

# Eradication of established intracranial rat gliomas by transforming growth factor $\beta$ antisense gene therapy

(glioblastoma/gene therapy/immunosuppression/cancer/antisense)

HABIB FAKHRAI\*<sup>†</sup>, OLIVER DORIGO\*, DANIEL L. SHAWLER\*, HONG LIN\*, DAN MERCOLA\*, KEITH L. BLACK<sup>‡</sup>,  
IVOR ROYSTON\*, AND ROBERT E. SOBOL\*

\*Sidney Kimmel Cancer Center, San Diego, CA 92121; and <sup>‡</sup>Division of Neurosurgery, University of California, School of Medicine, Los Angeles, CA 90024

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**ABSTRACT** Like human gliomas, the rat 9L gliosarcoma secretes the immunosuppressive transforming growth factor  $\beta$  (TGF- $\beta$ ). Using the 9L model, we tested our hypothesis that genetic modification of glioma cells to block TGF- $\beta$  expression may enhance their immunogenicity and make them more suitable for active tumor immunotherapy. Subcutaneous immunizations of tumor-bearing animals with 9L cells genetically modified to inhibit TGF- $\beta$  expression with an antisense plasmid vector resulted in a significantly higher number of animals surviving for 12 weeks (11/11, 100%) compared to immunizations with control vector-modified 9L cells (2/15, 13%) or 9L cells transduced with an interleukin 2 retroviral vector (3/10, 30%) ( $P < 0.001$  for both comparisons). Histologic evaluation of implantation sites 12 weeks after treatment revealed no evidence of residual tumor. *In vitro* tumor cytotoxicity assays with lymph node effector cells revealed a 3- to 4-fold increase in lytic activity for the animals immunized with TGF- $\beta$  antisense-modified tumor cells compared to immunizations with control vector or interleukin 2 gene-modified tumor cells. These results indicate that inhibition of TGF- $\beta$  expression significantly enhances tumor-cell immunogenicity and supports future clinical evaluation of TGF- $\beta$  antisense gene therapy for TGF- $\beta$ -expressing tumors.

Brain tumors are responsible for significant morbidity and mortality in both pediatric and adult populations (1). While glioblastoma multiforme tumors rarely metastasize outside of the nervous system, they diffusely infiltrate brain tissues (2) and cannot be cured by surgical resection. Surgery and high-dose radiation provide the best standard therapy; however, these treatments are not curative and prognosis remains poor (2). Therefore, development of new therapies for these tumors is essential.

Glioma cells express major histocompatibility complex class I and class II molecules (3), as well as tumor-associated antigens that have been demonstrated to stimulate anti-tumor immune responses and thus are good tumor vaccine candidates (4, 5). However, several studies have demonstrated the secretion of an immunosuppressive factor by glioma cells that was subsequently identified as transforming growth factor  $\beta$  (TGF- $\beta$ ) (6–9).

TGF- $\beta$  has been shown to exert several potent immunosuppressive effects (10, 11), including the inhibition of cytotoxic T-lymphocyte activation (6, 12–14). We hypothesized that genetic modification of glioma cells to inhibit TGF- $\beta$  expression may enhance their immunogenicity and make them more suitable for active tumor immunotherapy. We describe a form of immuno-gene therapy based upon antisense inhibition of the immunosuppressive factor TGF- $\beta$ . In an animal glial-tumor model, active immunotherapy with TGF- $\beta$  antisense-

modified tumor cells resulted in the elimination of established tumors. Our findings support the clinical evaluation of this approach for the treatment of gliomas and other TGF- $\beta$ -expressing tumors.

