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Comparative analysis of paracrine immunotherapy in experimental brain tumors

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Object. Local delivery of cytokines has been shown to have a potent antitumor activity against a wide range of malignant brain tumors. In this study, the authors examined the efficacy of treating central nervous system (CNS) tumors by transfecting poorly immunogenic B16/F10 melanoma cells with interleukin (IL)-2, IL-4, or granulocyte-macrophage–colony stimulating factor (GM-CSF) gene, and using these cells to deliver the cytokine locally at the site of the CNS tumor. The object was to determine which cytokine would possess the greatest antitumor activity and to further elucidate its mechanism of action.

Methods. The transfected B16/F10 cells were irradiated to prevent replication and injected intracranially into C57BL/6 mice (10 mice per group) along with nonirradiated, nontransfected B16/F10 (wild-type) melanoma cells. Sixty percent of mice treated with IL-2 ($p < 0.001$ compared with control) and 10% treated with IL-4 (median survival = 31 days, $p < 0.001$ compared with control) were long term survivors (> 120 days). The median survival for animals treated with GM-CSF was 22 days with no long term survivors ($p = 0.01$ compared with control). Control animals that received only wild-type cells had a median survival of 18 days (range 15–20 days). Histopathological examination of brains from animals killed at different times showed minimal infiltration of tumor cells in the IL-2 group, moderate infiltration of tumor cells in the IL-4 group, and gross tumor invasion and tissue necrosis in the GM-CSF group. Animals treated with IL-2 showed a strong CD8 T cell–mediated response, whereas IL-4 evoked a prominent eosinophilic infiltrate in the area of the tumor.

Conclusions. High levels of locally expressed IL-2 rather than IL-4 or GM-CSF stimulate a strong immunological cytotoxic antitumor response that leads to significant prolongation of survival in mice challenged with B16/F10 intracranial melanoma tumor cells. Consequently, IL-2 may be a superior candidate for use in paracrine immunotherapy.

KEY WORDS • immunotherapy • interleukin • histopathology • immunohistochemistry • brain tumor

Innovative strategies are urgently needed for the treatment of patients with malignant gliomas. Conventional therapy consists primarily of surgical debulking and radiation. Unfortunately, the median survival time for patients after surgical intervention alone is 6 months, and only 7.5% of patients survive for 2 years. The addition of radiation therapy can extend median survival to 9 months.[2,3,14] Despite recent advances in drug delivery to tumors of the CNS, little progress has been made in extending overall patient survival. Consequently, efforts aimed at developing new therapies have focused on new treatment strategies that specifically target tumor cells and spare normal cells. One such modality, immunotherapy, has shown promise in the spectrum of agents used against malignant brain tumors.

Abbreviations used in this paper: BBB = blood–brain barrier; CNS = central nervous system; GM-CSF = granulocyte-macrophage–colony stimulating factor; IL = interleukin; MHC = major histocompatibility complex.

The identification and cloning of cytokines has provided one important tool for manipulation of the immunological response to tumors. The generation of an effective immune response requires both the presentation of a foreign antigen to lymphocytes and an appropriate stimulatory molecule such as a cytokine. The rationale for using cytokines is based on their ability to produce a strong local inflammatory response that is specific to a particular cytokine. High local concentrations closely mimic the natural biology of cytokine action. In the context of this paracrine physiology, it has been hypothesized that cytokine gene–transduced tumor cells can alter the local immunological environment and thus enhance either antigen presentation or activation of tumor-specific lymphocytes.

