ABSTRACT

The ability of interleukin-4 (IL-4) to mediate an antitumor response to human gliomas was studied in vivo in nude mice. To allow the effect of IL-4 to be exerted over a relatively short distance and at an optimal concentration, a transfected tumor cell line expressing a high level of IL-4 was used in mixed tumor transplantation assays. There was a significant inhibition of growth of the U87 human glioma line when the IL-4-secreting cell line, LT-1, was implanted s.c. with the glioma in 5 nude mice when compared to contralateral control tumors consisting of the U87 glioma and IL-4-negative control cells. In addition, there was a prolongation of survival when U87 along with IL-4-secreting cells were implanted intracerebrally in 12 nude mice compared to 12 control nude mice implanted with U87 and IL-4-negative control cells and 11 control animals receiving U87 alone. Histological analysis 4 days after i.c. inoculation revealed the presence of a dramatic eosinophil infiltrate and tumor necrosis. The absence of viable glioma cells as well as resolution of inflammation 19 days after treatment suggests the potential for complete tumor regression without ongoing inflammatory sequelae resulting from cytokine treatment.

INTRODUCTION

The current treatment of primary malignant glioma, consisting of gross surgical resection followed by radiation therapy and/or chemotherapy, is cytoreductive in nature but has failed to substantially change the outcome of patients with glioblastoma (1). This has led to the investigation of other modalities of treatment including immunotherapy. Lymphocytic infiltrates have been described in primary brain tumors and were speculated to be a host-mediated response against the tumor (2). Because the presence of lymphocytic infiltrates has been associated with improved prognosis (3), many groups have attempted adoptive transfer of lymphocytes as an adjuvant treatment for brain tumors. Clinical trials have involved the intrathecal administration of autologous lymphocytes (4) or i.t. administration of lymphocytes (5–7). The development of recombinant human interleukin 2 and lymphokine-activated killer cells has generated renewed interest in adoptive immunotherapy (8–14). Although the treatment was well tolerated, these therapeutic attempts were unrewarding.

The failure to affect survival of these patients may be secondary to continued suppression of the T-cell-dependent arm of the immune response to the glioma. In addition to a diminished blastogenic response by peripheral blood leukocytes from these patients (15, 16), there is a decrease in the number of circulating lymphocytes with a specific reduction in the number of T-cells (17, 18). At least a portion of the depressed T-cell immunity is due to an increased monocyte suppressor function in patients with glioblastoma multiforme (19, 20). It is possible that immunotherapy of malignant gliomas requires a novel T-cell-independent approach or a multimodal approach to circumvent the immune suppression seen in these patients.

IL-4 is a lymphokine the pleiotropic functions of which implicate its candidacy for tumor immunotherapy against human gliomas. IL-4 is derived primarily from CD4+ T-cells (21). In vitro studies have demonstrated the ability of IL-4 to activate major histocompatibility complex-restricted and major histocompatibility complex-unrestricted cytotoxic lymphocytes (22, 23) and macrophages (24). IL-4 has been shown to have a potent antitumor effect in vivo against tumors of diverse histological origins including plasmacytomas, mammary adenocarcinomas, lymphomas, melanomas, sarcomas, and renal cell carcinomas (25, 26). Histological analysis has revealed eosinophil infiltration during tumor necrosis. The demonstration of an antitumor effect of IL-4 in nude mice suggested that IL-4 action was not T-cell dependent (25).

We sought to determine the effect that IL-4 would have in adjuvant immunotherapy for human glioma. The action of IL-4 has been clearly shown to be dose dependent, and tumor inhibition mediated by IL-4 is reversible by the in vivo delivery of anti-IL-4 antibody (25). To allow the effect of IL-4 to be exerted over a relatively short distance and at an optimal concentration, a transfected plasmacytoma cell line, LT-1, expressing a high level of IL-4 was used as a source of IL-4 in mixed tumor transplantation assays. LT-1, as a result of IL-4 expression, has been shown by itself to be nontumorigenic in immunocompetent and nude mice (25). To determine whether IL-4 would have an antitumor effect i.e., we stereotactically inoculated nude mice in the right frontal lobe with a cell suspension consisting of human glioblastoma cells (U87) and IL-4-producing cells (LT-1). Survival studies were performed to determine the therapeutic effect of IL-4-secreting cells and histology was examined to determine the cellular infiltrate associated with the antitumor effect.

