Time course analysis and modulating effects of established brain tumor on active-specific immunotherapy

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Object. There have been numerous attempts to establish an effective immunotherapy for the treatment of brain tumors. To date, reliable methods to manipulate the immune system for promoting brain tumor regression have been disappointing. Generation of active immune responses in most of these studies was only possible in the absence of viable tumor cells, suggesting that immunotherapy can only be used as preventive therapy. In few studies the investigators have demonstrated success in using immunotherapy to treat a preestablished intracranial tumor. Using the 9L intracranial glioma model, the authors sought to delineate the underlying mechanisms for these observations.

Methods. In animals vaccinated with irradiated 9L glioma cells and interferon-gamma 14 and 7 days prior to intracranial tumor cell challenge, a significant increase in survival was shown. In contrast, vaccinations applied 3 days prior to, at the time of (Day 0) or 7 days after intracranial tumor cell challenge failed to influence survival. Histological examination of brain tissue specimens obtained in animals vaccinated before or after tumor cell challenge showed no difference in the degree of peritumoral mononuclear cell infiltration. When activated spleen cells obtained from animals were assayed for cytotoxicity and proliferative capacity, only those spleen cells derived from animals vaccinated prior to intracranial tumor cell challenge showed enhanced activity.

Conclusions. These data support the presence of a strong modulatory effect of tumor on local and systemic antitumor immune response. This immunosuppression appears to be secondary to a direct effect on T-cell function. Reversal of this immunosuppression may be a useful adjunct to tumor vaccine therapy.

Key Words • glioma • cancer vaccine • immunotherapy • immunosuppression
the vaccine against preestablished tumor explains, in part, the universally poor clinical results obtained when attempting to prolong survival in patients with high-grade gliomas who undergo trials involving manipulation of the immune system.\textsuperscript{2,18,35} The ability to create antitumoral immunity in the presence of an established intracranial tumor is clearly of clinical relevance. Tumor-induced immunosuppression is a well-recognized phenomenon,\textsuperscript{15,21,26} but the degree to which it contributes to the failure of current tumor vaccine treatment strategies is unclear. In this study we explore the importance of this phenomenon in an experimental intracranial tumor model, investigating the mechanisms responsible for immune inhibition. By using the Fischer 9L glioma model, the present study was instigated to delineate the mechanisms underlying the ineffectiveness of antitumoral immunotherapy in treating a preestablished syngeneic intracranial tumor. In using an array of survival experiments, histological surveys, and in vitro cytotoxicity and proliferation assays, the antitumoral effects of immunization before or after intracranial tumor cell challenge were studied. Analysis of our results demonstrates that tumor cells do exert a modulating effect on the immune system, contributing to the decreased efficacy of vaccine therapy against preestablished brain tumors. Specifically, we show that tumor-induced immunosuppression results in a decrease of T-cell cytoxicity and proliferation potential with no significant decrease in local T-cell infiltration. Collectively, these results point to the possibility of reversing tumor-induced immunosuppression as an adjunct to tumor-specific immunotherapy in the treatment of brain tumors.

\textbf{MATERIALS AND METHODS}

\textbf{Cell Line and Experimental Animal}

The 9L gliosarcoma, a malignant glioma cell line syngeneic to the Fischer 344 rat, was used for the proposed studies. Because the 9L gliosarcoma cell line is derived from a chemically induced tumor, it may be more immunogenic than other tumor cell lines. However, the 9L gliosarcoma cell line is known to secrete TGF\textsuperscript{2,}\textsuperscript{2} a major immunosuppression factor secreted by human glioma cells, making it very similar to human gliomas.\textsuperscript{23} The tumor cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, 50 \mu M 2-mercaptoethanol and 2 mM glutamine. The cells were grown in a humidified incubator at 37°C with a 5% CO\textsubscript{2}/air atmosphere. The Fischer 344 male rats with a mean weight of 250 ± 20 g were maintained and treated according to the guidelines of the National Institutes of Health for the care and use of laboratory animals.

\textbf{Subcutaneous Vaccination}

An 0.8-ml dose of PBS containing 5 × 10\textsuperscript{6} i9L cells (10,000 cGy) and 10,000 U IFN-\gamma was injected subcutaneously into the right hind leg of the rats. After the first injection, the rats received daily subcutaneous injections of IFN-\gamma (10,000 U in 0.3 ml) at the original site for an additional 5 days. Interferon-\gamma was chosen as the adjuvant in these vaccination studies for three reasons. First, because IFN-\gamma can increase class I MHC and class II MHC molecule expression on tumor cells,\textsuperscript{33,37} it acts to enhance the development of cytotoxic T cells.\textsuperscript{19} Second, IFN-\gamma has been shown to induce a potent antitumoral immune response when used as an adjuvant\textsuperscript{14} or in a genetically engineered tumor vaccine.\textsuperscript{3,6} Third, in experiments performed in our laboratory,\textsuperscript{22} a vaccine of i9L cells plus IFN-\gamma as an adjuvant, administered prior to intracranial tumor cell challenge, resulted in the longest animal survival rate, compared with using IL-2, GM-CSF, or GM-CSF plus IFN-\gamma.