To date, authors of several studies have attempted to exploit the ability of cytokine gene–transduced tumor cells in the immunotherapy of tumors. For instance, Glick and colleagues[8] demonstrated a significant increase in survival in the mouse glioma model when tumor cells mixed with IL-2–secreting allogeneic fibroblasts were injected intra-

cerebrally. This work has been further corroborated by reports from our own laboratory in which IL-2 was previously shown to have potent antitumor activity against the B16/F10 intracranial melanoma model.[21] Two other cytokines, IL-4 and GM-CSF, were found to have potential therapeutic applications. For example, cells engineered to secrete IL-4 have been shown to cure animals with renal cell tumors.[9] In turn, GM-CSF has been shown to play a major role in the induction of long-lived systemic antitumor immunity capable of rejecting tumors in animals previously immunized with a gene-transduced tumor cell.[6,12]

In this study, our objective was to compare directly the antitumor efficacy of IL-2, IL-4, and GM-CSF in a single model and to elucidate each cytokine's mechanism of action. By using tumor cell lines transfected with genes for each of these cytokines, we examined their efficacy in treating mice with CNS tumors. We then performed immunohistochemical analysis of animal brains to provide a better understanding of the nature of the immune response.

MATERIALS AND METHODS

Tumor Cell Lines and Animals

The B16/F10 melanoma cells (National Cancer Institute-Division of Cancer Treatment and Diagnosis Tumor Repository [Frederick, MD]) were cultured in Dulbecco's modified Eagle Medium containing 10% fetal calf serum and penicillin/streptomycin. The B16/F10 cells were transduced with the murine IL-2, IL-4, and GM-CSF genes by using the MFG retroviral vector, as previously described.[6] The amount of IL-2, IL-4, or GM-CSF produced by the transformed tumor cells was measured before each experiment by a standard enzyme-linked immunosorbent assay technique (Endogen, Cambridge, MA). Cultured monolayers were harvested with trypsin and resuspended in Dulbecco's modified Eagle Medium before injection. Tumor cells were exposed to 5000 cGy from a 137-cesium source (Gammacell model 62 irradiator; Nordin International, Inc., Kanata, Ontario, Canada) discharging 1378 cGy/minute, immediately before injection to render them incapable of replication. The C57BL/6 female mice (6–12 weeks old) were obtained from Harlan (Indianapolis, IN).

Experimental Intracranial Model

Mice were anesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing 25 mg/ml ketamine hydrochloride, 2.5 mg/ml xylazine, and 14.25% ethyl alcohol diluted 1:3 in 0.9% NaCl. For stereotactic intracranial injections of tumor cells, the surgical site was shaved and prepared with 70% ethyl alcohol and prepodyne solution. After a midline incision, a 1-mm right parietal burrhole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were then placed in a stereotactic frame and cells were delivered by a 26-gauge needle to a depth of 3 mm over a period of 3 minutes. The total volume of injected cells was 5 μl. The needle was removed, the site was irrigated with sterile 0.9% NaCl, and the skin was sutured with 4.0 vicryl.

Intracranial Cytokine Studies

The efficacy of local paracrine intracranial immunotherapy was tested in three experimental groups (10 mice in each group). The antitumor activity of irradiated IL-2–, IL-4–, or GM-CSF–secreting tumor cells was compared to that in control animals that received coinjections of wild-type tumor combined with either irradiated wild-type tumor cells or 0.9% NaCl. All animals were treated with stereotactic intracranial injections of 100 live nonirradiated, noncytokine–producing B16/F10 melanoma cells. Injection of these cells has been shown to produce a large tumor at the injection site that is uniformly fatal, with a median animal survival time between 16 and 18 days.[21] Based on our previously published toxicity studies, each of the animals received 7.5×10^4 IL-2–producing cells (80 ng/10^6 cells/24 hours), 2.5×10^6 IL-4–producing cells (40 ng/10^6 cells/24 hours), or 10^5 GM-CSF–producing cells (60 ng/10^6 cells/24 hours).[22] The results are based on five independent sets of experiments performed over the course of this study.

Histological Evaluation

One set of 10 animals was set aside for the purpose of histopathological examination. The animals were killed on Days 1, 4, 9, and 14. The brains were removed, the tissue was fixed in 10% formalin, blocked in paraffin, sectioned in the coronal plane in 10-μm sections, and stained with hematoxylin and eosin. Immunohistochemical analysis using the peroxidase–antiperoxidase technique was also used with the following primary antisera: CD3, CD4, L26, or CD8. A murine lymph node was used as a positive control.

