In Vivo Delivery of Immunomodulatory Genes is a Promising Strategy for Solid Tumor Vaccination. A drawback is that it necessitates induction of a large effect from transgene expression in a small percentage of tumor cells. Although the B7 family is known to be the most potent of the costimulatory molecules, gene transduction of B7 alone has not been effective in inducing antitumor immunity in nonimmunogenic tumors by ex vivo methods, much less in vivo. We have developed a novel approach where a gene encoding soluble B7-1, a fusion protein of the extracellular domain of murine B7-1 and the Fc portion of human IgG1, is delivered to tumor cells in vivo in the context of an oncolytic replication-competent herpes simplex virus, and the gene product is secreted by tumor cells rather than expressed on the cell surface. Defective herpes simplex virus vectors containing the B7-1-immunoglobulin (B7-1-Ig) fusion transgene (dvB7Ig) were generated using G207 as a helper virus and tested in the poorly immunogenic murine neuroblastoma, Neuro2a, in syngeneic A/J mice. Intraneoplastic inoculation of dvB7Ig/G207 at a low titer successfully inhibited the growth of established s.c. tumors, despite the expression of B7-1-Ig being detected in only 1% or fewer of tumor cells at the inoculation site, and prolonged the survival of mice bearing intracerebral tumors. Immunohistochemistry of dvB7Ig/G207-inoculated tumors revealed a significant increase in CD4 and CD8 T-cell infiltration compared with control tumors inoculated with defective vector expressing alkaline phosphatase (dvAP/G207). The antitumor effect of dvB7Ig/G207 was not manifested in athymic mice. In vivo depletion of immune cell subsets in A/J mice further revealed that CD8+ T cells, but not CD4+ T cells, were required. Animals cured of their tumors by dvB7Ig/G207 treatment were protected against rechallenge with a lethal dose of Neuro2a cells but not SaI/N cells. The results demonstrate that the use of soluble B7-1 for immune gene therapy is a potent and clinically applicable means of in situ cancer vaccination.

INTRODUCTION

A major problem in cancer gene therapy is how total tumor destruction might be achieved when none of the current vectors is capable of infecting or transducing 100% of the tumor cells in vivo. One approach is to activate the host’s immune system to generate a specific antitumor response. Most human tumors, however, are considered poorly immunogenic, i.e., they express low levels of tumor-associated antigens in the context of MHC class I molecules (1, 2). Tumor immunogenicity in experimental animals is the capacity of a tumor to be rejected after transplantation in an immunized syngeneic host (3). In less immunogenic tumors, immunomodulatory therapies are less able to produce successful outcomes. In vivo delivery of immune stimulatory cytokine genes has led to growth retardation of established tumors in certain experimental animal models, but combinations with other cytokine or immunomodulatory molecules were often required to obtain a robust antitumor effect (4–7). Tumor specificity and induction efficacy of the immune response are especially important for certain neoplasms, such as brain tumors, which are immunosuppressive and exist in an immune-privileged site where nonspecific inflammation can be dangerous.

Induction of tumor-specific CTLs requires at least two signals: (a) tumor antigen(s) that are processed and presented by MHC class I and/or class II molecules on the surface of APCs; and (b) sufficient levels of costimulatory molecule(s) on the tumor cells or other APCs (8). The B7 family of membrane proteins [B7-1 (CD80) and B7–2 (CD86)] are the most potent of the costimulatory molecules and interact with CD28 and CTLA-4 (CD152) on the T-cell surface (9). Although the use of B7-transfected tumor cells as a vaccine has induced specific antitumor immunity in models of relatively immunogenic tumors (10–14), mere expression of B7-1 on poorly immunogenic tumor cells has proven ineffective, presumably because of the lack or low expression levels of MHC-bound tumor antigens (3). In vivo gene delivery and expression of B7-1 alone in established tumors failed to induce a sufficient immune response to inhibit tumor growth (15–17).

To address these problems, we hypothesized that a gene encoding dimeric soluble B7-1, if secreted by tumor cells rather than expressed on the cell surface, might induce a specific antitumor immune response via facilitation of T-cell activation by APCs that uptake and process tumor antigens, irrespective of MHC class I-bound antigen presentation by tumor cells. The presence of soluble B7-1 in the tumor environment might also reverse anergic T cells to an activated state. It has been shown that cross-linking of neighboring CD28, a co-stimulatory receptor on T cells for the B7 molecule, is essential for T-cell activation (18). The soluble B7-1 is designed so that two molecules of B7-1 (extracellular domain) are linked by the Fc portion of IgG1. Hence, soluble B7-1 should provide a stronger stimulation to T cells by cross-linking CD28 than normal monomeric B7-1 expressed on the cell surface.

