemia cells expressing M-CSF were rejected by syngeneic mice and these mice were immune to rechallenge with parental cells. Similar results were observed by Walsh et al. (22), using melanoma cells engineered to express M-CSF. Because dendritic cells do not possess M-CSF receptors (23), it is thought that this antitumor immune response is due to the Ag-presenting functions of the macrophages.

Expression of the M-CSF gene results in two different isoforms of the M-CSF protein due to alternative posttranscriptional splicing within exon 6. One form of the protein is secreted as a 45-kDa homodimeric glycoprotein, and the other form remains associated with the cell membrane (24). The secreted form (sM-CSF) induces proliferation and differentiation of monocyte progenitors; is responsible for the stimulation of the effector functions of macrophages such as cytokine production and enhanced tumoricidal activity; and may function as a chemoattractant for circulating monocytes. In the brain, sM-CSF is believed to induce proliferation and activation of microglial cells (25). The membrane-associated isoform (mM-CSF) has also been shown to be functional in that it is capable of stimulating macrophage colony formation of bone marrow stem cells (26). Recent work by Jadus et al. (27, 28) with T9 glioblastoma cells transfected with either the mM-CSF or the sM-CSF cDNA isoform has demonstrated that T9 cells expressing only the mM-CSF isoform, but not the secreted form, are killed *in vitro* by tumoricidal macrophages. This suggests that the membrane-associated cytokine may induce a stronger antitumor response than its soluble counterpart.

It is common to find microglial cells and macrophages in the cellular infiltrate of gliomas (29). Their role in these brain neoplasms is unclear. They often lack *in vitro* tumoricidal activity, and it has been suggested that they may even promote tumor growth by the production of growth factors and angiogenic factors (30). Many gliomas produce TGF- β , which inhibits the immune system (31) and may suppress the actions of macrophages (32). In the presented study, we investigated the ability of glioblastoma mutant tumor cells to induce systemic immunity after genetically engineering them to express either the soluble or the membrane-

associated isoforms of M-CSF in a syngeneic Fischer rat brain tumor model.

Materials and Methods

Cell lines and culture

All cells were cultured in complete media (CM) consisting of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Life Technologies), and were maintained as adherent monolayers in T-75-cm² culture flasks. Cells were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air, and passed biweekly using trypsin. The T9 glioblastoma tumor was originally induced by the repeated *i.v.* injection of *N*-nitrosomethylurea in a Fischer F344 rat and grows as an adherent cell line *in vitro* (33). T9 cells were provided by Dr. J. Yoshida (Department of Neurosurgery, Nagoya University, Japan). Previous work has shown that an *i.c.* injection of 1×10^5 T9 cells into the Fischer rat brain is 100% lethal in 20 to 25 days (34). The generation and characterization of T9/M-CSF clones were previously described (27). For these studies, T9 clones expressing mM-CSF included clones T9/mM-CSF(C2), T9/mM-CSF(F12), and T9/mM-CSF(F2), and a clone secreting M-CSF, T9/sM-CSF(H1). The 9L cells were obtained from Dr. Carol Kruse (University of Colorado Health Science Center, Denver, CO). It has been reported that T9 glioma cells are derived from the 9L glioma cell line (35). The Fischer rat-derived RT2 glioma was induced by the avian sarcoma virus, and the glioma cell line was provided by Dr. Yancy Gillespi (University of Alabama, Birmingham, AL) (36). The MadB106 breast adenocarcinoma cell line was induced by the *i.v.* injection of 9,10-dimethyl-1,2 benzantracene in a Fischer F344 rat and was obtained from Dr. Craig Reynolds (National Cancer Institute, Frederick, MD) (37). MadB106 cells develop lethal tumors when implanted *s.c.* or *i.c.* in Fischer 344 rats. NuTu-19 cells were provided by Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA). NuTu-19 cells are an ovarian carcinoma that spontaneously arose from ovarian tissue of a Fischer 344 rat (38) and is lethal when injected into Fischer rats (39).

Detection of mM-CSF by flow cytometry

Cells (5×10^5 cells) to be phenotyped were incubated with 2.5 μ l of the anti-M-CSF Ab (Oncogene Sciences, Manhasset, NY) or 2.5 μ l of an isotypic IgG1 Ab on ice for 1 h. The cells were washed twice with PBS containing 1% FBS and incubated in a 1/10 dilution of a FITC-labeled rabbit anti-rat Ab (Vector Laboratories, Burlingame, CA) for an additional hour on ice. The cells were washed three times. Ten thousand cells were analyzed on the EPICS Profile. Data were collected and then analyzed on the Multi2D program (Phoenix Flow Systems, San Diego, CA).

