

Recombinant Baculovirus Containing the *Diphtheria Toxin A* Gene for Malignant Glioma Therapy

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Abstract

Insect baculoviruses are capable of infecting mammalian glial cells in the central nervous system. We investigated in the current study the feasibility of using the viruses as toxin gene vectors to eliminate malignant glioma cells in the brain. We first confirmed that glioma cells were permissive to baculovirus infection, with variable transduction efficiencies at 100 viral particles per cell and ranging between 35% and 70% in seven human and rat glioma cell lines. We then developed a recombinant baculovirus vector accommodating the promoter of glial fibrillary acidic protein (GFAP) to minimize possible side effects caused by overexpression of a therapeutic gene in sensitive neurons. We placed the GFAP promoter into a baculovirus expression cassette, in which the enhancer of human cytomegalovirus immediate-early gene and the inverted terminal repeats of adeno-associated virus were employed to improve the relatively low transcriptional activity of the cellular promoter. This recombinant baculovirus significantly improved transduction in glioma cells, providing the efficiency in C6 rat glioma cells up to 96%. When used to produce the A-chain of diphtheria toxin intracellularly in a rat C6 glioma xenograft model, the baculovirus effectively suppressed tumor development. The new baculovirus vector circumvents some of the inherent problems associated with mammalian viral vectors and provides an additional option for cancer gene therapy. (Cancer Res 2006; 66(11): 5798-806)

Introduction

Gliomas are the most common type of i.c. tumors with tendency to invade aggressively in the brain (1). Gliomas originate from glial cells, predominantly from astrocytes, and are graded from 1 to 4, in increasing level of malignancy. Grade 4 gliomas, also termed glioblastoma multiforme, comprise nearly half of all gliomas and are the most frequent primary brain tumors in adults. Glioblastoma multiforme is currently almost incurable. Even with surgical intervention, radiotherapy, and chemotherapy, patients with glioblastoma multiforme usually die within a year, with only a few patients surviving longer than 3 years.

Gene therapy is viewed as a promising therapeutic modality to treat cancer, having been successfully applied using various types of viral vectors, gene expression regulation elements, and putative antitumor genes in animal models. The baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV)-based vectors, traditionally used as biopesticides to kill infected insects (2, 3), are

recently tested as a new type of delivery vehicle for transgene expression in mammalian cells (4). These viruses can enter but not replicate in mammalian cells. With mammalian expression promoters, recombinant baculoviruses provide high transduction efficiencies in many different types of cells and tissues, including several tumor cell lines (4, 5). One of the attractive advantages of using baculovirus AcMNPV as a cancer gene therapy vector is the large cloning capacity conferred by its 130-kb viral genome, which may be favorably used to deliver a large functional gene or multiple genes from a single vector. Baculovirus transduction results in transient gene expression. Although stable Chinese hamster ovary cells expressing green fluorescent protein (GFP) could be isolated following baculovirus transduction and antibiotic selection, it is not clear at what frequency chromosomal integration of delivered DNA sequences without antibiotic selection may occur in transduced cells (6). Thus, the virus seems to be a vector suitable for applications requiring short-term gene expression. Other empirical advantages of baculovirus vectors include the easy construction of a recombinant viral vector and the simple procedure of purifying large quantities of viruses with high titers. It would be possible to scale up the less labor-intensive process to pharmaceutical levels. However, despite a good understanding of all these attractive features of baculovirus, gene therapy application of the virus is still in its infancy, and no practically useful application in cancer therapy has yet been produced, even in preclinical animal studies.

In the current study, we developed a recombinant baculovirus vector accommodating the transcriptional regulatory sequence of the glial fibrillary acidic protein (GFAP) to drive the expression of a toxin gene in glioma cells. Although baculoviruses seem to be more prone to infect glial cells than neurons in the brain (7, 8), the use of a glial cell-specific promoter would further reduce potential side effects on neurons caused by overexpression of a therapeutic gene, especially when a toxin gene is used. However, a cell type-specific promoter usually displays a relatively weaker transcriptional activity than those widely used strong promoters derived from viruses [e.g., the enhancer/promoter of human cytomegalovirus (CMV) immediate-early gene; ref. 9]. To circumvent this shortcoming, appending a viral transcriptional regulatory element has proved to be effective for many cellular promoters like the β -actin promoter and the promoter of human platelet-derived growth factor B-chain (10, 11). Another reported approach that could improve gene expression driven by a cellular promoter is flanking an expression cassette of interest with inverted terminal repeats (ITR) from adeno-associated virus (AAV). Several groups have developed viral or plasmid vectors with this approach and reported improved efficiency of transgene expression in mammalian cells (12), *Xenopus* embryos (13), and fishes (14).

The above viral regulatory elements were incorporated into baculoviral vector backbone in this study to improve transgene expression from the GFAP promoter. We provide the evidence

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showing that, with the assistance of the engineered gene expression cassette, baculoviruses containing a cDNA encoding the A-chain of diphtheria toxin (DT-A; refs. 15–19) preferentially killed glioma cells *in vitro* and effectively inhibited the growth of glioma cells in the rat brain.

Materials and Methods

Recombinant baculovirus vectors. Seven recombinant baculovirus vectors with different expression cassettes were constructed using the transfer vector pFastBac1 (Invitrogen, Carlsbad, CA; Table 1). Two of them contain the CMV enhancer/promoter to drive expression of a firefly luciferase reporter gene (BV-CMV-Luc) and an enhanced GFP (EGFP) reporter gene (BV-CMV-EGFP), respectively. Three baculovirus vectors carry the GFAP promoter to drive expression of the luciferase gene, the first of which has an unmodified GFAP promoter (BV-GFAP-Luc); the second, a modified GFAP promoter produced by appending the CMV enhancer (–568 to –187 relative to the TATA box) upstream to the GFAP promoter (BV-CMV E/GFAP-Luc); and the third, an expression cassette produced by flanking the second cassette with AAV ITRs (BV-CG/ITR-Luc). The other two vectors were produced by replacing the luciferase gene in BV-CG/ITR-Luc with a *DT-A* gene or an *EGFP* gene and named BV-CG/ITR-DTA and BV-CG/ITR-EGFP, respectively.