To test the efficacy of soluble B7-1 in tumor immune gene therapy, we used defective HSV-1 vectors that encode a soluble B7-1 molecule. Defective HSV vectors consist of viral particles that contain tandem repeats of an ampiclon plasmid (encoding B7-1-Ig in this case), thereby delivering multiple copies of the transgene, and helper HSV. As a helper virus, we used G207, a replication-competent, multmutated HSV-1 that can replicate and spread in situ and exhibits direct oncolytic effect, yet is nonpathogenic (19, 20). G207 is effective in inhibiting the growth of multiple types of established malignant tumors in vivo because of selective tumor replication (19, 21–23). Although oncolytic viruses are promising approaches for cancer therapy, the therapeutic effect depends upon the extent of intratumoral viral replication, and some tumor cells poorly support G207 replication. However, in immunocompetent mice, intratumoral inoculation of G207 induces a tumor-specific immune response that is able to inhibit the growth of both inoculated and noninoculated tumors (24, 25).
antitumor immunity induced by G207 can be augmented by combination with defective HSV vectors encoding cytokines (26).

We demonstrated previously that s.c. or intracerebral tumors of N18, a subclone of C1300 murine neuroblastoma cells, in syngeneic A/J mice provided a good animal model to evaluate the therapeutic efficacy of HSV-1 vectors. A/J mice are the most susceptible inbred mouse strain to HSV-1 infection, and G207 exhibits a significant antitumor effect in the N18 tumor model, in part because of induced antitumor immunity (25, 27). However, one of the difficulties in applying immunotherapy to humans is the low immunogenicity of human tumors. Therefore, as a tumor model, we used Neuro2a murine neuroblastoma cells, a subclone of C1300, that is poorly immunogenic (28–30) and capable of reproducibly forming s.c. and intracerebral tumors with 100% efficiency. Neuro2a cells do not express MHC class II, B7-1, B7-2, or intercellular adhesion molecule-1 and only low levels of MHC class I molecules (28, 31). Neuro2a cells transduced with B7-1 have decreased tumorigenicity but failed to induce significant immunological protection (28–30). In contrast to these results with cells expressing B7-1, we show that intratumoral expression of soluble B7-1-Ig results in potent growth inhibition of established Neuro2a tumors in the skin as well as in the brain, which is mediated by a cellular immune response requiring CD8+ cells.

MATERIALS AND METHODS

Cells and Viruses. Neuro2a cells, a subclone of C1300 murine neuroblastoma cells derived from an A/J mouse (H-2b), and Vero (African green monkey kidney) cells were obtained from ATCC (Rockville, MD). N18 cells were provided by Dr. Kazuhiko Ikeda (Tokyo Institute of Psychiatry, Tokyo, Japan). Sa/N, a chemically induced sarcoma cell line from an A/J mouse, was provided by Dr. Suzanne Ostrand-Rosenberg (University of Maryland-Baltimore County, Baltimore, MD). Cells were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml Fungizone. G207 (19) was grown in Vero cells, and virus titers were determined as described previously (32).

Flow Cytometric Analyses. Single-cell suspensions of Neuro2a or N18 cells (1 × 10^6 cells) were used for flow cytometric analysis of cell surface markers performed according to the procedures described (25). The monoclonal antibodies used were PE-conjugated antimouse CD80 (clone 16-10A1), PE-conjugated antimouse CD86 (clone GL1), PE-conjugated anti-H-2Dd, and anti-H-2Kd (clone AF3–12.1), and peridinin-conjugated anti-I-A^b (clone 11-5.2), all purchased from PharMingen (San Diego, CA).