Animals

Inbred Fischer 344 rats weighing 150–175 g and ranging in age from 4 to 6 mo were obtained from Harlan (San Diego, CA) and used in all experiments. Animal transportation, use, and care were conducted in accordance with the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* and in accordance with the Animal Welfare Act (7 U.S.C. 2131 et. seq.). In addition, all procedures were performed in a licensed animal facility, University of California, Irvine, Public Health Service Animal Welfare Assurance A3416.01, National Institute of Health (10/29/87). They were housed in a climate-controlled vivarium and provided unlimited access to rat chow and water.

Tumor implantation

Intracranial tumor implantation. Animals were anesthetized by an *i.m.* injection of ketamine (87 mg/kg) and xylazine (6.5 mg/kg). The scalp hair was shaved, and a 15-mm incision was made with a scalpel over the cranial midline. Animals were placed in a stereotactic apparatus, and the bregma was located and used as a reference point for injections. A hand-held Dremel drill was used to create a shallow depression 3 mm to the right of the sagittal suture and 1 mm posterior to the coronal suture. Cells were washed twice in PBS, and a final cell suspension was made in PBS. Ten microliters of the appropriate tumor cell suspension were injected into the posterior parietal lobe of the brain at a depth of 4 mm using a Hamilton syringe and a 27-gauge needle secured to the arm of the stereotactic apparatus. The needle track was sealed with melted paraffin to prevent tumor cell extravasation, and the incision was closed with surgical staples.

Subcutaneous tumor implantation. Tumor cells for *s.c.* implantation were washed twice in PBS, and a final suspension was made in PBS. Tumors were induced by injecting a suspension of 1×10^7 cells in 100 μ l of PBS into the left flank. Tumor growth was monitored by daily measurements of tumor diameter using calipers.

Electron microscopy

Animals were implanted i.c. with 1×10^6 T9/mM-CSF(C2) or T9/LXSN cells. After 2 to 6 days, animals were euthanized, and their brains were carefully removed and preserved in Bouin's fixative (10% neutral buffered Formalin containing 2% glutaraldehyde). The site of tumor implantation was then identified and isolated for electron microscopy. Briefly, 1-mm sections of brain tissue from the implant site were embedded in epon, and 1-micron thick sections were prepared. Appropriate areas were then identified for the preparation of thin sections. Thin sections were cut, mounted on grids, and stained with osmium tetroxide. Sections were then viewed through a Zeiss electron microscope, and relevant areas were photographed.

Depletion of CD3⁺ T cells and passive transfer of T9 immunity

Spleens were harvested from animals that rejected T9/mM-CSF(C2) cells. Spleens were finely minced with scalpel blades; splenocytes were washed free from the white pulp of the minced spleen with 30 ml of CM and centrifuged for 10 min at $200 \times g$. RBC were lysed by resuspending splenocyte pellets in Tris-buffered ammonium chloride (5 ml/spleen) and incubating cells at room temperature for 2 min. Splenocytes were washed twice in 30 ml of CM and resuspended in PBS at a final concentration of 1×10^8 splenocytes/ml. Splenocytes were divided into a complement control and an experimental group and stored on ice. Mouse anti-rat CD3 IgM (1 mg/ml; Harlan Bioproducts for Science, Indianapolis, IN) was added to the experimental splenocytes at a 1/100 dilution and incubated on ice for 30 min. The splenocytes were next washed with 10 ml of PBS containing 1% FBS. Cedarlane Low-Tox-M rabbit complement (Accurate Chemical and Scientific, Westbury, NY) was added to both control and experimental splenocytes at a 1/10 dilution and incubated at 37°C for 1 h. Splenocytes were washed with PBS, and a small sample from each group was assessed for viability and cell depletion using a hemacytometer and trypan blue. Naive rats were injected i.p. with 1×10^8 complement control splenocytes or splenocytes depleted of CD3⁺ T cells suspended in 1 ml of PBS. The next day, animals were implanted i.c. with 1×10^5 T9 parental cells.

In vitro T cell immune responses

Two in vitro tests were used to determine whether anti-T9 T cell responses were generated in vivo. These tests included the mixed lymphocyte-tumor reaction (MLTR) and the CTL assay.

The MLTR was done using a proliferative-based assay (40). Sixty thousand unfractionated splenocytes were placed in each well of a 96-well U-bottom microtiter plate. Stimulator tumor cells were used at a 10 lymphocyte to 1 tumor cell ratio. The stimulator cells were rapidly freeze thawed before being cultured with the lymphocytes. The cultures were pulsed with $1 \mu\text{Ci}$ of [³H]TdR (Amersham, Boston, MA) for the last 8 h of the reaction on day 4, which is the optimal day of the reaction. When freeze-thawed killed T cells were plated alone and pulsed with [³H]TdR, they incorporated less than 200 cpm. The cultures were then aspirated on glass fiber filters using PHD cell harvester and counted on a Beckmann scintillation counter. Data are expressed as cpm of the average of triplicate cultures \pm SDs.