To generate BV-CMV E/GFAP-Luc, a CMV enhancer sequence amplified from pRC/CMV₂ (Invitrogen) was inserted into pFastBac1 between the sites of *NotI* and *XbaI*, and a GFAP promoter amplified from pDRIVE02-GFAP (Invitrogen, San Diego, CA) was subsequently inserted downstream of the CMV E between *XbaI* and *XhoI*. To construct BV-CG/ITR-Luc, BV-CG/ITR-DTA, and BV-CG/ITR-EGFP, an expression cassette from pAAV plasmid (20), containing a multiple cloning site, a reporter gene encoding luciferase, a SV40 polyadenylated signal, and two ITR sequences at both ends, was amplified and inserted into pFastBac1 between *AvrII* and *SalI*. The CMV E/GFAP promoter was then inserted into the sites of *KpnI* and *HindIII*. A luciferase cDNA from pGL3-basic vector (Promega, Madison, WI), an *EGFP* reporter gene from pEGFP-C1 vector (Clontech, Mountain View, CA), and a *DT-A* gene amplified from pCAG/DT-A-2 (kindly provided by Dr. Masahiro Sato, Tokai University, Japan) were inserted, respectively, into the downstream of the GFAP promoter between the sites of *HindIII* and *XbaI*.

Recombinant baculoviruses were produced and propagated in *Sf9* insect cells according to the manual of the Bac-to-Bac baculovirus expression system (Invitrogen).

Virus infection. For *in vitro* experiments, human glioma cell lines of BT325, U251, U87, H4, SW1783, and SW1088; rat C6 glioma cell line; and two nonglioma cell lines HepG2 and NIH3T3 were used. A stable C6 cell clone with the firefly luciferase gene under the control of the CMV promoter (C6-Luc) was generated to facilitate bioluminescent imaging in living cells. Cells were seeded in 96-well plates at a density of 1,000 per well or 48-well plates at a density of 20,000 per well for luciferase activity assay and in 12-well plates with a density of 100,000 per well for flow cytometric analysis. Cells were incubated with appropriate amounts of baculovirus vectors in serum-free DMEM at 37°C for 1 hour. After the incubation,

serum-free DMEM containing the viruses was replaced by normal growth medium, and the infected cells were further cultured at 37°C until being used for various assays.

For *in vivo* experiments, adult male Wistar rats (weighing 250–300 g) were used. Rats were anesthetized and positioned in a stereotaxic instrument with the nose bar set at 0. Rats were first inoculated with rat C6 or C6-luc cells (1×10^5 in 5 μ L) in the striatum on one side of the brain (anterior-posterior: +1.0 mm, mediolateral: +2.5 mm, and dorsoventral: –5.0 mm from bregma and dura) using a 10- μ L Hamilton syringe connected with a 30-gauge needle at a speed of 0.5 μ L/min. The needle was allowed to remain in place for another 5 minutes before being slowly retracted at the end of each injection. Three days later, 5×10^7 viral particles of BV-CG/ITR-EGFP or 5×10^6 of BV-CG/ITR-Luc in 3 μ L were injected into the same region and into the contralateral striatum in some animals. Rats were euthanized 2 days after viral injection, and the brain tissues were collected for gene expression analysis.

Transgene expression analysis. For luciferase activity assays, cultured cells were washed and permeabilized with 100 μ L of reporter cell lysis buffer (Promega). Ten microliters of the cell extract were used for luciferase assays with an assay kit from Promega. Measurements were made in a single-tube luminometer (Berthold Lumat LB 9507, Bad Wildbad, Germany) for 10 seconds. To measure the luciferase expression in brain tissues, samples were collected and homogenized in PBS (100 μ L PBS per 50 mg tissue) by sonication for 10 seconds on ice and centrifuged at 13,000 rpm for 10 minutes at 4°C. Ten microliters of the supernatant of homogenized tissues were used for luciferase assays.

Luciferase activity in a stable C6 cell clone with the firefly luciferase gene was monitored by luminescent imaging with the IVIS Imaging System (Xenogen, Alameda, CA) comprising a highly sensitive, cooled CCD camera mounted in a light-tight specimen box. Two to 5 minutes before cell imaging, luciferin-EF (Promega) at 150 μ g/mL in PBS was added to each well. Bioluminescence emitted from the cells was acquired for 30 seconds and quantified as photons per second using the Living Image software (Xenogen).

For flow cytometric analysis for EGFP expression, transduced cells were washed with PBS, trypsinized, dispersed in suspension, and subjected to analyses by FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). Untransduced cells served as negative controls. Three sets of independent transduction experiments were carried out for each assay.

The DT-A mRNA expression was determined using SuperScript One-Step Reverse Transcription-PCR (RT-PCR) with PlatinumTaq kit (Invitrogen). The primer sequences for amplification were 5'-AAATACGACGCTGCGG-GAT in the forward direction and 5'-GAAGGGAAGGCTGAGCACTA in the reverse direction. The amplification conditions were predenaturation at 50°C for 30 minutes and 94°C for 2 minutes followed by 38 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 1 minute and final extension at 72°C for 10 minutes. Aliquots of PCR products were electrophoresed on a 1% agarose gel, stained by ethidium bromide, and visualized by UV.

For immunohistochemical analysis of cell type specificity of transgene expression, anesthetized rats were perfused with 0.1 mol/L PBS (pH 7.4) solution followed by 4% paraformaldehyde in PBS. The brains were removed and post-fixed in the same fixative for 2 to 4 hours before being transferred into 20% sucrose in 0.1 mol/L PBS to incubate overnight at 4°C. Cryostat sections were cut at 30- μ m thickness for immune staining. A polyclonal anti-luciferase (Promega; dilution 1:150) or a polyclonal anti-GFAP (Promega; dilution 1:150) was used as the primary antibody to show inoculated C6 glioma cells and nearby astrocytes, whereas the expression of EGFP could be visually detected without staining. Anti-rabbit IgG TRITC conjugate (Sigma-Aldrich, St. Louis, MO; dilution 1:500) was used as the secondary antibody. Mounted sections were examined using a laser scanning confocal microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were seeded in 96-well plates at density of 10,000, 1 day before virus infection. The cells were infected with appropriate amounts of recombinant baculoviruses containing either the DT-A gene (BV-CG/ITR-DTA) or EGFP reporter gene (BV-CG/ITR-EGFP) in 50 μ L of serum-free DMEM and