Generation of Defective HSV-1 Vectors. Amplicon plasmid pSRGPT/B7IG, containing the B7-1-Ig gene driven by the CMV IE promoter and the Escherichia coli gpt gene driven by the SV40 promoter (Fig. 1), was constructed as follows. A 0.9-kb fragment containing human genomic IgG1 Fc (hinge-CH2-CH3) was inserted into pCDM8 (provided by Dr. David Simons, Smith Kline Beecham, London, United Kingdom) to generate the plasmid pG1. The cDNA coding the extracellular domain of murine B7-I was inserted into the EcoRI site of pG1, upstream of IgG1 Fc, to generate plasmid B7-1-plg. A 2.4-kb HindIII-Nool fragment containing the B7-1-Ig gene from B7-1-plg was inserted into the polynucleotid region of pCR3 (Invitrogen, Carlsbad, CA) to generate pCR-B7IG. A 3.8-kb BstXI-ApulI fragment containing the B7-1-Ig cassette from pCR-B7IG was inserted into the PvuII site of pSR-GPT to generate pSRGPT/B7IG. Plasmid pSR-GPT contains the 2.0-kb PvuII-BamHI gpt fragment from pSV2-gpt (provided from ATCC) inserted into the Ap/III site of plasmid pSRA-ori, containing the HSV-1 cleavage/packaging signal and an HSV-2 origin of replication (ori2; Ref. 33). The product of the B7-1-Ig gene was confirmed to exist as a dimer by gel electrophoresis. The control ampiclon plasmid pHCAP-gpt2 is similar to pSRGPT/B7IG, except that it contains the human placental AP gene instead of B7-1-Ig. A 2.2-kb EcoRI-Xbal fragment containing AP from pGEM4Z/PLAP513 (obtained from S. Udenfriend, Roche Institute of Molecular Biology, Nutley, NJ) was inserted into the polynucleotid region of pCR3 to generate pCRAP. A 3.2-kb HindIII-BbsI AP fragment from pCRAP was inserted into the SalI site of pSR-GPT to generate pHCAP-gpt2.

Defective HSV vectors (dV7Ig and dvAP) were generated as described previously (34), with the following modifications. Amplicon plasmids pSRGPT/B7IG and pHCAP-gpt2 were transfected into Vero cells using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD), superinfected with G207 at a MOI of 0.02, and grown in DMEM supplemented with 1% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml Fungizone, and also containing 25 µg/ml mycophenolic acid, 250 µg/ml xanthine, and 15 µg/ml hypoxanthine (35). Viruses were harvested when a complete cytopathic effect was observed and then passaged six times. We did not detect any difference in the replication of G207 attributable to transgene expression from the defective vector (B7-1-gp or AP). The final virus stocks were purified using a freeze-thaw/sonication regimen and removal of cell debris by low speed centrifugation, as described previously (32). Defective vector titers were determined using immunohistochemistry and AP histochemistry as described below. The G207 titers of the final stock were 3.6 × 10^7 pfu/ml (1.2 × 10^8 pfu/ml, concentrated) for dV7Ig and 3.0 × 10^7 pfu/ml (8.1 × 10^7 pfu/ml, concentrated) for dvAP. The defective vector:helper virus ratio of the final stocks was 1:40 for dV7Ig and 1:6 for dvAP. The difference in ratio was likely attributable to differences in detection sensitivity.

Cytchemistry and ELISA for Detection of in Vitro Vector Expression. Vero or Neuro2a cells (1 × 10^5 cells/well) were plated in 24-well plates and incubated at 37°C for 24 h. Cells were infected with defective HSV vectors and further incubated at 39.5°C. For detection of B7-1-Ig, cells were fixed 18 h after infection with 4% paraformaldehyde in PBS for 30 min, washed twice with PBS, once with PBS containing 0.1% Triton X-100, and treated with blocking solution (PBS containing 20% FCS and 0.1% Triton X-100) for 10 min. Cells were then incubated with a primary antibody, biotin-conjugated antimouse CD80 (B7-1) monoclonal antibody (clone 16-10A1; PharMingen; 1:50 dilution), or biotin-conjugated anti-human IgG Fc antibody (109-065-098; Jackson ImmunoResearch Laboratories, West Grove, PA; 1:50 dilution), for 2 h, washed three times with PBS/Triton X-100, further incubated with ExtrAvidin (Sigma Chemical Co., St. Louis, MO; 1:50 dilution) for 1 h, and washed three times with Tris-buffered saline. Immune complexes were visualized using a 3,3’-diaminobenzidine peroxidase substrate kit (Vector Laboratories, Burlingame, CA). For detection of AP, cells were fixed with 0.5% glutaraldehyde in PBS for 10 min, washed three times with PBS, and heated at 65°C for 60 min. Cells were then stained using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium AP substrate kit IV (Vector Laboratories).