For the CTL assay, T cells were enriched from the spleen using the BioTex (Edmonton, Canada) purification columns. Splenocytes from either nonimmune or immune rats were prepared into a single cell suspension, and 1×10^8 cells were passed through the column. Yields of the cells averaged 20%, of which >95% were CD3⁺ T cells. The CTL assay was performed as previously described (41). The ⁵¹Cr-labeled target cells (5×10^3 cells/well) were incubated with varying numbers of effector cells in a final volume of 200 μl of CM in V-bottom microtiter plates. E:T ratios started at 100:1 for the freshly isolated splenocytes or 40:1 for the MLTR-activated lymphocytes and were serially diluted 2-fold, four times. The plates were spun down after 6 h of incubation, and 100 μl of the culture supernatant was removed and counted for ⁵¹Cr using a Beckmann scintillation counter. Maximum ⁵¹Cr release was achieved by freeze thawing the cells three times in liquid nitrogen. The specific release was calculated using the standard cytotoxicity equation: $\{[(\text{experimental } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release})] / [(\text{maximal } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release})]\} \times 100$ (Ref. 41). Data are presented as the mean specific release of triplicate cultures \pm SD.

Statistics

The significance of difference in animal survival was determined by a one-tailed Fisher's exact test, and *p*-values <0.05 were considered significant. For the in vitro tests, Student's *t* tests were performed and *p*-values <0.05 were also considered significant.

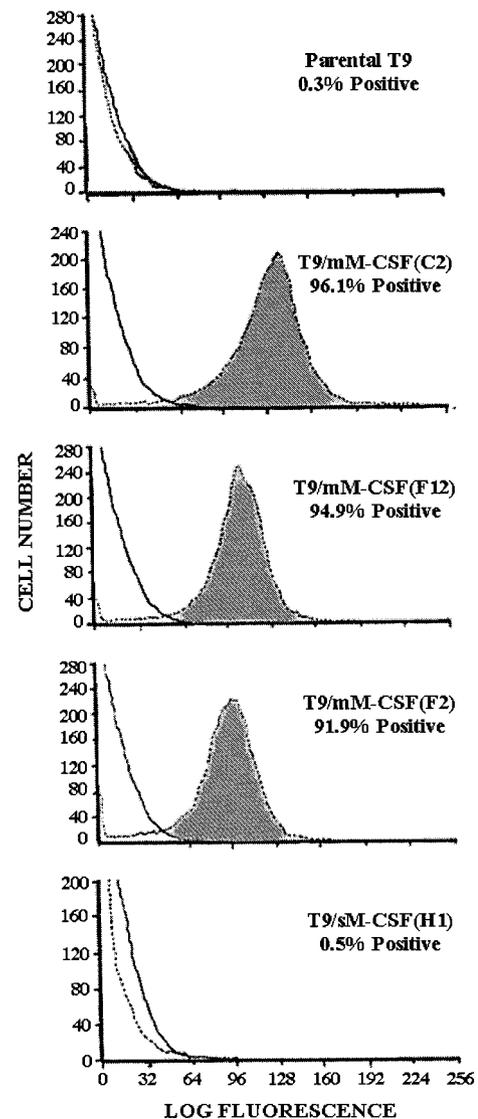


FIGURE 1. Flow-cytometric profile of parental T9, T9/mM-CSF(C2), T9/mM-CSF(F12), T9/mM-CSF(F2), and T9/sM-CSF(H1) cells. T9 cells were stained with an anti-M-CSF Ab, and 1×10^4 cells were analyzed on the flow cytometer. The data were then compared with the isotype controls using the Multi2D program.

Results

In vitro characterization of T9 cells expressing secreted or membrane-associated isoforms of M-CSF

T9 glioblastoma cells were transduced with a retroviral expression vector (LXSN) containing the cDNA gene for the secreted (T9/sM-CSF) or membrane-associated isoform (T9/mM-CSF) of M-CSF. Clone T9/sM-CSF(H1) was shown to secrete M-CSF, as determined by ELISA, at a level of 2000 pg/ml when 1×10^6 cells were cultured in 10 ml of media for 3 days. sM-CSF was shown to be biologically active by its ability to induce macrophage colonies from rodent bone marrow samples. T9/sM-CSF(H1) did not express any detectable mM-CSF, as determined by flow cytometry (Fig. 1). Several clones (T9/mM-CSF(C2), T9/mM-CSF(F12), and T9/mM-CSF(F2)) were selected for their expression of mM-CSF (Fig. 1). Flow-cytometric analysis indicated that clone T9/mM-CSF(C2) expressed a high level of M-CSF on its cell surface (9.2 arbitrary fluorescence units, as determined by the Coulter modified