\textbf{Intracranial Tumor Implantation}

Upon attainment of confluence, 9L glioma cells were trypsinized, washed three times in RPMI medium, and then resuspended in PBS. Using a Kopf stereotactic frame and a Hamilton syringe, a 10-\mu l dose of PBS containing 5 × 10\textsuperscript{3} of tumor cells was injected into the right frontal lobe of the rat. The number of glioma cells implanted was sufficient to cause the death of 100% of the rats within 30 days.\textsuperscript{14} Coordinates used for intracranial injection were 2 mm to the right and 2 mm anterior to the bregma, and 3 mm deep from the outer border of the cranium. After receiving the intracranial injections, the rats were monitored twice a day for neurological signs and weighed every 3rd day until the end of the experiment (60 days post–tumor cell implantation). All rats were killed once moribund or on Day 60. At the time of death, the brains of all rats were harvested for histological examination.

\textbf{Experimental Design}

To test the efficacy of tumor vaccine on preventing tumor formation, “prevention” and “treatment” experiments were conducted. In the “prevention” experiment, six rats per group were immunized subcutaneously with i9L cells, IFN-\gamma, PBS, or a mixture of i9L cells plus IFN-\gamma. One week after immunization animals were challenged with intracranial implantation of 5 × 10\textsuperscript{3} of viable 9L glioma cells. Survival in this group of animals was then compared with that of similar “treatment” group in which vaccination was administered on the same day of intracranial tumor cell challenge or 7 days after intracranial tumor cell challenge.

\textbf{Cytotoxicity Assay}

For these studies, rats either received vaccination with i9L plus IFN-\gamma on Day 0, intracranial tumor cells challenge on Day 0, intracranial tumor cell challenge on Day 0 followed by vaccination with i9L plus IFN-\gamma on Day 7, or PBS on day 0 (control rats). At various times after treatment, mononuclear cells from the spleens of the Fischer 344 rats were used as the source of effector cells for cytotoxicity studies. To isolate the mononuclear cells, spleens removed from two rats at each of the various time intervals after vaccination were studied. Pooled cell suspensions were then prepared by forcing the spleens through a 40-gauge stainless-steel screen mesh in a small quantity of RPMI-1640 medium. The cell suspensions were then subjected to gradient centrifugation in which a premixed gradient solution was used. Aliquots of the cell suspensions were then coincubated with mitomycin C–treated 9L glioma cells in RPMI 1640 medium, supplemented with 10% FBS, 50 \mu M 2-mercaptoethanol, 2 mM glutamine,
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1mM puruvate, and nonessential amino acids, under cell culture conditions for 5 days. To perform the cytotoxicity assay, $5 \times 10^4$ of 9L glioma tumor cells (target cells) were incubated with the stimulated mononuclear cells (effector cells) at an effector/target ratio of 10:1 for 4 hours at 37°C, in culture media containing 5% FBS. At the end of the incubation, mononuclear cell–mediated cytotoxicity was determined using the nonradioactive cytotoxicity assay kit. Released LDH in culture supernatants was measured by an ELISA plate reader. The data were calculated as: 

$$\text{CYTOTOXICITY} = \frac{(\text{EXR} - \text{ESR} - \text{TSR} + \text{CMB})}{(\text{TSR} - \text{VC} + \text{CMB})} \times 100$$

where EXR is experimental LDH release, ESR is effector cell spontaneous LDH release, TSR is target cell spontaneous LDH release, TMR is target cell maximum LDH release, VC is volume correction, and CMB is culture medium LDH background.

**Proliferation Assay**

Nonadherent mononuclear cells (responder cells) derived from the spleens of the rats were obtained as previously described. For these studies, animals received either vaccination with i9L plus IFN-γ on Day 0, intracranial tumor cell challenge on Day 0 followed by subcutaneous injection of PBS on Day 7, intracranial tumor cell challenge on Day 0 followed by i9L plus IFN-γ vaccination on Day 7, or vaccination with PBS on Day 0 (control rats). Seven days after the various treatments, the spleen was removed for the harvesting of mononuclear cells. These cells were incubated with mitomycin C–treated 9L tumor cells (stimulator) at a stimulator/responder ratio of 0.4:1 in the wells of a 96-well flat-bottomed microtiter plate (5 $\times$ $10^4$ responder cells/well). After 72 hours incubation, mononuclear cell proliferation was determined using a nonradioactive cell proliferation assay kit, as described by the manufacturer.