MATERIALS AND METHODS

Culture and Transfection. J558L is a heavy-chain-loss variant of the BALB/c plasmacytoma line J558 (27). LT-1 is the result of transfection of J558L with plasmid pLT.IIL4 which contains the entire 10.5 kilobase mouse IL-4 coding region and 3.5 kilobases of its 3' flanking sequences under the control of the promoter/enhancer from the long terminal repeat of the Moloney murine leukemia virus. As determined by IL-4 bioassay, this cell line produces approximately 50,000 units of IL-4 activity/106 cells over 48 h in vitro (25). C6-BAG is derived from the rat glioma cell line C6 by infection with the BAG retroviral vector which contains the ß-galactosidase gene (28). The U87 human glioma line was obtained from the American Type Culture Collection (Camden, NJ).

Animal Studies. Female nude mice (NCr/Sed nu/nu) were obtained from the Edwin L. Steele Laboratory for Radiation Biology at the Massachusetts General Hospital. Nude mice were anesthetized with 0.065 mg/g of sodium pentobarbital (Anesthesia Products Co., Arcadia, CA). Animal studies were done in accordance with guidelines for animal care by the Massachusetts General Hospital Subcommittee on Animal Care.

s.c. Tumor Transplantation. Transfected IL-4-producing cells (LT-1) and control plasmacytoma cells (J558L) were mixed with tumor cells (U87 or C6-BAG) using 1.0 x 106 cells of each type. The J558L cells were treated with 10 μg/ml of mitomycin C for 3 h to inhibit their growth prior to inoculation. LT-1 cells were treated with mitomycin C where indicated. Growth arrest was confirmed by culture of cells in vitro after mitomycin C treatment. The mixture of IL-4-producing cells (LT-1) and glioma cells (U87 or C6-BAG) was injected...
in a volume of 0.4 ml in the left lower abdominal quadrant. A mixture of control cells (J558L) and glioma was injected in the right lower abdominal quadrant of the same animal as an internal control. Tumor volumes were estimated as the product of tridimensional caliper measurements at biweekly intervals.

**ic. Tumor Implantation.** To determine whether IL-4 would have an antitumor effect i.e., we stereotactically inoculated a group of 12 nude mice in the right frontal lobe with tumor consisting of $2.0 \times 10^5$ U87 human glioma cells mixed with $2.0 \times 10^5$ LT-1 IL-4-producing cells in a total volume of 4 μl. Controls consisted of 1 group of 11 mice receiving $2.0 \times 10^5$ U87 cells alone and another group of 12 mice receiving U87 cells mixed with $2.0 \times 10^5$ J558L cells. The J558L cells and the LT-1 cells were treated with mitomycin C as described above prior to i.c. delivery with U87 glioma cells.

To determine whether a single inoculum of recombinant IL-4 would have an antitumor effect, 10,000 units of rIL-4 was coinjected i.e. with $2.0 \times 10^5$ U87 cells in a group of 10 nude mice. The control group consisted of 10 nude mice which were i.c. inoculated with $2 \times 10^8$ U87 cells alone. rIL-4 was purified as previously described (29).

**Histological Evaluation.** Tissue at the site of s.c. tumor cell inoculation was fixed in 10% formalin, blocked in paraffin, sectioned at 4 μm, and routinely stained with hematoxylin and eosin. Brain tissue was fixed in 10% formalin, blocked in paraffin, sectioned in a coronal plane in 10-μm sections, and stained with either hematoxylin and eosin or Giemsa, the latter for the detection of eosinophils and mast cells.