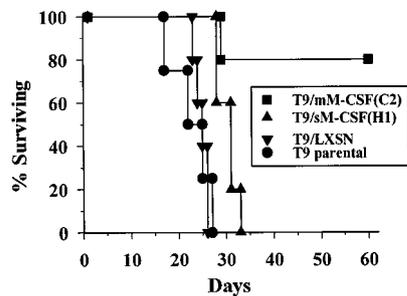


FIGURE 2. Survival of animals after i.c. implantation of T9 glioblastoma tumor cells either secreting or expressing M-CSF on their cell surface. Animals were implanted with 1×10^5 T9/mM-CSF(C2) cells ($n = 5$) or T9/sM-CSF(H1) cells ($n = 5$). Control animals were implanted with 1×10^5 parental T9 ($n = 4$) or T9/LXSN ($n = 5$) cells. Data represent one experiment of two experiments yielding similar results.

logarithmic scale using the raw data), whereas clone T9/mM-CSF(F12) expressed a much lower level of mM-CSF (3.75 arbitrary fluorescence units), and clone T9/mM-CSF(F2) even lower (2.5 arbitrary fluorescence units; isotype controls expressed 0.134 arbitrary units). T9/mM-CSF(C2) cells were effectively killed by rodent bone marrow macrophages in an *in vitro* coculture experiment, but T9/sM-CSF(H1) and parental T9 glioblastoma cells were not (27, 28). Clones T9/mM-CSF(C2) and T9/sM-CSF(H1) did not differ in their *in vitro* growth rate, measured by doubling time during logarithmic growth; expression of MHC class I, class II, and ICAM-1 (CD54) surface Ags (as determined by flow cytometry); or morphology from parental T9 or vector control cells (T9/LXSN, data not shown). Parental T9 and T9/LXSN cells did not express any detectable M-CSF on their cell surface and did not secrete detectable M-CSF protein.

Intracranial implantation of T9 cells expressing soluble or membrane-associated isoforms of M-CSF

Animals were implanted i.c. with 1×10^5 T9/mM-CSF(C2), T9/sM-CSF(H1), T9/LXSN, or T9 parental cells. The results shown in Fig. 2 indicate that animals injected with T9/sM-CSF(H1) cells have a slightly prolonged survival compared with control animals implanted with T9 parental or T9/LXSN cells, but ultimately succumb to progressive tumor growth. This was not the case, however, when animals were implanted with T9/mM-CSF(C2) cells, in which 80% (8/10) of the animals survived the intracranial challenge. These long-term survivors appeared normal and did not show any signs of neurological deficiency. Rejection of different T9/mM-CSF clones expressing different levels of M-CSF on their surface correlated with the level of membrane expression, as determined by flow cytometry. In this regard, clone T9/mM-CSF(F12), which showed a much lower level of expression than clone T9/mM-CSF(C2), was rejected in only 20% of animals (2/10), and clone T9/mM-CSF(F2), which showed even lower levels of expression than clone T9/mM-CSF(F12), was not rejected by any animals (0/10) when 1×10^5 cells were implanted i.c. (zref>49). Current studies with T9/mM-CSF(C2) cells suggest that extended long-term culture over several years may reduce the level of M-CSF expression on the surface of T9/mM-CSF(C2) cells, and consequently attenuate the induced i.c. antitumor response.

Macrophage infiltration of early T9/mM-CSF intracranial tumors

To analyze the early events in the destruction of T9/mM-CSF(C2) cells in the brain, animals were implanted i.c. with T9/LXSN or T9/mM-CSF(C2) cells, and after 2 to 6 days, their brains were

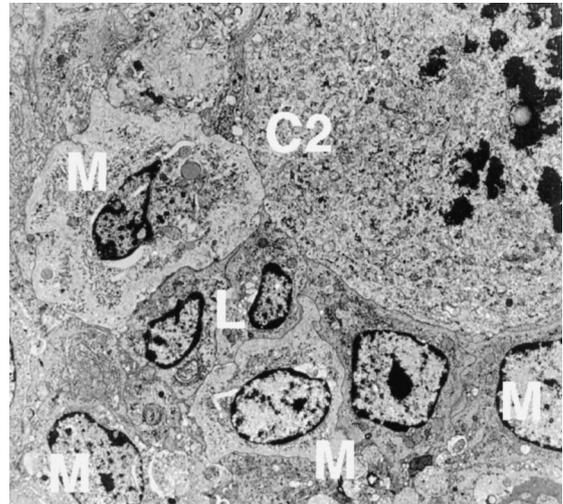


FIGURE 3. Electron microscopy of early (day 2) T9/mM-CSF(C2) i.c. gliomas. Electron micrograph of T9/mM-CSF(C2) implantation site showing two macrophage-like cells (M) in close contact with a T9/mM-CSF(C2) cell (C2) and with two lymphocytes (L). Magnification, $\times 6000$.