Statistical Analysis

For all efficacy studies, survival was the primary endpoint. All animals were monitored for any sign of neurotoxicity and underwent autopsy, when possible, to confirm that death was due to intracranial tumor. Survival was plotted using a Kaplan–Meier survival analysis and statistical significance was determined by the Kruskal–Wallis nonparametric analysis of variance followed by the nonparametric analog of the Newman–Keuls' multiple comparison test.[13]

RESULTS

Intracranial Paracrine Immunotherapy With IL-2 Rather Than IL-4 or GM-CSF Significantly Inhibits Tumor Cell Proliferation

All of the animals treated in this study were divided into one of the four groups receiving: IL-2, IL-4, GM-CSF, and control (Fig. 1 and Table 1). Sixty percent of mice treated with IL-2 ($p < 0.001$ compared with control) and 10% treated with IL-4 (median survival 31 days; $p < 0.001$ compared with control) were long-term survivors (>120 days). The median survival for animals treated with GM-CSF was 22 days ($p = 0.01$ compared with control). Control animals that received only wild-type cells had a median survival of 18 days (range 15–20 days). Within the experimental group, IL-2 was superior to IL-4 and GM-CSF (Table 2). Histopathological examination of

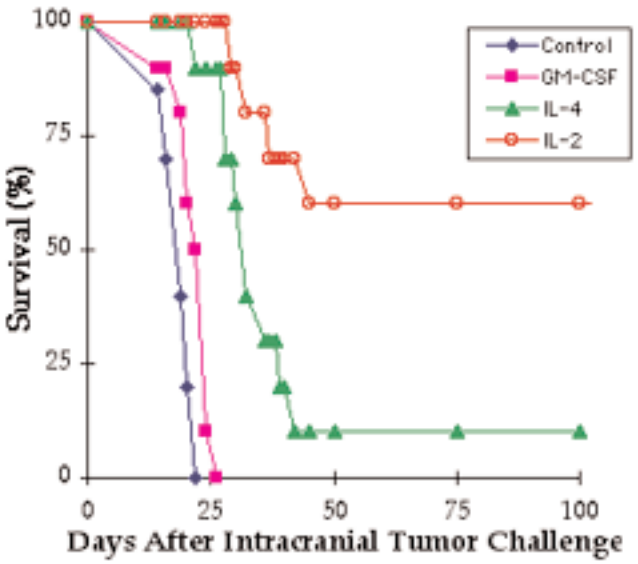

Fig. 1. Graph showing survival of mice after intracranial delivery of cytokine-transduced tumor cells and intracranial tumor challenge. Animals were treated with a single intracranial injection of irradiated B16/F10 melanoma cells engineered by gene transfer to secrete IL-2, IL-4, or GM-CSF. Control animals received intracranial injections of either normal saline or irradiated wild-type B16/F10 cells (noncytokine producing) (10 animals per group). All animals were challenged at the same time as their treatment with intracranial stereotactically coinjected nonirradiated wild-type B16/F10 melanoma cells. The results represent cumulative data for five independent sets of experiments. The mice receiving IL-2 and those treated with IL-4 showed prolongation of survival compared with controls ($p < 0.001$ for IL-4, $p < 0.001$ for IL-2).

brains in animals killed 14 days after injection of tumor cells showed that the majority of tumor cells were centered in the region of caudate/putamen, septal nuclei, and ventricular spaces. Furthermore, the tumor cells were noncohesive and associated with an inflammatory infiltrate. The brain surrounding the injection site showed some reactive changes with a mild leptomeningeal inflammatory infiltrate. There was minimal infiltration with tumor cells in the IL-2 group (< 1 mm), a moderate amount of tumor infiltration in the IL-4 group (1–2 mm), and a significant tumor volume in the GM-CSF group (> 2 mm) (Fig. 2).