Table 1. Vectors used in the current study

Name	Promoter	Transgene
BV-CMV-Luc	CMV	Luciferase
BV-CMV-EGFP	CMV	EGFP
BV-GFAP-Luc	GFAP	Luciferase
BV-CMV E/GFAP-Luc	CMV E+GFAP	Luciferase
BV-CG/ITR-Luc	CMV E+GFAP, ITR flanking	Luciferase
BV-CG/ITR-EGFP	CMV E+GFAP, ITR flanking	EGFP
BV-CG/ITR-DTA	CMV E+GFAP, ITR flanking	DT-A

incubated at 37°C for 3 hours. After the incubation, the serum-free DMEM containing the baculoviruses was replaced by 100 μ L of fresh growth medium, and the incubation of cells was continued at 37°C. At the indicated time points, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL in PBS, sterilized by filtration) were added to each well. After 4 hours of incubation at 37°C, the medium was removed, and 200 μ L of DMSO were added into each well to dissolve the crystals. The absorbance was measured in a microplate reader at 550 nm (Bio-Rad, Richmond, CA; Module 550).

Rat C6 tumor xenograft model and tumor growth monitoring. C6-luc cells were injected into the striatum on both sides of the rat brain (100,000 per side) using the protocol described above. Three days later, 1×10^7 viral particles in 3 μ L per side were injected into the striatum. BV-CG/ITR-DTA was injected into the left side, and BV-CG/ITR-EGFP, serving as a viral vector control, was injected into the right side. Tumor growth was monitored by either luminescent imaging of C6-luc cells in living animals or luciferase activity assays of brain tissues. Luminescent imaging was done with the IVIS Imaging System (Xenogen). Ten minutes before *in vivo* imaging, anesthetized animals were i.p. injected with D-luciferin (Promega) at 40 mg/kg in PBS. The animals were then placed onto a warmed stage inside the camera box. The detected light emitted from C6-luc cells was digitized and electronically displayed as a pseudo-color overlay onto a gray-scale animal image. Images and measurements of luminescent signals were acquired and analyzed using the Living Image software (Xenogen). Animals were euthanized 14 days after virus injection. For luciferase activity assays, tissue samples from two sides of the brain were collected and processed as stated above.

In the handling and care of animals, the *Guidelines on the Care and Use of Animals for Scientific Purposes* issued by the National Advisory Committee for Laboratory Animal Research, Singapore was followed. For any study that defines death of the experimental animals as the end point, the guideline requests to consider an earlier point in the study when the necessary data have been collected and the animals could be euthanized. The experimental protocols of the current study were approved by the Institutional Animal Care and Use Committee, National University of Singapore and Biological Resource Center, the Agency for Science, Technology, and Research, Singapore.

Results

Effective transduction of glioma cells by baculovirus vectors. The transduction efficiency of baculoviruses varies in different mammalian cells (5). Therefore, we first tested baculovirus-mediated gene transfer in seven glioma cell lines (i.e., C6, H4, SW1088, SW1783, U87, U251, and BT325) with two baculovirus vectors (BV-CMV-Luc and BV-CMV-EGFP) containing a luciferase and an EGFP gene, respectively, under the control of the ubiquitous CMV promoter. The broad activity of the CMV promoter facilitates the comparison of gene expression levels between glioma cell lines with different levels of malignancy. As shown with quantitative luciferase activity assays, BV-CMV-Luc dose-dependently transduced all the tested glioma cell lines with similar efficiency, except a somewhat higher level of transduction in U87 and a lower level in H4 at a multiplicity of infection (MOI) of 100 plaque-forming units (pfu) of viral particles per cell (Fig. 1A). Using BV-CMV-EGFP, we visually detected transgene expression in glioma cells as early as 4 to 6 hours after viral transduction. Flow cytometry analysis of these cells 24 hours after transduction indicated the percent transduced cells with intense green color ranging from 30% to 70% at a viral MOI of 100 (Fig. 1B). Increasing the MOI value to 200 resulted in only 10% improvement, indicating that the transduction efficiency in glioma cells had reached a plateau at an MOI around 100.

Improved gene transfer into glioma cells by baculovirus vectors with modified GFAP promoters. Using cell type-specific

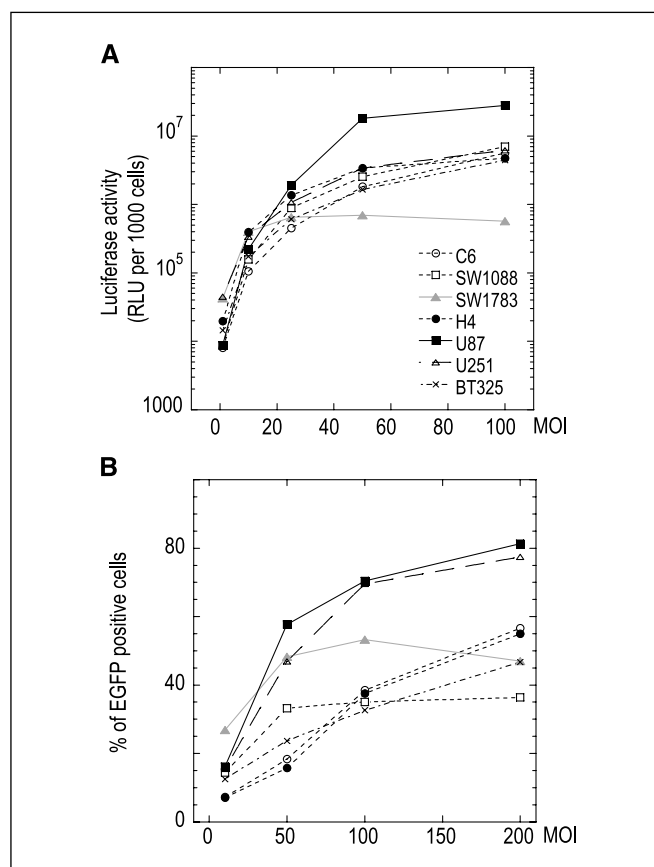


Figure 1. Baculovirus-mediated transduction in glioma cells. **A**, luciferase expression in glioma cells. Cells were transduced with BV-CMV-Luc with increased MOI from 1 to 100. Luciferase assay was carried out 1 day after infection. Expressed in relative light units (RLU) per 1,000 cells. **B**, EGFP expression in glioma cells. Cells were transduced with BV-CMV-EGFP with increased MOI from 10 to 200 and analyzed with flow cytometry 1 day later. Percentage of EGFP-positive cells.