removed and prepared for electron microscopy. Fig. 3 is a representative electron micrograph of the implantation site and shows the presence of numerous macrophage-like cells, many of which are in physical contact with T9/mM-CSF(C2) tumor cells. Two lymphocytes are also shown adjacent to the macrophage-like cells and the T9/mM-CSF(C2) cell. The appearance of macrophage-like cells was unique to T9/mM-CSF(C2) cells, as they were not seen in proliferating T9/LXSN or T9 parental gliomas. Electron microscopy of the T9/mM-CSF(C2) injection site 6 days after implantation showed marked numbers of inflammatory cells, including numerous lymphocytes, scattered macrophages, and evidence of marked perivascular cuffing and exocytosis, with no evidence of remaining viable tumor cells.

Intracranial rejection of T9/mM-CSF(C2) cells results in protection from parental T9 rechallenge

Animals that survived the i.c. challenges with T9/mM-CSF(C2) cells were subjected to i.c. rechallenges with either parental T9 cells or another syngeneic tumor. The results shown in Fig. 4 demonstrate that the initial rejection of T9/mM-CSF(C2) cells resulted in complete immunity to a subsequent i.c. challenge with 1×10^5 T9 parental glioma cells (Fig. 4A). However, no immunity was present against a syngeneic breast adenocarcinoma, MadB106 (Fig. 4B).

T cell immune responses generated against a glioma-specific Ag(s)

Passive transfer of 1×10^8 unfractionated spleen cells from T9/mM-CSF(C2)-immunized animals into naive recipients resulted in immunity to i.c. challenge with 1×10^5 T9 parental cells (3/4 survivors, Fig. 5). However, transfer of 1×10^8 immune spleen cells depleted of T cells by anti-CD3 Ab and complement abrogated the transfer of immunity (0/6 survivors).

Animals that rejected T9/mM-CSF(C2) cells were tested to determine what types of anti-T9 tumor T cell responses could be detected against various syngeneic tumor cells. Using a MLTR in 96-well microtiter plates, splenocytes from T9/mM-CSF(C2)-immunized rats proliferated in response to T9 cells, but did not respond to either syngeneic NuTu-19 ovarian cancer or MadB106 breast cancer cells (Fig. 6A). No responses were observed using

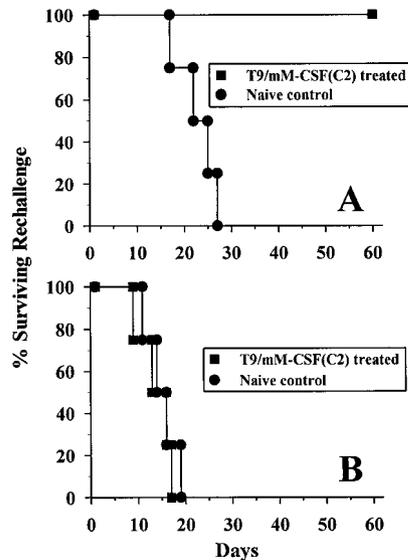


FIGURE 4. Intracranial rechallenge of animals that rejected T9/mM-CSF(C2) cells. Animals that had previously rejected an i.c. challenge of T9/mM-CSF(C2) cells or naive controls were subsequently rechallenged with A, 1×10^5 parental T9 cells, $n = 4$; or B, 1×10^5 MadB106 breast adenocarcinoma cells, $n = 4$.

splenocytes from naive nonimmune rats. Interestingly, splenocytes from T9/mM-CSF(C2)-immunized rats proliferated in response to several syngeneic rat gliomas, i.e., T9, 9L, D74, and RT2 (Fig. 6B). This suggests that a glioma-specific Ag(s) may exist and that T9/mM-CSF(C2) immunization may prime lymphocytes to respond in vitro to this glioma-specific Ag(s).

To determine whether cytotoxic T cells were present in the immunized spleen, CTL assays were performed (Fig. 7). In one representative experiment (of four performed), little or no tumor-specific CTL activity was detected using splenocytes from T9/mM-CSF(C2)-immunized rats, as measured by 6-h ^{51}Cr release assays. When purified T cells were isolated from the spleens of immunized rats, minimal CTL activity was observed even at a 100:1 E:T ratio. To determine whether CTLs could be generated in vitro, lymphocytes not used for the experiment in Fig. 6B were placed in culture for 1 wk in a MLTR using T9 stimulator cells. These lymphocytes were tested in a CTL assay against three rat glioma targets (Table I). The results showed that little or no CTL activity could be detected.