## MATERIALS AND METHODS

**Construction of Vectors and Genetic Modification of 9L Cells.** *TGF- $\beta$ 2 antisense vector.* To generate the TGF- $\beta$  antisense vector, a DNA fragment containing bases 1–870 of simian TGF- $\beta$ 2 cDNA (15) was ligated in reverse orientation in the *HindIII*–*XhoI* sites of the pCEP-4 vector (Invitrogen) (Fig. 1). Expression of the antisense molecule in pCEP-4 is driven by the cytomegalovirus promoter of the vector. The pCEP-4 vector also contains the hygromycin resistance gene driven by the herpes simplex virus thymidine kinase promoter, the Epstein-Barr virus origin of replication, and the gene for the Epstein-Barr virus nuclear-associated antigen protein 1. Genetic modification of 9L cells with the pCEP-4/TGF- $\beta$  antisense vector or an empty pCEP-4 control vector was performed by electroporation using a BTX (San Diego) electroporator (16). Pools of clones were selected with hygromycin at 200  $\mu$ g/ml (Sigma). TGF- $\beta$  secretion was measured by the previously described TF-1 cell bioassay (17). The growth of TF-1 cells (from Mire-Sluis, ref. 17) is inhibited in a dose-dependent manner by TGF- $\beta$  (17). To confirm the specificity of TF-1 growth inhibition by TGF- $\beta$ , the conditioned 9L supernatants were incubated with neutralizing concentrations of turkey anti-TGF- $\beta$  antiserum. Normal (nonimmune) turkey sera was used as a negative control. Standard curves generated with known concentrations of purified TGF- $\beta$  (Sigma) served as a positive control and permitted quantification of TGF- $\beta$  levels in the test samples. Antisense inhibition of TGF- $\beta$  has been maintained for >1 yr and has been confirmed by the TF-1 bioassay.

*IL-2 vector.* The construction of LNCX/IL-2 retroviral has been described (18). Virus-containing supernatant from PA317/LNCX/IL-2 packaging cell line was used to transduce the 9L and TGF- $\beta$ 2 antisense-modified 9L cell cultures as described (18). The levels of interleukin 2 (IL-2) in tissue culture supernatants of IL-2-transduced cells were measured by previously described ELISA, and the IL-2 biological activity was confirmed as described (18).

**Gene Therapy in the Rat 9L Glioma Tumor Model.** The 9L gliosarcoma cell line was from Carol Kruse (5). Intracranial implantation of 300 unmodified 9L cells in Fisher-344 rats results in >99% fatality by 5 weeks (19). In our studies  $5 \times 10^3$  logarithmic-phase 9L cells were injected intracranially into 5- to 6-week-old Fischer-344 rats (Harlan-Sprague-Dawley). Intracranial tumor implantation was done stereotactically 2 mm

Abbreviations: TGF- $\beta$ , transforming growth factor  $\beta$ ; IL-2, interleukin 2; NK, natural killer cell; IGF-1, insulin-like growth factor-1.

<sup>†</sup>To whom reprint requests should be sent at the present address: Division of Neurosurgery, University of California, School of Medicine, Los Angeles, CA 90024.

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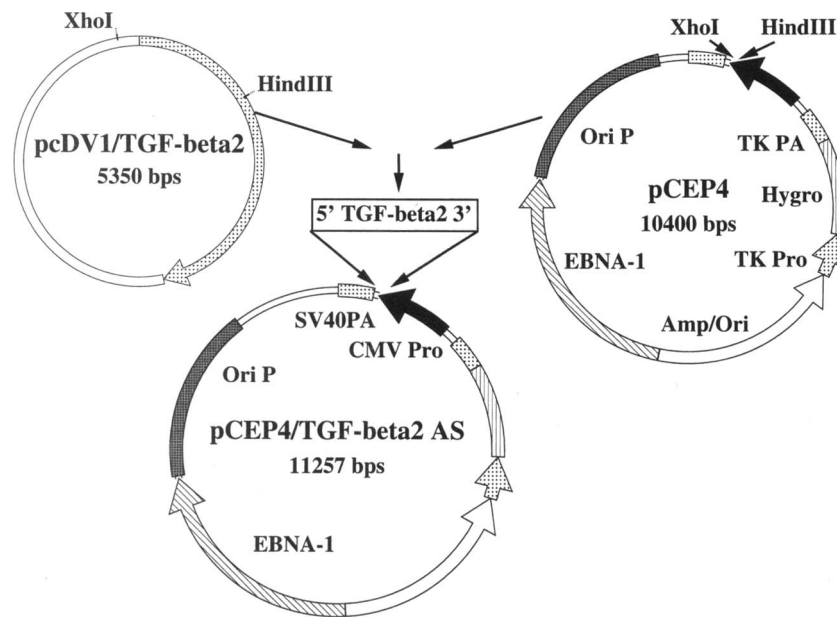