**RESULTS**

**s.c. Tumor Transplantation.** To assess the effect of IL-4 on glial tumors, the rat glioma cell line C6-BAG and the human glioma line U87 were first used in s.c. mixed tumor transplantation assays with the LT-1 and J558L cell lines. The rat glioma cell line, C6-BAG, when mixed with the IL-4-producing cell line, LT-1, was markedly growth inhibited compared to the control tumor consisting of C6-BAG with mitomycin C-treated J558L cells during the 27-day observation period (one-sided Wilcoxon rank test, $P < 0.005$). The U87 human glioma line when mixed with the LT-1 cell line exhibited complete inhibition in 5 of 6 animals during the 34-day observation period, whereas the contralateral J558L side all grew large tumors (one-sided Wilcoxon rank test, $P < 0.005$).

Histological evaluation of these mixed tumors 3 days postinjection revealed tumor necrosis and the presence of an inflammatory infiltrate consisting primarily of eosinophils and occasional macrophages in the C6-BAG/LT-1 mixed tumor as well as the U87/LT-1 mixed tumor. The control mixed tumors, C6-BAG/J558L and U87/J558L, displayed aggressive pleomorphic glial tumors without evidence of necrosis or an inflammatory infiltrate.

When the LT-1 cell line was treated with mitomycin C prior to injection, the U87 human glioma remained completely inhibited in 5 of 5 animals while the contralateral side containing the U87/J558L mix grew large tumors in 5 of 5 animals during the 22-day observation period ($P < 0.005$, one-sided Wilcoxon rank test) (Fig. 1). Tumor growth suppression suggested that the mitomycin-treated LT-1 cells remained metabolically active to secrete IL-4, and this was confirmed by in vitro IL-4 bioassay (data not shown).

**ic. Tumor Implantation.** Given the profound inhibitory effect of IL-4 on the glioma cell lines transplanted s.c., we wished to determine the efficacy of IL-4 in treating in situ intracerebral gliomas. For i.c. experiments, both J558L and LT-1 cells were treated with mitomycin C prior to inoculation.

After mixed i.c. implantation of U87 glioma with mitomycin C-treated LT-1 cells, there was a significant increase in the survival of these animals (median survival, 9.25 weeks) compared with two control groups: (a) a mixture of U87 cells and mitomycin C-treated J558L cells (median survival, 6 weeks); and (b) U87 cells alone (median survival, 8 weeks) (one-tailed Fisher exact test, $P = 0.0013$ versus J558L/U87 control group and $P = 0.0495$ versus U87 control group;...
group inoculated with U87 alone. By 11 weeks after inoculation, 8 of 10 nude mice were dead in both groups, and all mice were dead in both groups by the week 13.

Histopathological analyses of brains from nude mice implanted with tumors were performed in addition to survival studies. Six nude mice were stereotactically inoculated in the right frontal lobe with a mixed tumor consisting of U87 and mitomycin C-treated LT-1 or U87 and mitomycin C-treated J558L cells. One animal from each group was sacrificed 4 days after inoculation and 19 days after inoculation, respectively. One animal from the U87/LT-1 group was sacrificed at 7.5 weeks after inoculation. The brains were removed, fixed, sectioned, and microscopically examined.

At 4 days postinoculation, the U87/J558L control brain displayed a large tumor spanning the dorsal to ventral aspect of the right frontal lobe. A hematoxylin and eosin-stained section of the U87/J558L control is shown (Fig. 3A). There were islets of colonization of this pleomorphic glial tumor infiltrating into normal brain tissue. There was a very small area of necrosis at the dorsal aspect of the tumor and there was no evidence of inflammation. The left frontal lobe and posterior regions of the brain were normal in appearance.

In contrast, the U87/LT-1, IL-4-treated brain showed the majority of the tumor to be necrotic with nuclear debris within 4 days postinoculation. Hematoxylin and eosin staining revealed a dramatic granulocytic infiltrate throughout the tumor (Fig. 3B). Giemsa staining of coronal sections of an identically treated brain revealed that almost all of these cells were eosinophils (>99%) (Fig. 3C). The inflammatory infiltrate was limited to the area of tumor involvement and all other areas of the brain were normal.