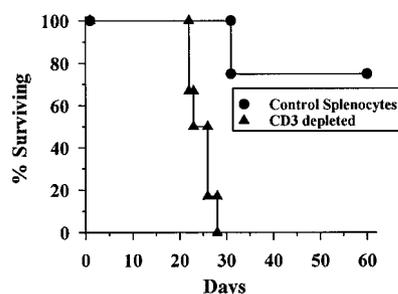


FIGURE 5. Passive transfer of T9 immunity by spleen cells from T9/mM-CSF(C2)-immunized rats is dependent upon CD3⁺ T cells. A total of 1×10^8 untreated or T cell-depleted spleen cells obtained from T9/mM-CSF(C2)-immunized rats were given i.p. to naive recipients and subsequently challenged i.c. with 1×10^5 parental T9 cells.

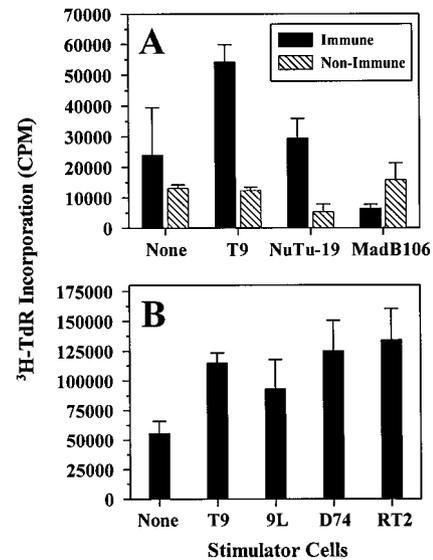


FIGURE 6. Splenocytes from T9/mM-CSF(C2)-immunized rats specifically proliferate in response to glioma Ag(s). Unfractionated splenocytes derived from either naive (nonimmune) rats or rats that rejected the T9/mM-CSF(C2) cells 3 wk earlier (immune) were tested in a MLTR assay against various syngeneic tumor cells. A, Immune control splenocytes used in the experiment shown in Fig. 5 were tested against inactivated parental T9 glioma, NUTU-19 ovarian cancer, or MADB106 breast adenocarcinoma tumor cells at a 10:1 lymphocyte:tumor cell ratio. The cultures were pulsed with [^3H]TdR on day 4 of the reaction, or B, immune splenocytes were allowed to proliferate against several inactivated syngeneic rat gliomas at a 10:1 lymphocyte:tumor cell ratio. The cultures were pulsed with [^3H]TdR on day 4 of the reaction. Bars represent SD.

Intracranial T9 gliomas are rejected with a s.c. injection of viable T9/mM-CSF(C2) cells

We observed that 100% of animals (10/10) injected s.c. with 1×10^7 viable T9/mM-CSF(C2) cells rejected their tumor in ~ 1 wk (Fig. 8). This was not the case, however, when animals were injected with T9/mM-CSF(H1) or T9/LXSN cells, each of which progressively formed large, ulcerating tumors. Animals that rejected the s.c. injection of T9/mM-CSF(C2) cells were completely resistant to i.c. rechallenge of 1×10^5 parental T9 cells (6/6 rats). Therefore, we investigated whether T9/mM-CSF(C2) cells could be used as a s.c. vaccine to induce an immune response that could protect an animal from a simultaneous implant of parental T9 cells in the brain. Animals were implanted i.c. with 1×10^4 or 1×10^5 parental T9 glioma cells and then received a single s.c. inoculation

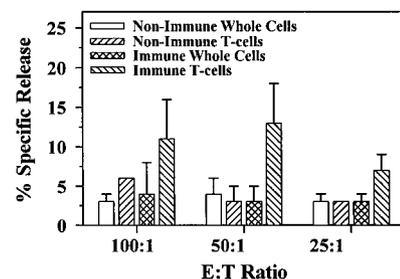


FIGURE 7. CTL activity of lymphocytes derived from T9/mM-CSF(C2)-immunized rats. Unfractionated whole splenocytes (Whole Cells) or BioTex column-purified T cells (T cells) derived from either a naive nonimmune rat (Nonimmune) or a rat that rejected the T9/mM-CSF(C2) cells 3 wk earlier (Immune) were tested in 6-h ^{51}Cr release assay against parental T9 target cells. Bars represent SD.

Table I. Lymphocytes stimulated in a MLTR do not display cytotoxicity against syngeneic rat glioma target cells^a

| E:T Ratio | Target Cells (% cytotoxicity ±SD) | | |
|-----------|--------------------------------------|-------|-------|
| | T9 | D74 | RT2 |
| 40:1 | 6 ± 2 | 3 ± 7 | 6 ± 1 |
| 20:1 | 4 ± 3 | 8 ± 3 | 4 ± 1 |
| 10:1 | 4 ± 3 | 6 ± 3 | 2 ± 2 |
| 5:1 | 2 ± 1 | 1 ± 9 | 2 ± 1 |