promoter to drive the expression of a therapeutic gene would ensure therapeutic efficacy in the cells of interest, whereas limiting side effects in nontarget cells. We constructed a baculovirus vector (BV-GFAP-Luc) with a GFAP promoter to drive the expression of a luciferase to restrict gene expression in glial cells in the brain (Fig. 2A). This vector, however, provided only low levels of transgene expression in the tested glioma cells, being 10 to several hundred-fold lower than those from the baculovirus vector with the CMV promoter (BV-CMV-Luc; Fig. 2A). We then placed two modified expression cassettes into the baculoviral vector (Fig. 2A). In one of the modified vectors, the CMV enhancer from human CMV was inserted upstream to the GFAP promoter (BV-CMV E/GFAP-Luc), and in another, the ITR sequences from AAV were used to flank the luciferase gene and the hybrid CMV E/GFAP promoter (BV-CG/ITR-Luc). As shown in Fig. 2A, the CMV enhancer in the context of baculovirus significantly improved the strength of the GFAP promoter in all tested glioma cell lines, resulting in the levels of gene expression close to those driven by the CMV promoter. With AAV ITR flanking, further improvement in gene expression, by at least 10-fold relative to BV-CMV E/GFAP-Luc, was observed. In five of seven tested glioma cell lines, the levels from BV-CG/ITR-Luc were even higher than those provided by BV-CMV-Luc (Fig. 2A).

In two nonglioma cell lines (HepG2 and NIH3T3), BV-CMV-luc provided transgene expression levels similar to those in the glioma cell lines infected with the same viral vector (Fig. 2A). In contrast, BV-GFAP-Luc, BV-CMV E/GFAP-Luc, and BV-CG/ITR-Luc displayed significantly lower activities than BV-CMV-Luc in HepG2 and NIH3T3 cells. As BV-CMV-Luc transduced the two nonglioma cell lines and those glioma cell lines with similar efficiency, the low levels of gene expression from the three viral vectors with the GFAP promoter in HepG2 and NIH3T3 cells was less likely caused by the difference in cellular uptake of viruses. These findings indicate the cell type specificity of the modified gene expression cassettes with the GFAP promoter.

Although the enzymatic activity of luciferase is a sensitive quantitative variable to evaluate gene transfer efficiency, especially in a study to compare the strength of the promoter in a particular cell line, the number of EGFP-positive cells would be more indicative of the percentage of transduced cells. Therefore, we

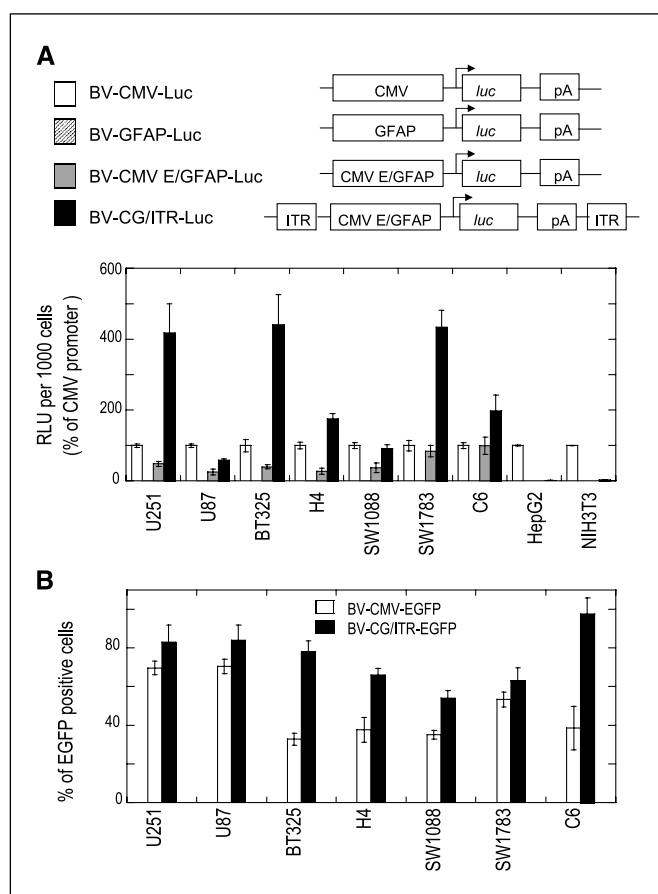


Figure 2. Modified GFAP promoters improved baculovirus-mediated transduction in glioma cells. **A**, baculoviral vectors with a luciferase reporter gene. Schematic diagram of the expression cassettes (top). **BV**, baculovirus; **CMV**, the promoter/enhancer of human cytomegalovirus immediate-early gene; **GFAP**, the promoter of the glial fibrillary acidic protein; **CMV E**, the enhancer of human cytomegalovirus immediate-early gene; **ITR**, AAV inverted terminal repeats; **luc**, luciferase gene; **pA**, SV40 polyA signal. Cells were infected with the baculoviral vectors at an MOI of 25. Luciferase activity assay was done 1 day after transduction. Expressed in relative light units (RLU) per 1,000 cells and reported as the percentage of relative light units produced by the vector with the CMV promoter. Columns, mean ($n = 4$); bars, SD. **B**, baculoviral vectors with an EGFP reporter gene. Cells were transduced with BV-CG/ITR-EGFP at an MOI of 100 and analyzed with flow cytometry 1 day later. Percentage of EGFP-positive cells. Results from the experiment with BV-CMV-EGFP in Fig. 1B are included for comparison.

constructed a baculoviral vector with the hybrid CMV E/GFAP promoter flanked by AAV ITR sequences to drive the expression of the *EGFP* gene (BV-CG/ITR-EGFP). Flow cytometry analysis showed a significant improvement in transduction efficiency over the experiments using the CMV promoter in all of the tested cell lines, with the percentage of transduced glioma cells ranging from 54% in SW1088 to 98% in C6 cells (Fig. 2B).