At 19 days postinoculation, a U87/LT-1 treated brain revealed a small area of neuronal loss and gliosis in the right frontal lobe indicative of a surgical scar. There was retraction of the right lateral ventricle secondary to the scar (Fig. 3D). There was no evidence of tumor or inflammation in the brain. Examination of a U87/LT-1 brain from an animal sacrificed at 7.5 weeks postinoculation revealed absence of tumor or inflammation even at this long-term point. In a U87/J558L control brain, examination 19 days postinoculation revealed a large ventral glial tumor near the surgical scar. The cells were pleomorphic with prominent nucleoli and several cells were seen undergoing mitosis. No necrosis or inflammatory infiltrate was seen.

**DISCUSSION**

We have demonstrated the potent antitumor effect of IL-4 on human glioma stereotactically implanted i.c. in nude mice. Tumor suppression *in vivo* was dependent on the local elaboration of IL-4 by transfected tumor cells. Tumor necrosis and a predominantly eosinophil infiltrate were observed in both s.c. and i.c. implanted tumors. The eosinophil infiltration was dramatic in the number of inflammatory cells present and resembled microabscesses in necrotic tumor tissue.
This infiltrate is characteristic of that observed with the local action of IL-4 in other tumors previously studied (25).

The involvement of only tumor tissue and lack of significant inflammation in normal brain tissue highlight the potential of localized IL-4 therapy to affect tumor and spare normal tissue. This may be secondary to a tumor specific interaction with the host effector cells induced by IL-4 or alternatively, a gradient effect in which cell killing is related to the level of IL-4 expression.

A statistically significant increase in survival was seen when human glioma cells were mixed with the mitomycin C-treated IL-4-secreting plasmacytoma line, LT-1. The demonstration of complete tumor inhibition at 19 days and 7.5 weeks post-i.c. inoculation of the U87 glioma and LT-1 would suggest that the local elaboration of IL-4 may result in complete cure of a glial tumor.

Recombinant IL-4 when cojected with U87 did not affect survival in nude mice. A majority of LT-1 cells (>90%) are s.c. killed by 48 h as a result of IL-4 expression (25). Ten thousand units of rIL-4 represents an estimate of the amount of IL-4 which is equivalent, or more likely greater, than that produced by the LT-1 cells in the i.c. experiments over 48 h. Thus, rIL-4 does not afford the antitumor effect of IL-4-producing cells. This difference in effect is explained by the short half-life of rIL-4 in vivo which is less than 5 min when administered i.v. IL-4-secreting cells offer sustained delivery over a period of time which is sufficient to induce the inflammatory reaction characteristic of IL-4-mediated antitumor activity.

In preliminary studies, the treatment of gliomas established for a period of 10 days prior to treatment with small numbers (2 × 10³) of LT-1 cells did not demonstrate a significant increase in survival. This may reflect an insufficient localized concentration of IL-4 in proximity to the established glioma. Established s.c. growing tumors have been demonstrated to be significantly inhibited in their growth by the direct i.t. administration of higher numbers of IL-4-producing cells than used in the present study (29).

Future experimentation will be directed at answering whether higher, sustained concentrations of IL-4 can promote the suppression of established gliomas in situ. The development of this protocol in an animal model may have direct implications for testing the clinical feasibility of this approach. Other methods of i.t. IL-4 delivery which may have potential efficacy in this regard include the direct injection of retroviral producer cells capable of targeting IL-4 to tumor tissue.

This is the first report of the antitumor effect of IL-4 against a tumor of the central nervous system in situ. Its action is associated with a marked eosinophil infiltrate as observed with the antitumor action of LT-1 cells. This infiltrate is characteristic of that observed with the local action of IL-4 in other tumors previously studied (25).

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