^a Spleen cells harvested from T9/mM-CSF(C2) immunized rats that were not used in the proliferation experiment shown in Fig. 6B were cultured for 1 wk in a bulk MLTR using T9 glioma cells as stimulator cells. The lymphocytes were then tested in a 6 h ⁵¹Cr release assay against syngeneic T9, D74, and RT2 glioma cells.

with either 1 × 10⁷ T9/mM-CSF(C2) or T9/LXSN cells. Fig. 9A demonstrates that a single s.c. vaccination with T9/mM-CSF(C2) cells was effective in protecting 100% (6/6) of the animals receiving a simultaneous i.c. injection of 1 × 10⁴ T9 parental cells. When 1 × 10⁵ parental T9 cells were given i.c., s.c. immunization with T9/mM-CSF(C2) cells was effective in saving 41% (7/17) of the animals (*p* = 0.04).

Discussion

There has been little advancement in the successful treatment of patients with glioblastoma multiforme, and traditional therapies, including surgery, radiation therapy, and/or chemotherapy, seldom alter the fatal progression of this disease. Novel approaches using immunotherapy are currently being explored for the treatment of brain cancer as well as other solid tumors. Recently, studies in animal models have demonstrated that tumor cells genetically engineered to express certain cytokines can elicit a strong antitumor response leading to tumor-specific immunity (3, 4, 15–18, 20, 21). In this study, we investigated the ability of T9 glioblastoma cells genetically engineered to express two different isoforms of M-CSF to induce an antitumor response when implanted into syngeneic Fischer rats.

The presented studies indicate that the response to M-CSF-transduced tumor cells is complex. T9 glioma cells expressing the secreted isoform of M-CSF failed to elicit an effective antitumor response when implanted either in the brain or s.c. This observation is similar to our experience with T9 glioma cells genetically engineered to secrete other cytokines such as TNF-α or IL-2, neither of which induced rejection of the tumor when implanted in the brain (Graf et al., unpublished). In contrast, T9 cells expressing the membrane-associated isoform of M-CSF are effectively rejected, and such animals develop tumor immunity, for they are completely

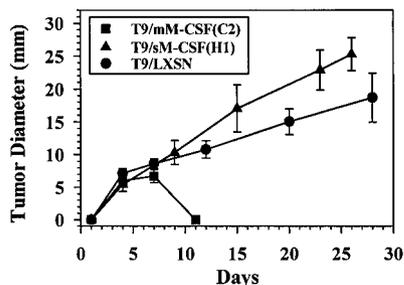


FIGURE 8. Subcutaneous growth of T9/mM-CSF(C2) cells. Animals were inoculated s.c. with 1 × 10⁷ T9/mM-CSF(C2), *n* = 10; T9/sM-CSF(H1), *n* = 4; or T9/LXSN, *n* = 4 cells, and monitored for tumor growth. Bars represent SD.

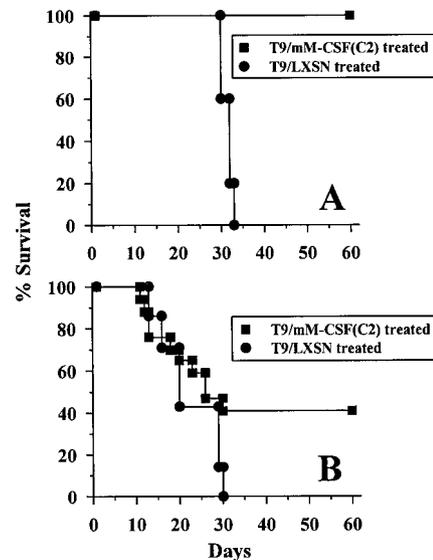


FIGURE 9. Survival of rats implanted with i.c. parental T9 gliomas and simultaneously vaccinated s.c. with T9/mM-CSF(C2) cells. A, Animals implanted in the brain with 1 × 10⁴ parental T9 cells and simultaneously given a s.c. vaccination with 1 × 10⁷ viable T9/mM-CSF(C2) (*n* = 6) or T9/LXSN (*n* = 5) cells. B, Same as in A, except 1 × 10⁵ parental T9 cells were implanted i.c. (*n* = 17 in the T9/mM-CSF(C2)-treated group, and *n* = 7 for the T9/LXSN-treated group).

resistant to i.c. rechallenge with parental T9 cells. This suggests that a membrane-bound cytokine is more potent than its soluble counterpart. Moreover, immunity was T cell mediated because spleen cells could transfer immunity to naive recipients and this was abrogated by the depletion of CD3⁺ T cells. Although little CTL activity against parental T9 cells was seen, lymphocytes from T9/mM-CSF(C2)-immunized rats did specifically proliferate in response to several syngeneic glioma cell lines, including parental T9 cells.