A DT-A expressing baculovirus construct inhibits protein synthesis and cell growth in cultured glioma cells. To explore the feasibility of using baculoviruses for glioma therapy, a new recombinant baculovirus vector was constructed containing a cDNA encoding DT-A under the control of the hybrid CMV E/GFAP promoter and flanked by the AAV ITRs (BV-CG/ITR-DTA). Although DT-A is highly toxic, viral particles with high titers were successfully generated in *Sf9* cells, most likely due to the tight transcriptional control of the GFAP promoter in the insect cells. The expression of the *DT-A* gene from the baculovirus vector was confirmed by RT-PCR with *DT-A* gene-specific primers in U251 glioma cells (Fig. 3A).

We then tested whether BV-CG/ITR-DTA can block protein synthesis in glioma cells by evaluating its effect on co-expressed luciferase proteins using a method first reported by Maxwell et al. (15). Six glioma cell lines were transduced with BV-CMV-Luc together with either BV-CG/ITR-DTA or BV-CG/ITR-EGFP. Forty-eight hours later, cell lysates were prepared and assayed for luciferase activity. Even at a viral MOI of 10, a significant decrease in luciferase activity was observed in all the tested glioma cell lines, with about 50% inhibition in BT325 to almost 90% inhibition in SW1088 cells (Fig. 3B). Transduction of glioma cells with a higher concentration of the control virus decreased luciferase activity in some cell lines, but the more dramatic effects were obvious when the viruses expressing DT-A were used.

To examine the DT-A inhibition effect over time, we used the sensitive IVIS cooled CCD camera system to monitor progressive change in luciferase activity over 6 days in stable rat C6 glioma cells engineered to express firefly luciferase (C6-Luc). After transduction of C6-Luc cells with BV-CG/ITR-DTA, luciferase activity decreased from 59% of the control on day 2 to 32% on day 6 at a viral MOI of 50 and from 38% of the control on day 2 to 14% by day 6 at an MOI of 100 (Fig. 3C). Inhibition of protein synthesis might eventually result in cell death, which would be another cause of deduction in luciferase activity on day 6. Transduction with an MOI of 10 resulted in 20% to 30% of inhibition on day 2 and 3 but not at later time points, probably due to rapid cell growth of nontransduced C6-Luc cells (Fig. 3C).

To test the direct cytotoxic effect of BV-CG/ITR-DTA, cell viability assays were done in C6-Luc and human U87 glioma cells, as well as two nonglioma HepG2 and NIH3T3 cell lines. These cells were transduced with BV-CG/ITR-DTA or BV-CG/ITR-EGFP at an MOI of 100 pfu of viral particles per cell, and the cell viability assay was carried out 6 days after viral transduction. As shown in Fig. 3D, transduction of the viruses with the DT-A expression cassette resulted in 90% of growth inhibition in C6-luc cells and 40% in U87 glioma cells but not obviously in HepG2 and NIH3T3 cells.

Baculovirus-mediated expression of reporter genes in glioma cells inoculated in the rat brain. To investigate the possibility of using baculoviruses for *in vivo* glioma gene therapy, we tested in an animal glioma xenograft model the expression of the *EGFP* gene and the *luciferase* gene. In the first experiment, rat C6-Luc glioma cells were inoculated into the rat striatum and