**Histological Detection of Mononuclear Cell Infiltration**

To detect mononuclear cell infiltration at the site of intracranial tumor cell challenge, two series of experiments were conducted. The first involved immunization prior to tumor cell challenge (prevention experiment), and the second involved immunization after tumor cell challenge (treatment experiment). In the first study, two rats in each group were injected subcutaneously with 9L cells and IFN-γ on Day 0, challenged intracranially on Day 7, and killed on Day 12, 17, or 22. For the treatment study, rats in each group were intracranially challenged on Day 0, vaccinated on Day 7, and killed on Day 12, 17, or 22. The brain from each animal was removed, fixed in 10% formalin, and embedded in paraffin for histological examination. Four-micrometer tissue sections were stained with hematoxylin and eosin, according to standard procedures, and evaluated for the presence of mononuclear cell infiltration by a neuropathologist blinded to the treatment groups.

**Statistical Analysis**

Survival estimates and median survivals were determined using the method of Kaplan and Meier. Survival data were compared using nonparametric log-rank analysis. Student’s unpaired t-test was used to determine the differences between the various groups in the proliferation and cytotoxicity assays. Statistical significance was determined at the 0.01 level.

**Sources of Supplies and Equipment**

The 9L gliosarcoma cell line was kindly provided by Dr. Kruse (University of Colorado Health Sciences Center, Denver, CO) We obtained RPMI-1640 medium from Life Technologies, Inc. (Bethesda, MD). The Fischer 344 male rats were acquired from Charles River Laboratories (Wilmington, MA). The IFN-γ was produced by R&D Systems (Minneapolis, MN). Both nonradioactive assay kit (CytoTox 96 and CellTiter 96 AQ) were manufactured by Promega (Madison, WI).

**RESULTS**

**Efficacy of Tumor Vaccine in Preventing Tumor Development**

We studied the effect on survival of vaccinating rats with a mixture of i9L cells and IFN-γ 7 days prior to intracranial tumor cell challenge (Fig. 1). The MST of control animals immunized with IFN-γ alone (28.5 days) was indistinguishable from that for animals receiving PBS alone (26.8 days). In contrast, prolonged survival was observed in rats immunized with i9L or i9L plus IFN-γ (MST 45.8 days and 56.5 days, respectively). All animals surviving to Day 60 were killed, and histological examination showed no evidence of residual intracranial tumor. These results suggest that immunizing animals with either i9L or a mixture of i9L and IFN-γ resulted in the generation of an effective antitumoral immune response. However, the protective effect of i9L and IFN-γ was greater than that conferred by i9L alone.

To test the effect of time of vaccine administration relative to tumor cell challenge on animal survival, animals were immunized with i9L plus IFN-γ 14, 7, 5, 3, or 0 days prior to intracranial tumor cell challenge. Groups tested consisted of five rats each. Results indicated that the survival of rats immunized 5, 3, and 0 days prior to tumor challenge was not statistically different from that in those receiving PBS alone (Fig. 2). In contrast, in rats immunized 14 and 7 days prior to tumor challenge significant protective immunity was demonstrated (MST 46.0 days and 54.2 days, respectively; Fig. 2). Histological examination of all rats that survived until Day 60 revealed no evidence of viable intracranial tumor cells. These results suggest that the optimum time period required to develop an effective antitumoral immune response in rats receiving a subcutaneously injected tumor vaccine is 7 days or longer.

**Efficacy of Tumor Vaccine in Treating Established Tumor**

Experiments were conducted to determine whether vaccinating rats with i9L plus IFN-γ is effective in extending the survival of animals with preestablished intracranial 9L gliomas. In these studies, rats were immunized at various time intervals after intracranial 9L glioma tumor challenge. The survival of rats receiving immunization 0 or 7 days after tumor challenge was not significantly different.
from that in rats receiving PBS alone (p = 0.7092 and 0.3680, respectively; Fig. 3A and B).