For detection of secreted soluble B7-1, ELISA was performed on conditioned medium (0.3 ml/well) collected 68 h after dv infection. Infected Neuro2a cells were incubated at 39.5°C, a nonpermissive temperature for
G207 replication, to prevent cytolysis by the helper virus. Conditioned medium was also collected from Vero cells transiently transfected with 0.5 µg/well of plasmid DNA (B7.1-pIg, cB7TIG, and pSRGPT/B7IG) and incubated at 37°C for 48 h. A 96-well plate was coated with 0.1 ml/well anti-human IgG (Fc) antibody (clone HP-6001; Sigma; 1:10,000 dilution in PBS) overnight at room temperature, blocked with PBS containing 0.1% BSA at 37°C for 2 h, and washed three times with PBS containing 0.05% Tween 20. Conditioned medium or human IgG (Sigma) serially diluted in medium as standards was added to wells and incubated at 4°C overnight, followed by three washes. For detection, biotin-conjugated goat anti-human IgG (Fc) antibody (109-065-098; Jackson ImmunoResearch; 1:12,500 dilution; 0.1 ml/well) was added and incubated at room temperature for 2 h. After three washes, wells were incubated with ExtrAvidin (1:1000 dilution) for 2 h. Wells were then incubated with tetramethylbenzidine (KPL, Gaithersburg, MD) for 15 min, and the reaction was stopped by the addition of 0.1 ml sulfuric acid (0.18 M). The absorbance at 450 nm was determined using a microplate reader (Bio-Rad Laboratories, Hercules, CA). The detection limit of the ELISA was determined to be 0.1 ng/ml.

**Animal Studies.** Female A/J or nude (BALB/c nu/nu) mice, 6 weeks of age, were purchased from the National Cancer Institute (Frederick, MD) and housed in groups of four or fewer. For injections and surgical procedures, each mouse was anesthetized with an i.p. injection of 0.20 – 0.25 ml solution consisting of 86% saline, 9% sodium pentobarbital, and 5% ethanol. All animal procedures were approved by the Georgetown University Animal Care and Use Committee.

**Tumor Therapy.** s.c. tumors were generated by injecting 5 x 10⁶ Neuro2a cells in 50 µl of serum-free medium s.c. into the left flank of 6-week-old mice. When s.c. tumors reached ~6 mm in diameter, usually in 5–7 days with a 100% take rate, 20 µl of G207, defective HSV vector, or mock-infected extract were inoculated intraneoplastically (day 0). Mock-infected extract was prepared from virus-buffer-infected cells using the same procedures as those used for virus inoculum (19). The treatment was repeated on day 3. Animals whose s.c. tumors were cured by dB71g/G207 inoculation were rechallenged with a s.c. injection of 5 x 10⁶ Neuro2a or Sal/N cells in 50 µl of serum-free medium into the right flank region. Tumor growth was determined by measuring the tumor volume (length x width x height) twice a week.

Intracerebral tumors were generated by injecting 5 x 10⁴ Neuro2a cells in 5 µl serum-free medium stereotactically into the right frontal lobe of A/J mice. After 5 days, 5 µl of defective HSV-Ig vector or mock-infected extract were inoculated stereotactically at the same coordinates, and survival was followed.

**Histological Examination.** Animals were sacrificed on days 2 and 5; tumors were removed and snap frozen in isopentane cooled with dry ice, and cryostat sections of 10 µm in thickness were prepared. The sections were fixed in cold acetone for 15 min and treated with 0.3% hydrogen peroxide in methanol for 5 min, followed by PBS containing 20% FCS and 0.1% Triton X-100 for 10 min. The sections were incubated for 2 h with primary antibodies; biotin-conjugated anti-human IgG Fc antibody (Jackson 109-065-098: 1:50 dilution), antimonium CD4 antibody (PharMingen 01061D: 1:100 dilution), antimonium CD8a antibody (PharMingen 01041D: 1:25 dilution), and anti-mouse Mac-3 antibody (PharMingen 01781D: 1:100 dilution). The sections were then washed three times with PBS Triton X-100, incubated for an additional 1 h with biotin-conjugated anti-rat IgG antibody (Jackson 212-065-082: 1:400 dilution), and washed three times with PBS. The sections were then developed with 0.03% 3,3-diaminobenzidine tetrahydrochloride plus 0.01% hydrogen peroxide in 50 mm Tris-HCl buffer (pH 7.4). Sections were counterstained with hematoxylin before mounting. The percentage of B7.1-Ig-positive cells was determined by counting the number of positive cells in three areas of ~1000 cells in the vector inoculation region on enlarged photographs taken under low magnification. Sections fixed in 2% paraformaldehyde in PBS were used to perform X-gal histochemistry as described (25) and AP staining as described above.