TABLE 1

*Statistical analysis of experimental data after intracranial delivery of cytokine-transduced tumor cells and intracranial tumor challenge**

Cytokine	Median Survival (Days)	No. & % Long Term Survivors	p vs. Control
IL-2	NA	30, 60%	$p < 0.001$
IL-4	31	5, 10%	$p < 0.001$
GM-CSF	22	0	$p = 0.24$
control	18	0	NA

* NA = not applicable.

The Immune Response in Mice Treated With IL-2 is Composed of CD8 T Cells

To understand the antitumor effects of IL-2 better, we stained animal brains with different tissue markers. Whereas all animals treated with IL-2 reacted strongly with the T-cell marker CD3, none of them showed any evidence of an L26 B-cell response. Furthermore, when examined for CD4 or CD8 markers, the brains of the mice treated with IL-2 stained negatively for CD4 and positively for CD8 (Fig. 3). These results showed no variation in the type of immune infiltrate over a course of 2 weeks.

Mice Treated With Intracranial Il-4 Mount an Eosinophilic Response to Tumor Cells

Although animals treated with IL-4 did not survive as long as those treated with IL-2, they lived longer than animals treated with GM-CSF and those in the control group. Histopathological examination of the brains of animals treated with IL-4 showed no evidence of infiltration with CD3, L26, CD4, or CD8 cells (Fig. 3). Nevertheless, these animals showed a strong eosinophilic infiltrate in the area of tumor injection that appeared as early as Day 1 after tumor injection and disappeared by Day 14 following tumor injection (Fig. 4).

Tumor Cell Infiltrates of Mice Treated With GM-CSF Show no Immune Response

Mice treated with GM-CSF had the poorest survival and greatest tumor burden at death. Examination of the brains of animals treated with GM-CSF showed a lack of immune response, with no evidence of staining for CD3, L26, CD4, CD8, or eosinophils.

DISCUSSION

The role of the immune system in the response to tumors of the CNS remains a matter of debate. For many years, it was believed that the BBB effectively prevented any interaction between the immune system and the brain parenchyma. In recent years, however, the results of numerous studies indicate an active although unclear role of the immune system in the CNS. The breakdown of the BBB that occurs with both malignant and metastatic brain tumors clearly causes infiltration of inflammatory cells within the tumor confines.[1,16] Moreover, considerable evidence has accumulated indicating that activated T cells can pass through an intact BBB and enter the CNS.[10,22] The identification of tight connections between the CNS and the immune system through cervical lymphatics has further demonstrated that both the afferent and efferent

TABLE 2

Comparative statistical analysis of experimental data between different cytokine groups

Cytokine	p Value
IL-2 vs IL-4	0.01
IL-2 vs GM-CSF	< 0.0001
IL-4 vs GM-CSF	< 0.0001