FIG. 1. Schematic diagram of pCEP-4 TGF- $\beta$  antisense vector. EBNA-1, Epstein-Barr nuclear-associated antigen 1; TK, thymidine kinase; Hygro, hygromycin resistance gene; CMV Pro, cytomegalovirus promoter; SV40, simian virus 40.

anterior and 2 mm lateral to the junction of the bregma and sagittal sutures. Subcutaneous immunizations were initiated 5 days after tumor implantation. Animal groups received a total of four immunizations on a twice-a-week schedule with cell preparations described in Table 1. All tumor-cell preparations used for immunizations were irradiated with 6000 cGy before treatment. In a repeat experiment, animals received two immunizations of antisense-modified 9L cells. Light microscopic histologic evaluation of the implantation sites was performed on hematoxylin/eosin-stained tissue sections. A subset of the surviving animals from these experiments were subjected to a second intracranial tumor challenge in the contralateral hemisphere with either  $1 \times 10^5$  or  $1 \times 10^6$  unmodified 9L cells. The second tumor challenge was administered 12 weeks after the completion of initial therapy. All comparisons of the proportion of animals with tumors between treatment groups were evaluated for statistical significance by contingency table analyses using Fisher's exact test (20).

**Cell-Mediated Cytotoxicity Assays.** Standard chromium-release assays (21) were used to assess the effects of immunization with TGF- $\beta$  antisense- and IL-2 gene-modified tumor cells. Lymph node effector cells from immunized animals were harvested and stimulated *in vitro* with irradiated 9L stimulator cells at a 30:1 effector-to-stimulator ratio for 5 days in the presence of recombinant human IL-2 at 50 units/ml. Target cells ( $5 \times 10^6$  9L) were labeled with 400  $\mu$ Ci (1 Ci = 37 GBq) of Na  $^{51}$ Cr overnight at 37°C. The natural killer-sensitive

A2T2C4 cell line (from Donald Bellgrau, ref. 5) was also used as a target line to test for nonspecific cytotoxicity (5). Target cells ( $1 \times 10^4$ ) were mixed with effector cells at effector target ratios of 100:1, 33:1, 11:1, and 3.7:1 for a 4-hr  $^{51}$ Cr release assay. The percentage of specific lysis was calculated by using the following formula:  $[(\text{cpm}_{\text{exp}} - \text{cpm}_{\text{bkgd}})/(\text{cpm}_{\text{max}} - \text{cpm}_{\text{bkgd}})] \times 100$ , where exp is experimental and bkgd is background.

## RESULTS

**Characterization and Genetic Modification of 9L Gliosarcoma Cells.** TGF- $\beta$  secretion by 9L cells was measured by using the TF-1 erythroleukemia cell line, the growth of which is inhibited by TGF- $\beta$  in a dose-dependent manner. As shown in Fig. 2, conditioned supernatant from unmodified 9L cultures inhibited proliferation of TF-1 cells. This inhibition was neutralized by TGF- $\beta$  antiserum, but not by control serum, indicating that the inhibition of TF-1 proliferation was due to TGF- $\beta$  in the 9L supernatant. To suppress TGF- $\beta$  expression, 9L cells were transfected with the pCEP-4/TGF- $\beta$  antisense vector by electroporation followed by selection in medium containing hygromycin. Compared to unmodified 9L cells, supernatant from TGF- $\beta$  antisense-modified 9L cells caused less inhibition of TF-1 cell proliferation (Fig. 2). From a TGF- $\beta$  standard curve, it was determined that TGF- $\beta$  antisense-modified 9L secreted 750 pg of TGF- $\beta$  per  $10^6$  cells per 24 hr,