**In Vivo Depletion of Lymphocyte Subsets.** Tumor-bearing A/J mice were injected i.p. with 250 µg of antimonium CD4 monoclonal antibody (clone GK1.5; ATCC) or 50 µg of antimonium CD8a monoclonal antibody (clone 2D3; ATCC). A similar dose of normal rat IgG (Sigma) was used for controls. The monoclonal antibody treatments were given 1 day before defective HSV vector inoculation and 2, 6, and 12 days after inoculation. To ensure complete depletion, spleen cells were collected from sentinel mice treated by the same regimen, on days 6 and 12, and from mice sacrificed because of tumor burden, and assessed for lymphocyte subsets by flow cytometric analysis as described (25). The depletion of CD4 + and CD8 + T cells was consistently >98%.

**RESULTS**

Defective HSV Vectors Expressing Soluble B7-1-Ig Fusion Protein. To generate a soluble bivalent B7-1 molecule, the extracellular domain of murine B7-1 was fused with the Fc portion of human IgG1. An ampiclon plasmid containing the B7.1-Ig cDNA (Fig. 1) was constructed and used to generate a defective HSV vector dB71g with G207 as helper virus. Expression of B7.1-Ig could be detected in dB71g-infected Vero cells with antibodies against murine B7.1 (Fig. 2A) or human IgG (Fc) (Fig. 2B). Similar numbers of positive cells were detected with both antibodies. No immunoreactivity for murine
untransfected CHO cells. Also, biotinylated CTLA-4-Ig bound to B7-1-Ig immobilized on nylon filters.

B7-1-Ig Expression Inhibits s.c. Tumor Growth in Immunocompetent Mice. The therapeutic efficacy of dvB7lg/G207 was evaluated in A/J mice bearing established s.c. Neuro2a tumors. To determine the impact of the helper virus G207 on tumor growth inhibition, tumors of ~6 mm in diameter were inoculated intraneoplastically with increasing doses of G207 (10^5, 10^6, and 10^7 pfu). Two injections of G207 caused a dose-dependent inhibition of tumor growth, with 10^6 and 10^7 pfu resulting in a 66 and 44% reduction, respectively, at day 13 compared with mock (Fig. 3; P < 0.01 and P < 0.05, respectively; unpaired t test). G207 at 10^7 pfu did not result in any inhibition of tumor growth compared with mock-infected animals (Fig. 3).

To study the effect of B7-1-Ig expression on tumor growth, we used dvB7lg/G207 at a lower titer of G207 (2 × 10^5 pfu) that was ineffective alone. DvAP/G207, the negative control, caused no inhibition of tumor growth compared with mock, as was expected from the prior results with G207 alone. In contrast, dvB7lg/G207 caused a significant inhibition of tumor growth compared with mock or dvAP/G207 (Fig. 4; P < 0.01 and P < 0.05, respectively, on day 14; unpaired t test), although none showed tumor regression in this study.

In a separate experiment using the same protocol, s.c. Neuro2a tumors were harvested for histological evaluation on days 2 and 5 after treatment (2 mice/group). X-gal staining revealed abundant but localized expression of lacZ from the helper virus G207 in dvAP/G207- and dvB7lg/G207-inoculated tumors (Fig. 5A). In situ expression of B7-1-Ig was detected immunohistochemically in all dvB7lg/G207-inoculated tumors examined at the site of vector inoculation (Fig. 5B). The percentage of B7-1-Ig-positive cells at the inoculation site was 1% or less. The dvAP/G207-inoculated tumors all contained AP positively stained cells (Fig. 5C) but were negative for human IgG (Fc) (Fig. 5D).

Immune Cell Infiltrates in Treated Tumors. The presence of immune cells in the tumor was examined by immunostaining for CD4, CD8, and Mac-3. On day 2 in the dvB7lg/G207-inoculated tumors, there was a moderate amount of CD4, CD8, and Mac-3. On day 2 in the dvB7lg/G207-inoculated tumors, there was a moderate amount of CD4+ T-cell infiltration in the “inoculation region” (injection site and closely adjacent area as
demonstrated by positive X-gal staining) and a high amount in the “surrounding region” (the rest of the tumor that surrounded the inoculation region). By day 5, the number of CD4\(^+\) T cells further increased, particularly in the inoculation region (Fig. 6A). CD8\(^+\) T cells were scattered throughout the dvB7Ig/G207-inoculated tumors on day 2, and increased considerably in the inoculation region by day 5, although to lesser extent than the CD4\(^+\) T cells (Fig. 6B). In contrast, in dvAP/G207-inoculated tumors, only a small number of CD4\(^+\) T cells and a rare CD8\(^+\) T-cell were observed, mainly in the inoculation region on both days 2 and 5,

Fig. 5. HSV vector expression in vivo. s.c. Neuro2a tumors were inoculated with 2 \(\times\) 10\(^5\) pfu dvB7Ig/G207 (A and B) or dvAP/G207 (C and D) and isolated 2 days later. X-gal staining (blue) reveals lacZ expression from the helper virus G207, indicating the site of vector inoculation (A). Immunohistochemical staining (brown) for human IgG (Fc) demonstrates the expression of B7-1-Ig in dvB7Ig/G207-inoculated tumors in the region corresponding to vector inoculation (B). In the dvAP/G207-inoculated tumor, vector expression is demonstrated by AP staining (C; purple), but the same region is negative for human IgG (Fc) immunostaining (D). A, counterstained with H&E; B and D, counterstained with hematoxylin; C, counterstained with carmalum. ×90.