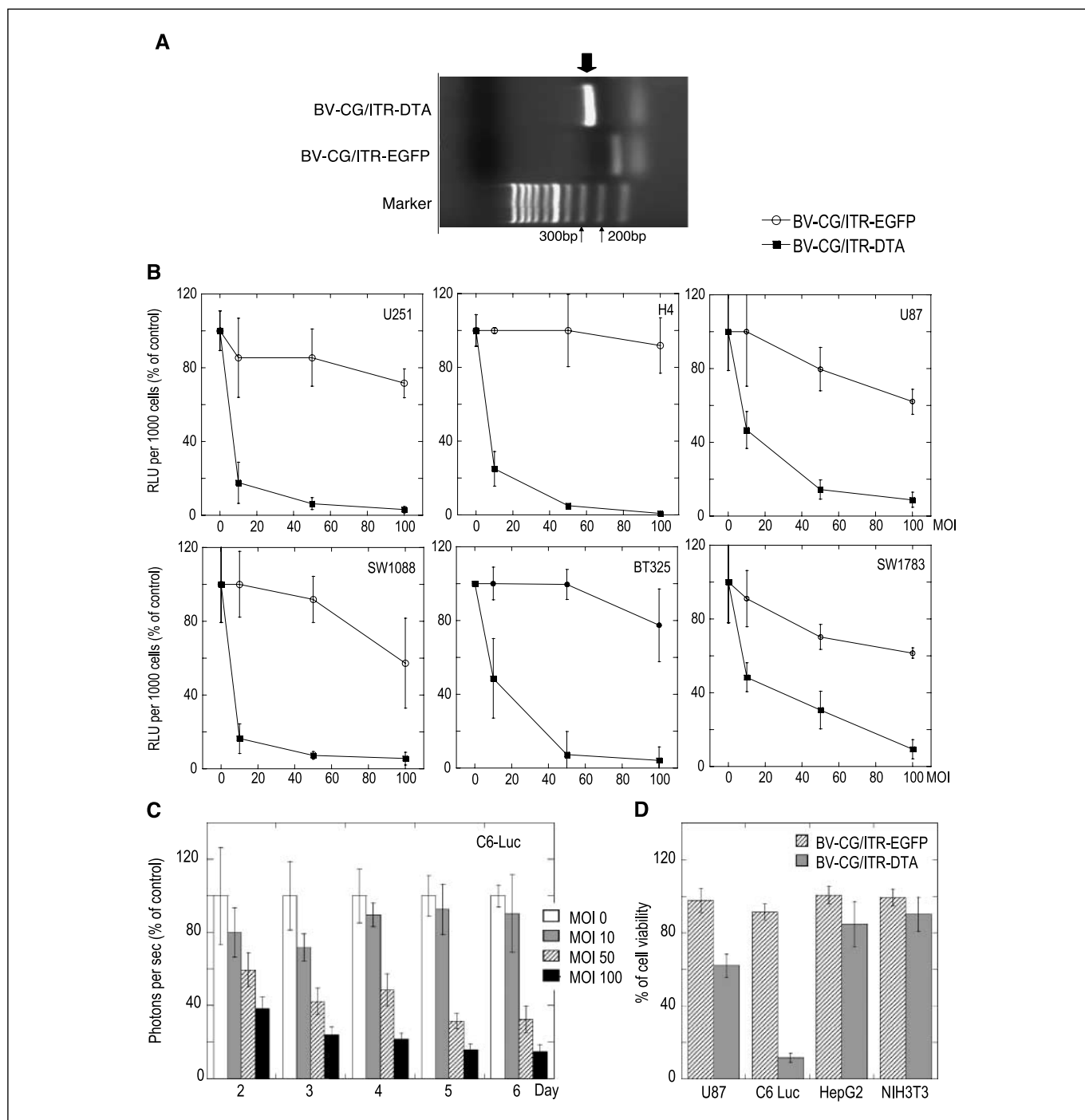


Figure 3. *In vitro* effects of baculovirus vectors carrying the *DT-A* gene. Cells were transduced in quadruplicate. **A**, RT-PCR analysis of *DT-A* expression in U251 cells 48 hours after transduction at an MOI of 100. *Down arrow*, a clear PCR band with a predicated size of 250. **B**, protein synthesis inhibition, as shown by decrease of luciferase activity, was assayed 48 hours after transduction with increased MOI from 10 to 100 in six glioma cell lines. **C**, after transduction of BV-CG/ITR-DTA in C6-Luc cells, time-dependent effects over 6 days were examined using the IVIS imaging system. **D**, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 6 days after viral infection at an MOI of 100 in four cell lines.

allowed to grow for 3 days to establish a glioma xenograft model. BV-CG/ITR-EGFP was then injected into the glioma tumor at the same position, and immunostaining was carried out 2 days later. As shown in Fig. 4A, luciferase-positive C6 cells expressed detectable EGFP. In the sections immunostained with antibodies against GFAP, the solid gliomas were delineated by a rim of reactive astrocytes with strong GFAP signals. Many of these reactive

astrocytes also expressed high levels of EGFP, displaying bright green fluorescence (Fig. 4A, *right*). We did not observe any EGFP-positive cells in the normal region outside the gliosis rim.

In the second *in vivo* experiment, we aimed to compare the transgene expression levels in glioma cells and normal astrocytes. We inoculated C6 glioma cells without the *luciferase* gene into the one side of the rat striatum. Three days later, we injected the same

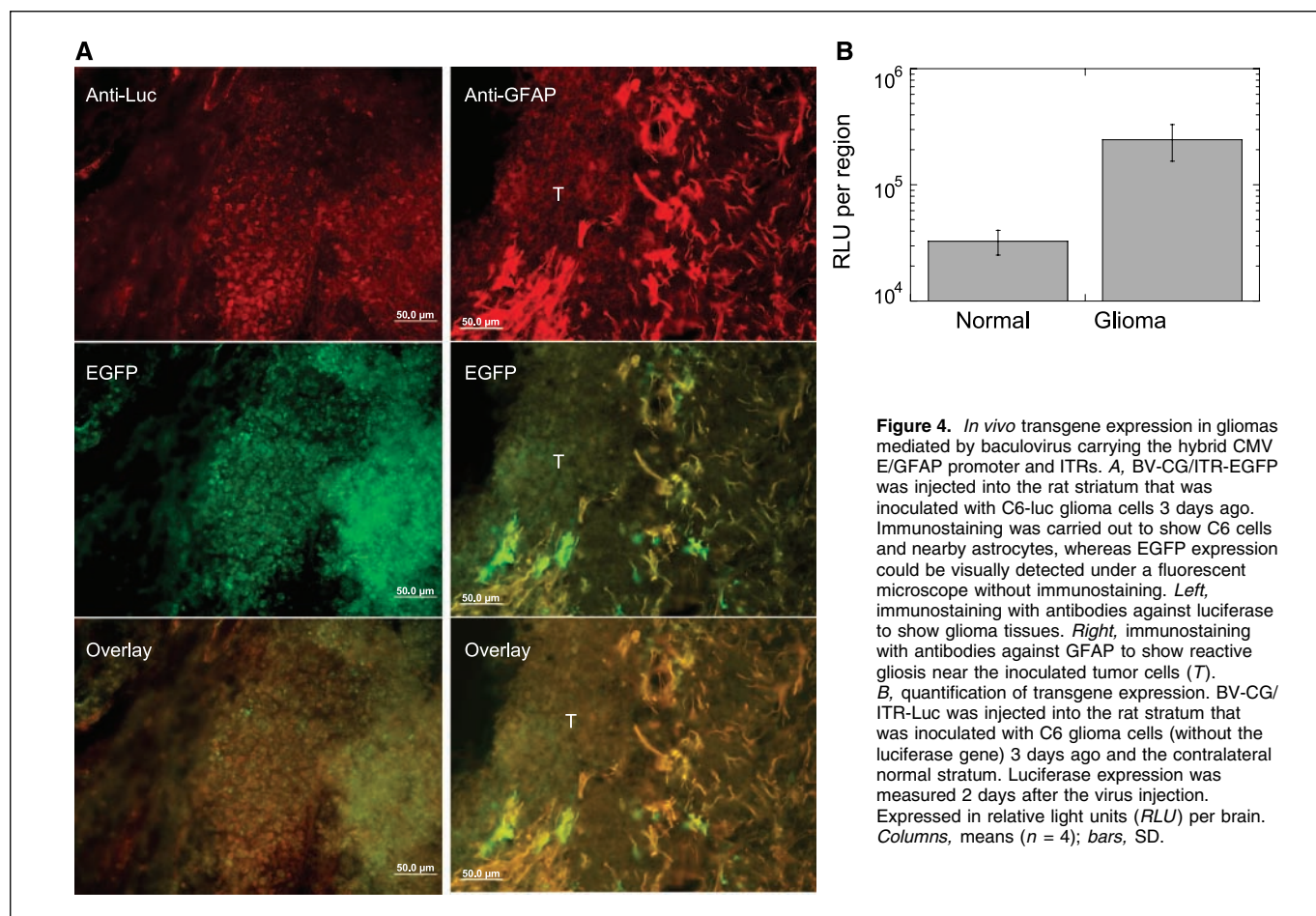
amount of BV-CG/ITR-Luc into the C6 cell-inoculated brain region and the contralateral side of the rat brain, respectively. Two days after viral injection, we collected the brain tissues and measured luciferase activity. We detected a 10-fold higher level of luciferase expression in C6-inoculated brain region than that in normal brain (Fig. 4B).