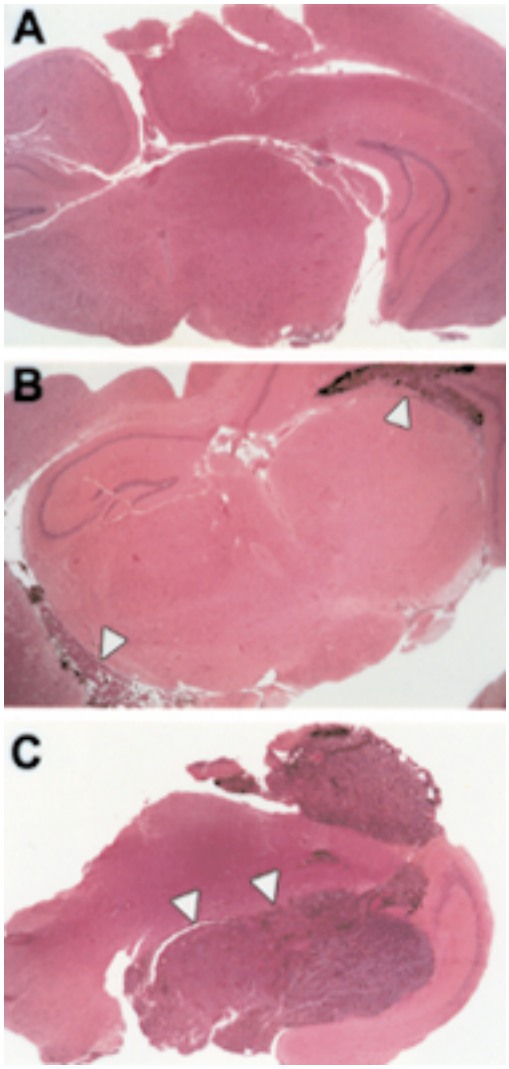

Fig. 2. Photomicrographs of animal brains 2 weeks after injection of tumor cells. A: There was minimal infiltration with tumor cells in the IL-2 group (< 1 mm). B: There was a moderate amount of tumor infiltration in the IL-4 group (1–2 mm). C: There was a significant tumor volume in the GM-CSF group (> 2 mm). The tumor cells were centered mostly in the region of caudate/putamen, septal nuclei, and ventricular spaces. H & E, original magnification × 20.

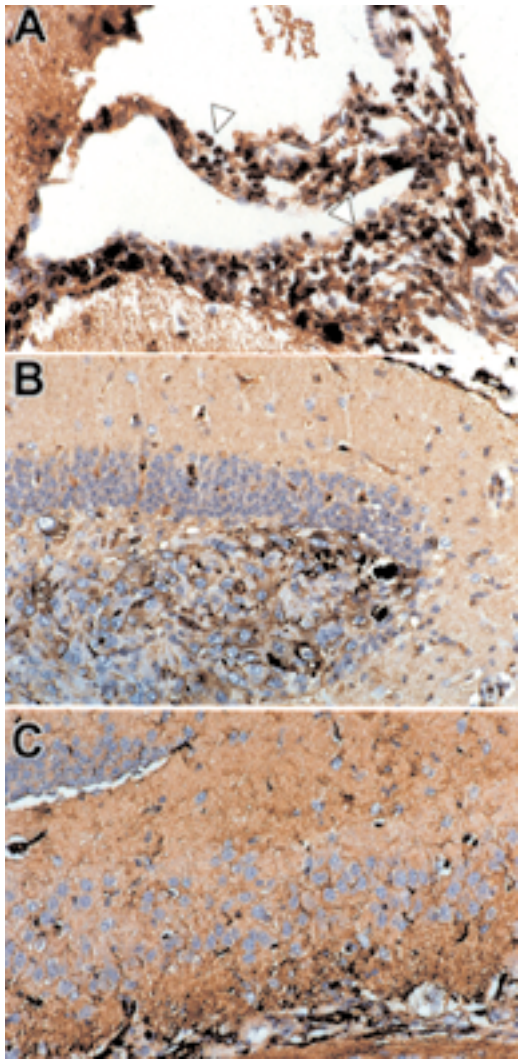

Fig. 3. Photomicrographs showing the results of immunohistochemical analysis of animal brains stained for the presence of the T-cell marker CD8. Animals treated with (A) IL-2, (B) IL-4, or (C) GM-CSF showed the presence of CD8 cells *(arrowheads)* only in the IL-2 treatment group. There was no evidence of CD8 cells in either the IL-4 or the GM-CSF group. Original magnification × 300.

arms of the immune system are functional and that passage of lymphocytes into the CNS can occur via expression of specific adhesion molecules.[5,23] Taken together, the results of these studies clearly indicate that the immune system plays an important although not fully understood role in the body's natural response to brain tumors.