T9 glioma rejection appeared to be related to the level of expression of mM-CSF on the tumor cell membrane. Although 80% of the animals rejected an i.c. implant of clone T9/mM-CSF(C2), which expressed the highest level of mM-CSF, only 20% could reject clone T9/mM-CSF(F12), which expressed a much lower level of mM-CSF, and no animals rejected clone T9/mM-CSF(F2), which expressed the lowest level of mM-CSF. These differences were not due to changes in the levels of expression of MHC proteins because each of these clones showed similar levels of MHC class I and class II Ags, which were analogous to the levels expressed on T9 parental and T9/LXSN cells.

Histological analysis of the T9/mM-CSF(C2) tumor site 2 days after i.c. injection revealed infiltration of the tumor by numerous macrophage-like cells, many of which were in close physical contact with T9/mM-CSF(C2) cells. This was followed several days later by a marked influx of lymphocytes with evidence of perivascular cuffing and exocytosis. Although further work is needed, our studies suggest that the macrophage-like cells that may represent endogenous microglial cells are responsible for tumor destruction. It has been shown that microglial cells are tumoricidal (42, 43). These macrophage-like cells may be responsible for initiating the primary events leading to the destruction of the T9/mM-CSF(C2) cells. It is intriguing to note that only the T9/mM-CSF tumors were destroyed, while the T9/sM-CSF tumors were not, indicating that M-CSF must be present on the surface of the glioma cell to elicit the tumoricidal responses. Jadus et al. (27, 28) have suggested that the membrane-bound M-CSF molecules may provide a molecular bridge between the macrophages and the tumor cells and directly

induce the tumoricidal effects of the macrophages. However, while macrophages were the initial cells infiltrating T9/mM-CSF(C2) tumors, this was later followed by influxes of inflammatory cells, including numerous lymphocytes with the development of systemic T cell immunity. In this regard, passive transfer experiments demonstrated that glioma-specific immunity could be transferred to naive rats by splenocytes collected from T9/mM-CSF(C2)-immunized rats, and this was abrogated by depletion of CD3⁺ T cells. Moreover, the immunity induced by T9/mM-CSF(C2) cells was specific for glioma cells, as these rats could not reject i.c. challenges of a syngeneic breast adenocarcinoma. It is tempting to postulate that infiltrating macrophages have two roles in this model. First, they kill the T9/mM-CSF(C2) cells in a nonspecific manner through the M-CSF/M-CSF receptor bridge; and second, they phagocytize tumor debris and present processed tumor Ags to T cell precursors, thereby inducing T cell immunity. We believe that endogenous brain-associated macrophage/microglial cells are responsible for this immunizing effect because dendritic cells do not possess M-CSF receptors (23), whereas microglial cells do express M-CSF receptors (44, 45) and are known to act as APCs (46, 47).

T9/mM-CSF(C2) cells (but not T9/sM-CSF(H1) or T9/LXSN cells) were also rejected when injected s.c. This indicates that the host's immune cells are capable of rejecting mM-CSF-transduced cells outside of the brain. Subcutaneous rejection resulted in systemic immunity because these animals were completely resistant to a subsequent rechallenge with parental T9 cells in the brain. Based on this, we investigated whether a single s.c. inoculation of T9/mM-CSF(C2) cells could generate an effective systemic anti-tumor response that could protect an animal from a simultaneous intracerebral implant of parental T9 cells. Using this model, we were able to save 100% of animals given an i.c. implant of 1×10^4 parental T9 cells and 41% of the animals given an i.c. implant of 1×10^5 parental T9 cells. Thus, the result was clearly related to the dose of tumor in the brain. It is noteworthy that in these experiments, viable T9/mM-CSF(C2) cells injected s.c. formed a palpable tumor nodule that grew for ~5 to 7 days, at which point the nodule underwent accelerated regression in all animals. It is during this period of regression, beginning at about day 7, that the generation of tumor-specific immunity would most likely occur. At this point, the i.c. parental T9 glioma has undoubtedly become well established in the brain.

In summary, we have demonstrated in a syngeneic Fischer rat T9 glioblastoma model that tumor cells expressing mM-CSF can elicit an immune response resulting in systemic tumor immunity, whereas T9 glioblastoma cells secreting M-CSF fail to elicit such a response. It appears that macrophages are responsible for the initial destruction of T9/mM-CSF cells; however, glioma-specific immunity is transferred by T lymphocytes. Furthermore, we demonstrated that s.c. inoculation of T9/mM-CSF(C2) cells is effective in treating an i.c. T9 glioma. These findings may be important in the clinical application of mM-CSF-transduced glioma cells for the treatment of malignant brain tumors, in which such vaccines could be used to prevent tumor recurrence after initial surgical resection. **Note.** During the review of this manuscript, Soo Hoo et al. (48) reported that P815 tumor cells engineered to express the membrane form of GM-CSF were much more immunogenic and were able to elicit an effective antitumor immune response in a syngeneic mouse model.

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