Fig. 6. Immune cell infiltration in tumors inoculated with B7-1-Ig-expressing vectors. s.c. Neuro2a tumors were inoculated with 2 \(\times\) 10\(^5\) pfu dvB7Ig/G207 (A–C) or dvAP/G207 (D–F) on days 0 and 3. Tumors were harvested on day 5 and immunostained for CD4 (A and D), CD8 (B and E), or Mac-3 (C and F). In the dvB7Ig/G207-inoculated tumors, an abundant infiltration of CD4\(^+\) T cells (A) and a moderate number of CD8\(^+\) T cells (B) were observed in the vector inoculation region, whereas in the dvAP/G207-inoculated tumors, only a few CD4\(^+\) T cells (D) and CD8\(^+\) T cells (E) were found. Mac-3-positive macrophages were found ubiquitously in both dvB7Ig/G207- and dvAP/G207-inoculated tumors (C and F). Counterstained with hematoxylin. ×100.
compared with either mock- or dvAP/G207-inoculated animals (Fig. 7, right). Depletion of CD8 \(^+\) T cells alone did not affect tumor growth (Fig. 9, right). On the other hand, depletion of CD4 \(^+\) T cells did not affect the antitumor activity of dvB7Ig/G207 (Fig. 9, left). There was a significant inhibition of tumor growth after dvB7Ig/G207 inoculation compared with mock-inoculated, CD4 \(^+\) T-cell-depleted controls (P < 0.001 on day 13, unpaired t test). The inhibition of tumor growth in CD4 \(^+\) T cell-depleted animals was as efficient as in nondepleted animals, and CD4 \(^+\) T-cell depletion itself had no effect on tumor growth (Fig. 9, left). This suggests that the antitumor immune response elicited by B7-1-Ig requires CD8 \(^+\) T cells but not CD4 \(^+\) T cells. Unfortunately, Neuro2a cells were found unsuitable for in vitro standard CTL assays (data not shown).

### B7-1-Ig Treatment Confers Tumor-specific Protective Antitumor Immunity.

To investigate whether the B7-1-Ig treatment confers protective antitumor immunity with memory, five A/J mice whose s.c. Neuro2a tumors were cured (no tumor regrowth during 3 months of follow-up) by intraneoplastic dvB7Ig/G207 inoculations (3 of 8 animals cured under CD4 \(^+\) T-cell depletion and 2 of 16 animals cured under mock depletion from Fig. 9) were rechallenged with a s.c. injection of a lethal dose of Neuro2a cells (5 \(\times\) 10\(^6\)). All 6 naive A/J mice, used as controls, showed continuous tumor growth. In contrast, all mice cured by B7-1-Ig treatment showed tumor regression after an initial temporary growth, indicating the presence of protective antitumor immunity.

To test whether the protective antitumor immunity is specific to Neuro2a cells, the survivors from the s.c. Neuro2a rechallenge study above were further challenged with a s.c. injection of SaI/N, A/J-derived sarcoma cells (5 \(\times\) 10\(^6\)). All mice cured by B7-1-Ig, as well as all 6 naive A/J mice used as controls, showed tumor formation at 4 weeks after implantation, indicating that the antitumor immunity was specific to Neuro2a.

### DISCUSSION

Because B7-1 has been recognized as one of the most potent costimulatory molecules for T-cell activation, there has been a large effort to use the B7-1 gene in immunotherapy for cancer. In the majority of studies, B7-1-transduced tumor cells have been used for active immunization. Transduction of the B7-1 gene alone induces protective immunity against immunogenic tumors (10–14) but fails to induce regression of established tumors (36–39). The ability of B7-1...
expression to induce tumor immunity is directly related to the immunogenicity of the tumor cells (3). B7-1 transduction has been ineffective in poorly immunogenic tumors, and coexpression of other cytokines, cell adhesion molecules, MHC molecules, or tumor antigens was often necessary to enhance antitumor immunity (3, 40–44).