In our laboratory, we have focused on using melanoma cells transduced with genes for different cytokines, which have been irradiated to prevent replication, as vehicles for delivery of the cytokine locally to the brain tumor. The B16/F10 melanoma cell line is an attractive tumor model because it is poorly immunogenic and expresses low levels of MHC Class I and no MHC Class II molecules.[6] As such, the B16/F10 tumor can be recognized by CD8 cytotoxic T cells although it cannot directly present tumor antigens to CD4 cells because this presentation is MHC Class II restricted. Because cytokines work best in a paracrine fashion, local to the site of their release, we have exploited this fact in the delivery of cytokines to brain tumors. We have previously shown that paracrine IL-2 has a potent antitumor response against the B16/F10 melanoma model.[21] In addition, we have demonstrated synergy between locally delivered IL-2 and local chemotherapy.[17] Investigators from other laboratories have shown some degree of efficacy with locally delivered IL-4 as well as GM-CSF;[4,6,9,20] however, a direct comparison of these different cytokines has hitherto not been described previously.

In this investigation, we compared the efficacy of IL-2, IL-4, and GM-CSF in the treatment of B16/F10 melanoma both via efficacy studies and by histopathological ex-

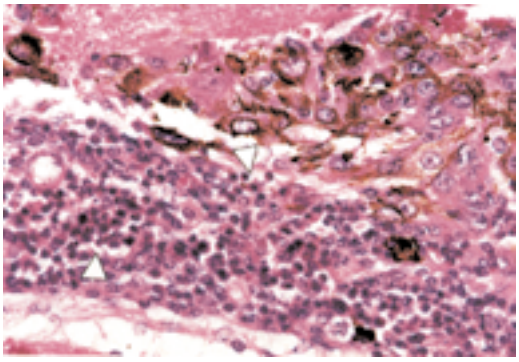

Fig. 4. Photomicrograph of stained sections of animals treated with IL-4, showing the presence of an eosinophilic infiltrate *(arrowhead)*. H & E, original magnification × 300.

amination of animal tissue. Through histological and immunohistochemical examination of animal brains, we were able to show significant differences in the action of the three cytokines. By taking advantage of these differences and developing strategies that further enhance the immune response, we hope to develop new immunotherapeutic approaches for the treatment of CNS tumors.

Interleukin-2 Protects Animals From Intracranial Tumor Challenge via a CD8 T-Cell Response

Of the three ILs used in this study, IL-2 was clearly superior to both IL-4 and GM-CSF against B16/F10. Mice treated with IL-2 not only survived longer, but more than 60% of the IL-2 group were long-term survivors (> 120 days). Examination of the brains of animals treated with IL-2 showed very little to almost no tumor infiltration at 2 weeks posttumor challenge. In stark contrast, animals treated with IL-4 or GM-CSF showed progressive increases in tumor volume. When the brains of animals treated with IL-2 were examined immunohistochemically, several important findings were noted. First, all of the animals in the IL-2 group mounted an inflammatory response, as shown by the presence of polymorphonuclear cells and lymphocytes. Second, these lymphocytes consisted primarily of T cells rather than B cells. And finally, only the CD8 T cells were found in the tumor cell infiltrates.

The discovery of CD8 T cells in the infiltrate of tumor cells treated with IL-2 was of particular interest. It clearly showed that the immune system, when appropriately stimulated, can mount an immune response against CNS tumors. This is a T-cell–mediated response and not based on immunological memory as would be expected if B cells were present. Furthermore, only CD8 T cells, and not CD4 T cells, appear to play a role in the antitumor response. Whether this is because the B16/F10 melanoma cells express only MHC Class I molecules or there is an intrinsic defect in normal MHC Class II–restricted antigen presentation in the CNS is a matter of debate. However, recent work by Elliott, et al.,[7] has implicated a defect in IL-2 secretion and in the expression of the high affinity IL-2 receptor in the CD4 T cell subpopulation. This defect is not amenable to exogenous IL-2 administration and is likely caused by immunosuppressive substances secreted by tumors, among them transforming growth factor–β, IL-10, insulin-like growth factor, and prostaglandin-2.[11,15] Taken together, these findings suggest that overcoming the CD4 anergy while preventing the effects of the immunosuppressive substances might enhance not only antigen presentation in the CNS, but also the antitumor activity of the cytotoxic antitumor lymphocytes.