Table 1. Effect of immunization with irradiated TGF- $\beta$  antisense and IL-2 gene-modified tumor cells on survival of rats with intracranial tumors

Inoculum	Survival* no./total no. (%)	P value†
Saline	0/10 (0)	NS
Unmodified 9L cells	2/10 (20)	
Retroviral control-modified 9L cells	2/10 (20)	NS
pCEP-4 control Vector-modified 9L cells	0/5 (0)	NS
LNCX-IL2-modified 9L cells	3/10 (30)	NS
TGF- $\beta$ antisense-modified 9L cells	11/11 (100)	<0.001
TGF- $\beta$ antisense- + IL-2-modified 9L cells	7/7 (100)	<0.002

\*NS, not significant. Survival at 12 weeks after tumor implantation.

†Fisher's exact test comparing inoculation with modified 9L cells to inoculation with unmodified 9L cells.

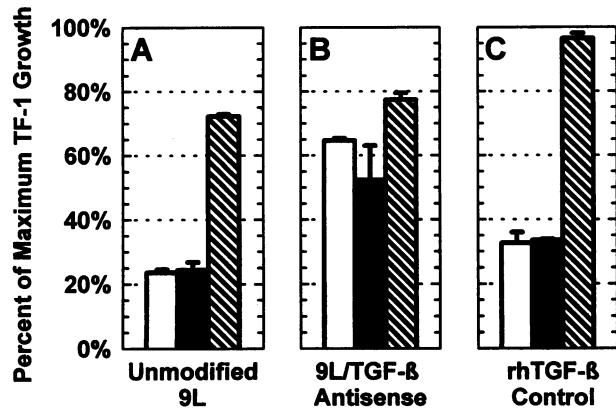


FIG. 2. Suppression of TGF- $\beta$  secretion by TGF- $\beta$  antisense-modified 9L cells. Conditioned supernatants from cultured unmodified 9L (Left), TGF- $\beta$  antisense-modified 9L (Middle), or purified TGF- $\beta$  at 1 ng/ml (Right) were incubated with TF-1 cells (white bars). The growth of TF-1 cells is inhibited in a dose-dependent manner by TGF- $\beta$ . To confirm the specificity of TF-1 growth inhibition by TGF- $\beta$ , the supernatants were incubated with neutralizing concentrations of turkey TGF- $\beta$  antiserum (hatched bars). Normal (non-immune) turkey serum was used as a negative control (black bars).

which is  $\approx$  5-fold less than from unmodified 9L cells, which secreted 3500 pg of TGF- $\beta$  per  $10^6$  cells per 24 hr.

Transduction of rat 9L gliosarcoma cells with the LNCX/IL-2 retroviral vector resulted in secretion of 1015 BRMP (18) units of IL-2 per  $10^6$  cells per 24 hr as measured by IL-2 ELISA. The biological activity of the secreted IL-2 in the supernatant of the transduced cells was confirmed by CTLL-2 cell bioassay (data not shown).

**Treatment of Established Tumors with Genetically Modified Tumor Cells.** Unmodified 9L cells ( $5 \times 10^3$ ) were stereotactically implanted in the forebrain of Fisher 344 rats. Immuno-gene therapy was initiated 5 days later, and the tumor-bearing animals were immunized s.c. a total of 4 times on a twice-a-week schedule and followed for survival. Immunizations with irradiated TGF- $\beta$  antisense-modified tumor cells showed exceptional efficacy in animals with established tumors (Table 1). Treatment of tumor-bearing animals with TGF- $\beta$  antisense-modified 9L cells resulted in a significantly higher number of animals surviving for 12 weeks (11/11, 100%) compared to immunizations with the empty pCEP-4 control vector-modified (0/5, 0%), retroviral control vector-modified 9L cells (2/10, 20%), unmodified 9L cells (2/10, 20%), or 9L transduced with LNCX/IL-2 (3/10, 30%) ( $P < 0.001$ ). Treatment of tumor-bearing animals with 9L cells that had been modified with both LNCX/IL-2 and TGF- $\beta$  antisense vectors also resulted in 100% 12-week survival ( $n = 7$ ) (Table 1). Similar results were also obtained in a repeat experiment where 6/6 animals immunized with TGF- $\beta$  antisense-modified 9L cells survived for 12 weeks. Histologic evaluation of hematoxylin/eosin-stained brain tissue sections by light microscopy revealed no evidence of residual tumor cells in the animals that survived for 12 weeks.