Inhibition of glioma xenograft growth in the rat brain by the DT-A expressing baculovirus construct. Having shown the potent cytotoxicity in cultured glioma cells induced by baculoviruses with the *DT-A* gene and the efficient transduction of baculovirus in glioma xenograft, we then explored the *in vivo* antiglioma effect of BV-CG/ITR-DTA in the rat brain. Rat C6-Luc glioma cells were used to facilitate noninvasive quantitative evaluation of tumor growth in living animals with the IVIS *In vivo* Imaging System. C6-luc cells were inoculated into the striatum on both sides. Three days later, BV-CG/ITR-DTA was injected into the left side and BV-CG/ITR-EGFP as the control into the right side. Tumor cell growth in the rat brain was monitored 0, 3, 7, and 14 days after virus injection. Figure 5A shows the easily detected luminescent activity from the inoculated C6-luc cells on the control side from day 3 onward and the continuous increase of the luminescent intensity during a 14-day experiment period in one rat. On the DT-A-injected side of the same animal, there was no detectable luciferase activity. Quantitative results from these rats were summarized in Fig. 5B, showing an obvious growth inhibition of C6 glioma cells by just one injection of BV-CG/ITR-DTA.

Tumor growth in the brain was also examined by measuring tissue luciferase activity. Brain tissues from both sides of C6-luc-inoculated rats were collected at day 0 (3 days after tumor cell inoculation) and day 14 after *i.t.* injection of baculoviruses. At the time of viral injection (day 0), luciferase activities from two sides of the brain were very close. Two weeks after viral injection, the activity on the control side injected with BV-CG/ITR-EGFP was 30-fold higher than that on the BV-CG/ITR-DTA-injected side (Fig. 5C), indicating that the DT-A expression from the baculoviral vector effectively inhibited growth of tumor cells in the brain.

Discussion

Under the control of a cell type- or tumor-specific promoter, the *DT-A* gene has been tested in cancer therapy (15–19). This bacterial protein is highly toxic when introduced into the cytoplasm of eukaryotic cells. It inhibits protein synthesis by catalyzing ADP ribosylation of the diphthamide group of cellular elongation factor 2 and kills cells through an apoptosis pathway (21). The high toxicity of DT-A in mammalian cells necessitates the use of a promoter with tumor selectivity to control its expression and to avoid any unintended deleterious effects on non-target cells. In this study, we have used the GFAP promoter to restrict the expression of the *DT-A* gene to glial cells. In combination with the *i.t.* injection procedure, the use of the cell type-specific promoter would significantly reduce the risk of disturbing neuronal functions due to expression of the toxin



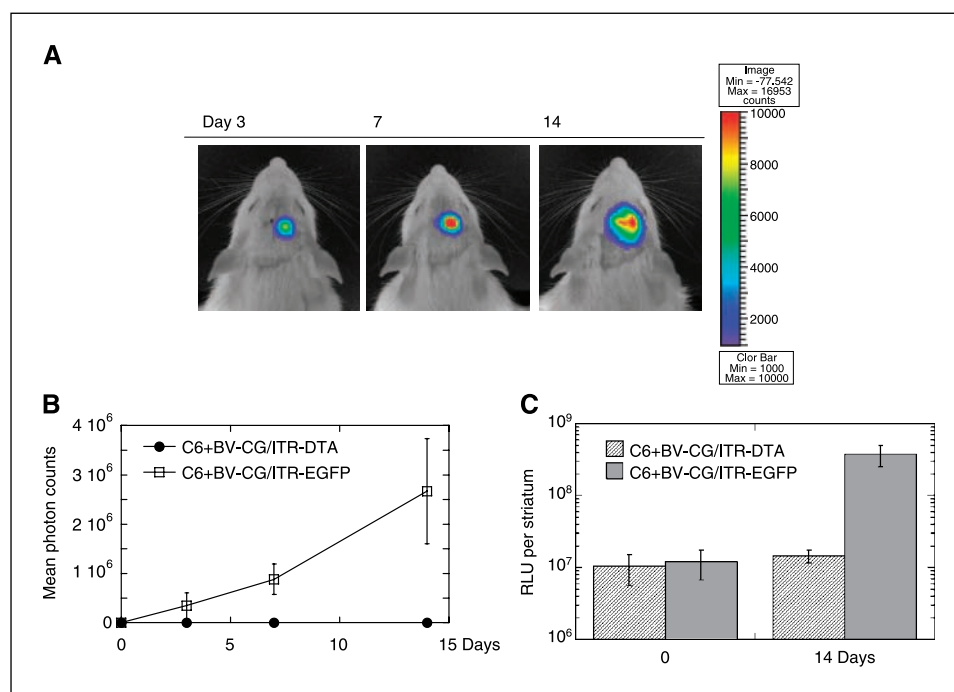


Figure 5. *In vivo* assays of C6 glioma growth in the rat brain. Anesthetized rats were inoculated with C6-luc cells to each side of the brain followed by injection of BV-CG/ITR-DTA (left) and BV-CG/ITR-EGFP (right) 3 days later (designated as day 0). *A*, *in vivo* bioluminescent images of the brains with inoculated C6-luc cells 3, 7, and 14 days after virus injection in a living animal. The luminescent light emitted from the side injected with the control viruses was easily detected and increased over time. No light could be detected on the BV-CG/ITR-DTA injected side. *B*, quantification of *in vivo* bioluminescence. Points, mean photon counts over time; bars, SD. Photon counts at day 0 were not much different from background bioluminescence. $n = 6$ at days 3 and 7 and $n = 5$ at day 14, as one rat died at day 12. *C*, measurement of tumor growth by luciferase activity assays of brain tissues collected at day 0 ($n = 3$) and day 14 ($n = 5$). Columns, mean relative light units (RLU) per brain; bars, SD.

gene. Although leakage of the viral vectors may infect nearby astroglial cells, these cells are not functionally crucial, and the expression of a toxin gene in normal astroglial cells could be tolerable.