Interleukin-4 Induces an Eosinphilic Immune Response That Prolongs the Survival of Mice With CNS Tumors

Although not as effective as IL-2, IL-4 also appears to play role in inducing some antitumor activity (Table 1). Examination of the brains of animals treated with IL-4 showed no evidence of polymorphonuclear cells or lymphocytes. However, as previously described, there was a marked eosinophilic infiltrate present throughout the course of the immune response. For IL-4–transduced tumors, the presence of an eosinophilic infiltrate has been attributed to the expression of a vascular cell adhesion molecule on local vascular endothelial cells by IL-4. Vascular cell adhesion molecule appears to be the most important ligand for the VLA-4 receptor, which is expressed on circulating eosinophils.[18] That eosinophils can play a direct role in an antitumor response is not well supported but IL-4 has been shown to confer potent antitumor effects. For example, Golumbek, et al.,[9] showed that administration of IL-4 can cure animals with renal cell tumors, and Yu, et al.,[24] reported that IL-4 prolongs survival of mice with glial tumors. In this setting, one can speculate that the eosinophilic infiltrate induced by IL-4 can mediate the immune response in one of two ways. Either it serves to promote the direct killing activity of eosinophils via the basic granular proteins and the peroxidase–halide generating system or the eosinophils serve as antigen-presenting cells in the CNS. Evidence for the latter comes from studies by Huang, et al.,[12] who showed that the generation of systemic, although not necessarily CNS, immunity by IL-4 may be due at least in part to the enhanced presentation of tumor antigens by influxing macrophages and eosinophils.

No Benefit From GM-CSF in the Treatment of Mice Challenged With Tumor Induction

Although GM-CSF has been previously shown to be beneficial in the treatment of systemic cancers,[4,6,20] our results do not support a role for direct administration of GM-CSF in the CNS. In this study, animals treated with GM-CSF did not survive longer than control animals. Histopathologically, brains of animals treated with GM-CSF showed no evidence of any immune infiltrate, which would account for the lack of the immune response and therefore the animals' poor survival. Previous work from our laboratory suggests that this cytokine is most effective when administered as a subcutaneous vaccine rather than directly to the brain.[21] This is because of the ability of GM-CSF to promote the recruitment and differentiation of professional antigen-presenting cells such as dendritic cells at the vaccine site.[12,19] As such, its application would be better in the context of an immune vaccine, where the priming of immune cells with both a foreign antigen and GM-CSF has been shown to have potentially remarkable antitumor effects.

CONCLUSIONS

To aid in the development of promising new immunotherapies targeted at cancer, we have examined the effects of three important cytokines delivered directly to B16/F10 intracranial melanoma tumors. Although both IL-2 and IL-4 appear to have antitumor capacities, IL-2 is clearly superior to IL-4. We have found no role for locally administered GM-CSF in our study. Consequently, we propose that further efforts related to the development of cytokine-based therapies should focus on IL-2, and in particular, determination of the mechanism by which this cytokine stimulates a subset of CD8 T cells. The ability to enhance the intrinsic properties of IL-2 and block the immunosuppressive effects of various tumors could offer a powerful approach in the continually evolving therapies against both systemic and CNS tumors.

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Disclosure

Dr. Brem is a consultant to Guilford Pharmaceuticals, Inc., and to Aventis Pharmaceutical Products, Inc. The Johns Hopkins University and Dr. Brem own Guilford stock, the sale of which is subject to restrictions under University policy. The terms of this arrangement are being managed by the University in accordance with its conflict of interest policies.

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