The present findings illustrate several features that may be important for the clinical application of B7-1-Ig immunotherapy. A significant inhibition of tumor growth was achieved by direct inoculation of vectors expressing soluble B7-1-Ig into established tumors. Although ex vivo methods using modified tumor cells as cancer vaccines have proved effective in experimental models, the need to harvest, transport, and reinfuse tumor cells on a patient-to-patient basis has limited their feasibility in clinical situations (45, 46). In vivo transduction of the B7-1 gene by means of either adenovirus or vaccinia virus vectors has been ineffective in reducing the growth of established tumors, even in immunogenic tumor models (15–17). In contrast, a defective HSV vector expressing B7.1 with a replication-deficient HSV helper virus was effective at curing established s.c. EL4 lymphoma tumors in C57BL6 mice (47). The efficacy of B7-1-Ig after in vivo gene delivery suggests that expression of a soluble gene product by a small percentage of tumor cells may lead to a substantial antitumor effect. Herein, a relatively small amount of vector (2 × 10^5 pfu G207 and 5 × 10^5 defective particle units of dvB7lg) exhibited a significant antitumor effect, despite <1% of tumor cells at the inoculation site expressing B7-1-Ig as detected by immunohistochemistry. At these doses, G207 alone was unable to inhibit tumor growth. It is anticipated that higher doses of dvB7lg/G207 would improve antitumor efficacy in this system. B7-1-Ig gene therapy was shown to be effective in a poorly immunogenic tumor, without coexpression of other immunomodulatory genes. This is an advance over prior studies using Neuro2a cells, where transduction with B7-1 reduced tumorigenicity to some extent but failed to induce significant antitumor immunity (28–30). These features of dvB7lg/G207 support the therapeutic feasibility, because most human tumors, including gliomas, express low levels of MHC class I and are considered poorly immunogenic, with a few exceptions such as melanomas (1, 2).

B7-1-Ig gene therapy also significantly prolonged the survival of mice bearing brain tumors. Although the brain has been considered as relatively immune privileged, immune-based approaches have been tried with some success in experimental brain tumor models (48–52). Inoculation of replication-defective adenovirus vectors encoding B7-1-Ig into the brain of AO rats caused an infiltration of CD4^+ and CD8^+ T cells with large perivascular cuffs,^4 which occurred in a significantly larger area and for a longer period of time than the inflammation caused by control vectors encoding *Escherichia coli lacZ* or no transgene (53). This indicates that *in situ* expression of B7-1-Ig can induce strong immune responses in the brain, as well as in the s.c. tumors. Clinical treatment of brain tumor patients would require elicitation of strong antitumor immune responses without significant induction of nonspecific inflammation. *In vivo* delivery of the B7-1-Ig gene may provide a potent and practical strategy for immunotherapy of brain tumors.

*In vivo* depletion of lymphocyte subsets revealed that the antitumor activity of locally secreted B7-1-Ig requires CD8^+ T cells but not CD4^+ T cells. Infiltration of CD4^+ and CD8^+ T cells was significantly increased in tumors inoculated with dvB7lg/G207 compared with tumors inoculated with control vectors. These findings support several possible mechanisms for the action of B7-1-Ig immunotherapy:

(a) Most types of tumor cells do not express B7 molecules (3, 54). Anergy or tolerance to tumor cells may occur as a result of CD8^+ T cells receiving a signal of MHC-bound tumor antigen but lacking the second costimulatory signal (18). The state of anergy may be reversed by providing T cells with costimulatory signals. Soluble B7-1 when secreted from tumor cells may activate T cells in an anergic state that are infiltrating or surrounding the tumor.

(b) MHC class I-restricted tumor antigens are usually not presented by the tumor itself but by dendritic cells or bone-marrow-derived APCs (55). APCs are capable of efficiently presenting antigens derived from apoptotic cells or virus-infected cells, stimulating class I-restricted CD8^+ CTLs (56). The use of oncolytic HSV as a helper virus may have played an important role in providing tumor antigens to APCs. It has been suggested that viral infection induces cross-primering of MHC class I-restricted CD8^+ cells by APCs, which supports the significance of using an oncolytic helper virus (55, 56). It has also been shown that oncolysis after virus replication can act as a potent adjuvant for the induction of antitumor immunity (25, 57, 58). However, APCs that infiltrate tumors can lack B7 molecules and therefore may have reduced T-cell stimulatory activity (59). Expression of soluble B7-1-Ig by tumor cells may allow those tumor cells to function as APCs and, more importantly, could provide professional APCs for increased T-cell stimulatory activity attributable to high levels of B7-1 in the tumor environment. It has been demonstrated previously that B7-1-transduced tumor cells are capable of directly priming naive CTLs *in vivo* to some extent, but the dominant mechanism of CTL priming is still through the uptake and presentation of tumor antigens by bone marrow-derived APCs (60). This CTL priming by tumor-infiltrating APCs may be further facilitated when B7-1 is expressed in soluble form and secreted by tumor cells into the surrounding environment. This priming should occur irrespective of antigen presentation by tumor cells themselves.