Some of the surviving animals that had not been sacrificed for histologic evaluation were subjected to a second intracranial tumor challenge in the contralateral hemisphere with either  $10^5$  or  $10^6$  parental tumor cells 12 weeks after the completion of initial treatment. All nine animals challenged with  $10^5$  unmodified tumor cells survived for a 6-mo observation period, whereas all five unimmunized control animals died by 5 weeks. In the animals rechallenged with  $10^5$  tumor cells, histologic evaluation by light microscopy revealed no evidence of residual tumor at either the first or second tumor implantation sites (Fig. 3A). After challenge with  $10^6$  unmodified 9L cells, most of the animals immunized with TGF- $\beta$

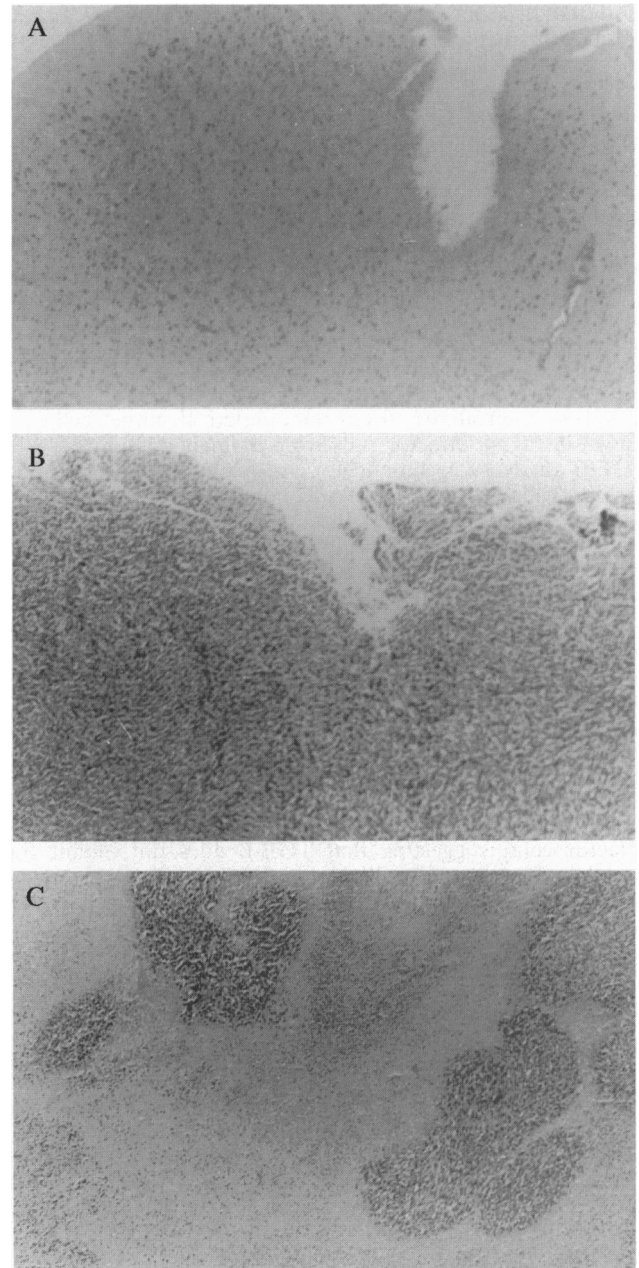


FIG. 3. Histological evaluation of tumor-implantation sites. Representative hematoxylin/eosin-stained brain tissue sections evaluated by light microscopy in surviving animals subjected to a second intracranial tumor challenge in the contralateral hemisphere with either  $10^5$  or  $10^6$  unmodified 9L cells. (A) Absence of tumor at the implantation sites 6 mo after rechallenge with  $10^5$  tumor cells. (B) Large tumors characterized by uniform, diffuse infiltration were observed at the second tumor implantation site after rechallenge with  $10^6$  tumor cells in animals immunized with either unmodified or IL-2 gene-modified 9L cells. (C) Smaller tumors were seen in animals immunized with TGF- $\beta$  antisense-modified tumor cells after rechallenge with  $10^6$  tumor cells. These tumors were characterized by extensive necrosis. ( $\times 50$ ).