A number of mechanisms can be proposed to explain why the vaccination failed to impact survival under these circumstances. It is possible that: 1) a single vaccination of the animal in the presence of viable intracranial tumor cells is insufficient to generate a potent antitumoral immune response; or 2) an established brain tumor may exert an inhibitory influence on the development of an antitumoral immune response. To examine the former possibility, an additional experiment was performed in which rats received four vaccinations of i9L plus IFN-γ—one each on Days 5, 8, 11, and 14 after intracranial tumor cell challenge. If our first possibility is correct, the increased number of vaccinations should enhance the immune response and extend the survival of rats. This did not appear to be

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**Fig. 1.** Effect of vaccination with i9L plus IFN-γ on survival of rats challenged with intracranial tumor cells. Rats (six per group) were immunized subcutaneously in the hind leg with either i9L, IFN-γ, PBS, or a mixture of i9L plus IFN-γ. One week after the initial immunization, rats were challenged intracranially with $5 \times 10^3$ of viable 9L glioma cells. The p values refer to comparison with PBS-treated rats.

**Fig. 2.** Effect of time of vaccine administration on survival of rats challenged with intracranial tumor cells. Rats (five per group) were immunized with i9L plus IFN-γ at 14 (−14), 7 (−7), 5 (−5), 3 (−3), and 0 days before intracranial tumor cell challenge (Day 0). Control rats received an equivalent volume of PBS on the same day of intracranial tumor cell challenge. The p values refer to comparison with PBS-treated rats.
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infiltration. We then examined its effect on host mononuclear cell proliferative capacity and cytotoxicity.

**Determination of Mononuclear Cell Infiltration at the Site of Tumor Cell Challenge**

To examine if established brain tumors can exert a repressive effect on the antitumoral immune response, the extent of mononuclear cell infiltration at the intracranial site of tumor cell implantation was examined following various vaccination regimens (that is, the prevention and treatment protocols). In the prevention experiments (when vaccination was applied 7 days prior to intracranial tumor cell implantation), extensive mononuclear cell infiltration into the tumor and the surrounding brain was observed 10 days after tumor cell challenge (Table 1). Five days after this observation (that is, 15 days after tumor cell challenge), tumors in the vaccinated rats showed marked signs of regression. In the control rats that received PBS, only a few mononuclear cells were observed at the tumor edge. The tumors in this group of animals continued to grow and showed no sign of regression even 15 days after tumor cell challenge (Table 1).

In the treatment group (that is, when vaccination was initiated 7 days after tumor cell challenge), extensive infiltration of the tumor and the surrounding brain by mononuclear cells was also noted. However, it took slightly longer for these immune cells to appear when compared with the prevention protocol (17 days and 10 days, respectively). Despite this mononuclear cell infiltration, tumors in these rats continued to grow (Table 2). In both the prevention and treatment studies, challenging the vaccinated animals with an intracranial injection of PBS did not result in any appreciable mononuclear cell infiltration at the site of intracranial injection.

Together, these data demonstrate that subcutaneous immunization prior to (prevention) or after (treatment) intracranial tumor cell challenge does not result in a significant difference in local peritumoral mononuclear cell infiltration. However, as shown earlier, when vaccination is applied 7 days or more prior to tumor cell challenge, intracranial tumor cell growth is significantly inhibited. Thus, our data indicate that local intracranial suppression of mononuclear cell infiltration is not the mechanism by which the tumor inhibits the antitumoral immune response. With the tumor exhibiting no appreciable effect on the number of locally infiltrating peritumoral mononuclear cells, this underscores the possibility that the tumor may be influencing the function of the locally infiltrating peritumoral mononuclear cells.

**Tumor-Induced Immunomodulating Effect on Cytotoxic and Proliferation Activity of Systemic Mononuclear Cells**

To examine if established brain tumors indeed exert an immunomodulating effect, the cytotoxic and proliferative activity of mononuclear cells obtained from the spleens of immunized and nonimmunized rats, with or without established intracranial tumor, were studied. If an established tumor exerts an immunomodulatory effect on T lymphocytes, mononuclear cells obtained from rats vaccinated 7 or 14 days prior to T-cell harvest should exhibit greater cytotoxicity toward target 9L cells than those vaccinated 7 days after tumor cell challenge (Fig. 4). Simi-
larly, mononuclear cells obtained from rats vaccinated 7 days prior to T cell harvest should also show greater proliferation than those obtained from a nonvaccinated group or a group vaccinated after initiation of tumor cell challenge (Fig. 5). In both cases, our data clearly demonstrate that mononuclear cells obtained in animals vaccinated prior to tumor cell challenge exhibited greater cytotoxic and proliferative activity toward target 9L glioma cells. Minimal cytotoxic or proliferative activity was observed in mononuclear cells obtained in animals that received no immunization or in animals vaccinated 7 days after intracranial tumor cell challenge. These data are consistent with the results demonstrated in our earlier survival experiments and support the premise that the antitumoral immunity seen is not the result of increased peritumoral T-cell infiltration but, rather, the development of increased tumor-specific cytotoxic T-cell activity. Furthermore, our data support the premise that established tumor exerts an inhibitory effect on the proliferative and cytotoxic activity of mononuclear cells subjected to systemic vaccine therapy.