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4 A. P. Byrnes, unpublished data.
(c) It has been suggested that induction of antitumor immunity by B7-1-transduced tumor cells may be attributable to increased lysis of B7-1-expressing tumor cells, facilitating subsequent antigen uptake and processing by APCs (60). Several groups have reported that the expression of B7-1 by tumor cells promotes natural killer cell-mediated cytolysis (61–63). In our study in nude mice, the presence of natural killer cells in the absence of T cells was not sufficient for locally secreted B7-1-Ig to exhibit antitumor activity. G207, the replication-competent helper virus in this study, is currently in a clinical trial for patients with recurrent malignant glioma (64). Although the results with G207, both experimental and clinical, show promise for the development of a new field of tumor therapy using oncolytic HSV vectors, one concern is that the oncolytic antitumor effect depends on whether tumor cells support viral replication. Intratumoral inoculation of a B7-1-Ig-expressing HSV vector, together with G207 at a low dose, exhibited significant antitumor activity in Neuro2a tumors, whereas the same dose of G207 with a control vector showed no effect. Therefore, B7-1-Ig immunotherapy can be combined with oncolytic viral therapy to enhance the antitumor effect when the oncolytic virus alone is ineffective in eradicating tumors.

A strategy for generating soluble forms of membrane proteins generally involves designing a fusion protein consisting of a functional extracellular domain of the membrane protein and a protein with a secretion signal. For example, a soluble form of CTLA-4, CTLA-4-Ig, which binds to B7 molecules with high affinity and blocks T-cell activation, has been used to induce graft tolerance or to treat autoimmune disease in experimental animals (65–68). Human IgG1 (Fc) is often fused to the extracellular domain of a mouse protein (65, 69), as was done with the B7-Ig construct. The human Fc region induces a strong humoral response when recombinant CTLA-4-Ig protein is injected systemically in mice, whereas injection of an adenovirus vector expressing CTLA-4-Ig failed to induce an antibody response (70). This difference in humoral response was reflected in the decreased biological activity of recombinant CTLA-4-Ig in suppressing inflammation compared with the adenovirus vector-derived CTLA-4-Ig or CTLA-4-mouse Ig fusion protein (70). It remains to be determined whether an immune response is generated against human Fc in the B7-Ig construct and whether this would be beneficial or detrimental to antitumor activity.

Recently, biotinylated soluble B7-1 has been used to target tumor antigens in vivo for the purpose of adhering B7-1 to the cell surface of existing tumors. This had an antitumor effect in poorly immunogenic TS/A mammary adenocarcinoma (71). While our manuscript was being prepared, Sturmhoefel et al. (72) reported on the use of recombinant soluble B7-IgG fusion protein for tumor therapy. Repeated intra-footpad administration of B7-IgG caused regression or growth suppression of established tumors, which was dependent on CD8+ T cells and independent of IFN-γ expression by the host. Their results support our findings on the potent antitumor activity of soluble B7-1. Although morbidity was not observed in mice receiving systemic administration of B7-IgG, the approach must be carefully considered. There are potential advantages of local vector administration over systemic protein administration. Above all, the activated T cells are likely to be highly specific to tumor cells, as a result of tumor-secreted soluble B7-1 functioning through the proposed mechanisms described above. Local vector administration may provide higher concentrations and more localized distribution of soluble B7-1 in the tumor than systemic administration. Systemic soluble B7-1 administration, on the other hand, may cause activation of T cells primed to various types of antigens. Such nonspecific activation may trigger immune responses against irrelevant antigens, or normal tissues and organs, potentially causing an undesirable toxicity or an autoimmune disease.

Secretion of soluble B7-1 is a phenomenon that does not occur physiologically, and further studies are needed to better understand the mechanisms by which soluble B7-1 induces potent antitumor immunity. Nevertheless, the observation that in vivo expression of soluble B7-1 stimulated a significant antitumor immune response with high efficacy in established poorly immunogenic tumors affords a new strategy of immunotherapy for tumors. B7-1-Ig immune gene therapy should be considered for further preclinical testing and possible future clinical application in patients with solid tumors, including those in the brain.

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