antisense-modified tumor cells (5/5) or combined antisense- and IL-2 gene-modified 9L cells (5/6) survived  $\geq 4$  weeks, compared to 0/5 animals immunized with control vector-modified or IL-2 gene-modified 9L cells, which had all died by 3 weeks after tumor challenge. Overall, animals immunized with TGF- $\beta$  antisense-modified 9L had significantly better 4-week survival after rechallenge with  $10^6$  unmodified 9L cells than did animals immunized with either control vector-

modified or IL-2 gene-modified 9L cells (10/11 vs. 0/5,  $P < 0.02$ ). At necropsy of the animals rechallenged with  $10^6$  tumor cells, histological evaluation of brain sections by light microscopy revealed large tumors at the second tumor implantation sites in the animals that were immunized with IL-2 gene-modified or unmodified 9L cells (Fig. 3B). The animals immunized with TGF- $\beta$  antisense-modified tumor cells generally had smaller tumors at the second tumor injection sites, which were characterized by extensive necrosis (Fig. 3C). None of these animals had evidence of residual tumor at the original tumor-implantation site.

**Cell-Mediated Cytotoxicity Assays.** Standard chromium-release assays were used to assess the antitumor immune responses generated by immunization with inocula described in Table 1. Immune lymphocytes from the lymph nodes of these rats were stimulated *in vitro* with unmodified 9L cells and tested for their ability to kill unmodified 9L target cells. As shown in Fig. 4, effector cells from animals immunized with TGF- $\beta$  antisense-modified 9L cells exhibited  $\approx$  3- to 4-fold greater killing of 9L target cells compared to effector cells from animals immunized with unmodified 9L or LNCX/IL-2 modified 9L cells. Effector cells from animals immunized with 9L cells modified with both TGF- $\beta$  antisense and LNCX/IL-2 exhibited intermediate killing (Fig. 4). In a different experiment, immune lymphocytes from the lymph nodes of rats immunized with unmodified 9L cells were stimulated *in vitro* with unmodified or TGF- $\beta$  antisense-modified 9L cells and tested for their ability to lyse unmodified or TGF- $\beta$  antisense-modified 9L target cells. As shown in Fig. 5, effector cells stimulated by TGF- $\beta$  antisense-modified 9L exhibited  $\approx$  3-fold greater killing of 9L target cells than effector cells stimulated by unmodified 9L cells. Unmodified and TGF- $\beta$  antisense-modified 9L target cells were equally susceptible to killing by effector cells, suggesting that TGF- $\beta$  does not inhibit the function of activated cells. In both of these experiments, effector cell cytolytic activities against natural killer cell-sensitive A2T2C4 target cells always paralleled the results seen with 9L target cells (data not shown).

## DISCUSSION

We have demonstrated that the efficacy of tumor cell vaccines may be significantly enhanced by genetic modification to