**DISCUSSION**

In this study we have demonstrated that subcutaneous vaccination with 9L glioma cells plus IFN-γ, in an intracranial brain tumor model, can generate a potent protective antitumoral effect against subsequent intracranial 9L glioma challenge in the Fischer 344 rat. These findings are consistent with the work of previous studies in which the investigators used a variety of cytokines including IL-12, IL-2, GM-CSF, IL-6, and IL-3. Our model differs from those of previous investigators in that we purposefully chose not to depend on cytokine gene transfection but rather the coadministration of irradiated tumor cells and cytokine subcutaneously. This is a subtle but important difference when considering the adaptation of this work to human use. The clinical use of gene-transfected tumor vaccines poses several potential difficulties. First among these is the need to culture glioma cells obtained from patients prior to treatment. It is not always possible to establish stable cultures from patients with gliomas, and in the process of establishing these stable cell lines a certain degree of growth selection inevitably occurs. This carries the risk of changing the antigenic nature of the tumor, which in turn raises the concern that the cultured tumor may no longer be representative of the patient’s tumor. Second is the need to then transfec these stable cell lines, which requires valuable time that the patient may not have. In our tumor model, freshly harvested tumor cells are irradiated and immediately available for use, independent of viability. This greatly simplifies the ability to translate promising results into human clinical trials.

In the present study, we have also demonstrated that the development of an intracranial antitumor immune response follows a very specific time course. Our data indicate that at least 5 days are required for the immune system to develop an effective antitumoral immune response, with the optimum response being obtained when vaccination was applied 7 days prior to tumor cell challenge. This is the first examination of the time course for an intracranial antitumor immune response from a systemic vaccina-
tion has been examined. The results, not unexpectedly, are consistent with the appearance of graft-specific CD8+ cytotoxic T lymphocytes detected after 7 to 10 days in a mixed leukocyte reaction or MHC-restricted CD8+ cytolytic T lymphocytes detected 5 to 10 days following viral infection. The failure to generate an effective immune response at shorter intervals between administration of vaccine and initiation of tumor cell challenge or after tumor cell challenge is probably multifactorial. Our data support the presence of a tumor-associated immunomodulating factor. This tumor-associated immunomodulating factor does not appear to have a significant effect on local intracranial peritumoral immune cell infiltration but, rather, affects the inherent tumor-specific cytotoxicity and proliferative capacity of these immune cells. Tumor-induced immunosuppression has been well documented in

Fig. 4. Bar graph showing cytotoxicity analysis of spleen cells obtained in primed and unprimed rats. Effector cells derived from animals vaccinated 7 days and 14 days before harvesting displayed greater cytotoxicity than those harvested in animals challenged intracranially with 9L glioma cells alone or those vaccinated 7 days after intracranial tumor cell challenge. Data are presented as the mean ± standard error of the mean from two separate experiments.

Fig. 5. Bar graph showing proliferation analysis of spleen cells obtained in primed and unprimed rats. Mononuclear cells derived from rats vaccinated 7 days prior to killing showed greater proliferation than those from either the control rats, those challenged intracranially with 9L glioma cells, or those that received vaccination 7 days after intracranial tumor cell challenge. Data are presented as the mean ± standard error of the mean from two experiments.
patients with malignant glioma and in tumor-bearing ani-
mals. The responsible factor, however, remains un-
known. Tumor cells have been shown to release a num-
ber of immunomodulating factors that function to suppress
the antitumoral immune response. Candidate
factors in gliomas include TGFβ2, IL-10, and prosta-
glandin E2.

Transforming growth factor–β2, the most potent of
these factors, has been shown to be secreted in a high per-
centage of malignant gliomas. Not unlike that demonstrat-
ed in this study, TGFβ2 has been shown to suppress T-cell
activity. Preliminary studies in our laboratory focusing
on the local inhibition of TGFβ2, through the use of anti–
TGFβ2 antibodies, is being explored as a promising ad-
joint to the use of tumor vaccines. The future of tumor
vaccine therapy in the treatment of malignant glioma may
very well be a multimodal approach in which tumor-spe-
cific immune stimulation is combined with inhibition of
tumor-induced immunosuppression.

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