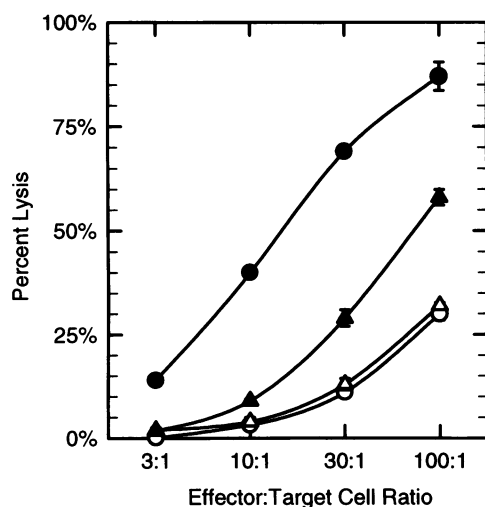


FIG. 4. Enhanced generation of cytotoxic effector cells by immunization with TGF- $\beta$  antisense-modified 9L cells. Mononuclear cells from lymph nodes of immunized rats were stimulated *in vitro* for 5 days with irradiated unmodified 9L and tested for their ability to kill unmodified 9L target cells in a chromium-release assay. Effector cells from animals immunized with TGF- $\beta$  antisense-modified 9L cells (●) or TGF- $\beta$  antisense-LNCX-iIL2-comodified 9L cells (▲) displayed greater tumor cytotoxicity compared to immunizations with unmodified 9L cells (○) or LNCX-iIL2-modified 9L cells (△).

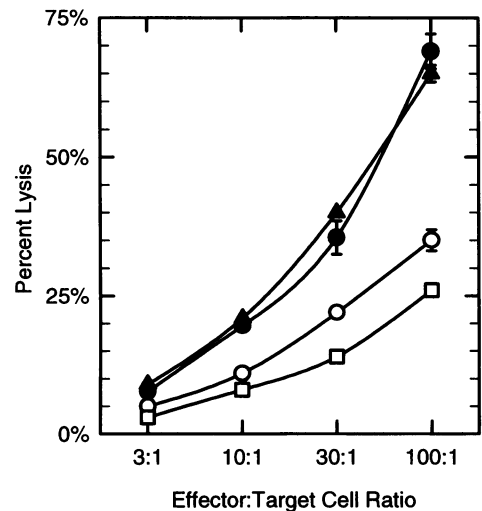


FIG. 5. Increased *in vitro* stimulation of cytotoxic effector cells by TGF- $\beta$  antisense-modified 9L cells. Mononuclear cells from lymph nodes of rats immunized with unmodified 9L cells were stimulated with irradiated TGF- $\beta$  antisense-modified or unmodified 9L cells for 5 days *in vitro* and tested for their ability to kill TGF- $\beta$  antisense-modified or unmodified 9L target cells in a chromium-release assay. Increased stimulation of cytotoxic effector cells was observed when TGF- $\beta$  antisense-modified 9L cells (solid symbols) were used for stimulation compared to unmodified 9L cells (open symbols): ●, TGF- $\beta$  antisense-modified 9L stimulator cells and unmodified 9L target cells; ▲, TGF- $\beta$  antisense-modified 9L stimulator cells and TGF- $\beta$  antisense-modified 9L target cells; ○, unmodified 9L stimulator cells and unmodified 9L target cells; and □, unimmunized control with unmodified 9L stimulator cells and unmodified 9L target cells.

suppress tumor-cell TGF- $\beta$  expression. In the rat 9L glioma model, s.c. immunization with TGF- $\beta$  antisense-modified tumor cells was highly effective in the treatment of established tumors and resulted in complete remissions in all treated animals. Histological evaluation of the tumor-implantation sites in surviving animals revealed no evidence of residual tumor cells after therapy.

Suppression of cellular immunity has long been observed in patients with glioblastoma (8–10). Incubation of peripheral blood lymphocytes with the supernatant of glioma tumor cells renders the lymphocytes unresponsive to mitogenic activation (6, 7, 22). This initially described glioblastoma-derived suppressor factor was later identified as TGF- $\beta$  (23). TGF- $\beta$  is a potent immunosuppressive agent that inhibits many immune system functions (10, 11). In particular, TGF- $\beta$  inhibits the activation of cytotoxic T cells and B cells and through a feedback mechanism results in deactivation of NK and LAK cells (6, 12–14, 24–27, 30). Immune suppression mediated by TGF- $\beta$  appears to be due, in part, to impairment of high-affinity IL-2-receptor function and expression (6, 12–14, 28). These are important elements for the initiation and maintenance of T-cell activation and function (14). TGF- $\beta$ -induced impairment of IL-2 receptors may account, in part, for the decreased efficacy of IL-2 gene therapy compared to TGF- $\beta$  antisense gene therapy observed in our studies. Our findings suggest that inhibition of immunosuppressive factors such as TGF- $\beta$  may be an important component of future efforts to develop genetically engineered tumor-cell vaccines for the treatment of gliomas and other tumors associated with the expression of immunosuppressive agents.

The results of our *in vitro* cytotoxicity assays were consistent with the *in vivo* animal tumor model studies and support the hypothesis that TGF- $\beta$  antisense modification renders glial tumor cells more immunogenic and suitable for active immunotherapy. *In vitro* stimulation of effector cells with irradiated TGF- $\beta$  antisense-modified 9L cells increased cy-

cytolytic activity compared to stimulation by unmodified 9L cells. In addition, effector cells from animals immunized with TGF- $\beta$  antisense-modified 9L cells had higher cytolytic activity compared to animals immunized with IL-2 gene-modified tumor cells or unmodified 9L cells. This increase in cytolytic activity was also observed against natural killer cell-sensitive target cells, consistent with previous reports that TGF- $\beta$  also suppresses natural killer cell activation and proliferation (27). The use of TGF- $\beta$  antisense-modified 9L as target cells in these assays had no effect on effector cell-mediated killing, suggesting that TGF- $\beta$  does not suppress the function of activated effector cells. This finding is consistent with previous reports that the immunosuppressive effects of TGF- $\beta$  are most pronounced on afferent components of immune response induction (26, 29, 30). These observations support the view that immunizations with tumor cells genetically modified to suppress TGF- $\beta$  may be efficacious against established tumors that express TGF- $\beta$ .

The use of gene therapy for the treatment of gliomas is still at an early development stage, and different approaches have been tested by other investigators. The transfer of the herpes simplex virus thymidine kinase gene into tumor sites *in vivo* followed by administration of the pro-drug ganciclovir has resulted in the eradication of intracranially implanted tumors in a subset of experimental animals (31). On the basis of these studies, clinical trials of herpes simplex virus thymidine kinase gene therapy in glioblastoma patients have been initiated. However, the inefficiency of *in vivo* gene transfer may limit the efficacy of herpes simplex virus thymidine kinase gene therapy against infiltrating glial tumors. In this regard, tumor cells that escaped ganciclovir therapy produced lethal tumors in other experimental models of this approach (31). Some glial tumors express insulin-like growth factor 1 (IGF-1) resulting in an immature stage of differentiation that may contribute to their poor immunogenicity and escape from immune surveillance (32, 33). Immunization of Wistar rats with C6 glial tumor cells genetically modified with antisense vectors to IGF-1 or IGF-1-receptor expression resulted in the induction of antitumor immune responses that rejected lethal doses of unmodified parental tumor (32–34). These protective immune responses were not observed after immunization with unmodified wild-type tumor cells (32–34). These findings and our own results suggest that glial tumors may have acquired mechanisms to escape immune destruction, which may be circumvented by inhibiting the expression of differentiation or immunosuppressive factors.

Our results may have important implications for the development of whole-cell tumor vaccines for the active immunotherapy of other tumors. TGF- $\beta$  is produced by many normal cells, and it is overexpressed by the most common forms of cancer, including most colon, breast, and prostatic carcinomas (35–37). Our preliminary results in another animal model, murine ovarian teratoma, also demonstrate the requirement for inhibiting TGF- $\beta$  expression in generating efficacious antitumor immunity by tumor-cell immunizations (O.D. *et al.*, unpublished work). Our encouraging results in these animal tumor models support the development of this approach in clinical trials for the treatment of TGF- $\beta$ -expressing tumors. It is possible that other immunosuppressive factors secreted by tumor cells, such as interleukin 10 (38, 39) or prostaglandin E<sub>2</sub> (39, 40), may exert similar negative effects on active tumor immunotherapy. Our findings suggest that strategies to inhibit the expression of these immunosuppressive factors may be important in the development of future immuno-gene therapies for cancer.

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