The GFAP promoter is inactive in insect cells, as revealed by our observations that whereas a baculovirus construct accommodating the *EGFP* gene driven by the viral CMV promoter produced green fluorescence in insect cells, the construct using the GFAP promoter did not produce EGFP-positive insect cells.³ This cellular specificity has made it possible for us to propagate high titers of recombinant baculoviruses containing the *DT-A* gene. We have thus shown the usefulness of the baculovirus/insect cell system to generate a recombinant toxin gene-expressing viral vector suitable for targeted toxin or suicide gene expression in tumor cells.

To enhance the transcriptional strength of the GFAP promoter, we have modified the promoter by incorporating two types of viral transcriptional regulatory elements. We showed the feasibility of using the CMV enhancer to positively stimulate the GFAP promoter in a baculovirus vector. After incorporating AAV ITRs into our baculovirus vectors harboring the hybrid CMV E/GFAP promoter, further improvement in expression levels was achieved. We also showed that the glial cell specificity of the GFAP promoter was retained after all the modifications. Interestingly, our recombinant baculoviruses accommodating the engineered expression cassette could drive a higher level of gene expression in a glioma cell-inoculated region than in the normal brain region. This may be due to the actively proliferating state of tumor cells and/or gliosis in the surrounding tissue in response to the damage caused by tumor growth. In the histologic analysis of the brain inoculated with glioma cells followed by *i.t.* injection of baculoviruses, we indeed observed high levels of EGFP expression in the gliosis rim surrounding the tumors, which could probably serve as a barrier to prevent leakage of viruses into the normal tissues. These features

indicate a great potential of the developed viral vector in glioma gene therapy. The specificity of the therapeutic vector in the treatment of gliomas could be improved further if a promoter active only in gliomas becomes available.

We have used rat C6 glioma cells to establish a xenograft animal model. The cell line seems highly susceptible to baculovirus transduction. When the CG/ITR expression cassette was used, almost all C6 cells (98%) were transduced. With such high transduction efficiency and the potent DT-A effect, one would expect to see a significant growth inhibition of C6 cells in the animal model. Consistent with the *in vivo* observation, our *in vitro* experiments showed as well a significant growth inhibition and cell death in C6 cells (Fig. 3C and D). We feel that failure to eliminate more C6 cells in our animal study is mainly due to the penetration efficiency of injected viruses in tumor mass being less than satisfactory. With multiple injections of viruses in several locations of the tumor, we might be able to observe further decrease in luciferase signals. As DT-A cannot penetrate the cell membrane without the assistance of the B chain of DT, we believe that a bystander effect of the DT-A is less likely. However, we cannot exclude the possibility that DT-A released from dead cells is taken up by nearby tumor cells through non-receptor-mediated endocytosis.

Many different types of viral vectors have been developed and tested for the delivery of therapeutic genes into malignant gliomas (22). In terms of transduction efficiency, baculoviral vectors tested in the current study are well compatible to other commonly used viral vectors, such as AAV, adenoviruses, and lentivirus. For example, a recent study based on flow cytometric analysis showed that AAV2 was superior to AAV1, transducing up to 80% of cells at an MOI of 5,000 and 90% at an MOI of 50,000 in five human glioma cell lines (23). Enger et al. did flow cytometric analysis to quantify AAV2-GFP- and adenovirus-GFP-mediated transduction with an MOI of 100 in six glioma lines and reported transduction efficiency variable between the cell lines from 1.5% to 81%, with most of the values <50% (24). It has been reported that lentiviral vectors

³ Unpublished observation.

pseudotyped with different virus envelopes displayed transduction efficiency from 50% to 90% in U87 glioma cells at MOI of 1 (25).

Adenovirus, AAV, and lentivirus are infectious human viruses. A common problem associated with the use of human viral vectors is the preexisting immunity directed against them in the vast majority of the population. Specific antibodies against adenoviruses are detectable in 97% of individuals, and those against AAV2 are detectable in 35% to 80% of individuals, depending on age group and geographic location (26). Such immunity would eliminate viral particles and impair viral transduction, decreasing both the levels and the duration of transgene expression. The absence of preexisting immunity against nonhuman viruses is well established. Being insect viruses that do not replicate in mammalian cells, wild-type baculoviruses are much less likely to penetrate within the human body and stimulate the immune system. This would significantly reduce the chance of vector neutralization caused by preexisting antiviral humoral and cellular immunity in patients.

Early generation adenovirus vectors elicit a strong innate immune response *in vivo* with their capsid proteins and viral gene products (26, 27). It is likely that baculovirus does not express its own viral proteins in mammalian cells, and it does not seem overly pathogenic to mammalian cells (2, 5, 28, 29). However, the viruses do have immunostimulatory capacities to induce the expression or promote the release of inflammatory cytokines of IFN, tumor necrosis factor- α , interleukin-1 α (IL-1 α), IL-1 β , and IL-6 (30–32). Once injected into the body, baculoviruses can elicit significant innate immune responses. This property has been exploited to protect animals from a lethal challenge with encephalomyocarditis virus (30) and influenza virus (32). A recent study has shown that it is the viral genome that induces the innate immune response through the toll-like receptor 9 signaling pathway and suggested the possibility that the virus may be harnessed therapeutically to induce a host immune response against various infectious diseases (33).

First-generation adenovirus vectors also induce adaptive immunity, featured as an immune response specifically directed against the products of viral genes. Even in the absence of viral gene

products, adenoviral capsid proteins can still induce the infiltration of CD8⁺ T cells, leading to cell-mediated destruction of the infected cells involving secretion of IFN- γ and induction of apoptosis or lysis (26, 34). For the treatment of cancer, this immunogenicity may actually augment the antitumor effects of specific virus-mediated gene transfer approaches (22, 34). The initiation of an adaptive immune response is critically dependent on the activation, functional maturation, and migration of dendritic cells. Baculoviruses can activate these cells via the interaction with intracellular toll-like receptor 9 (33). Moreover, muscle injection of baculoviruses in mice markedly stimulated the production of neutralizing antibodies against the injected viruses, which had probably caused the clearance of transduced muscle fibers (35). In the context of cancer therapy, whereas this adaptive immune response and the innate immune response elicited by baculoviruses will compromise the expression of therapeutic genes, the immune responses might also act partially like those elicited by first generation adenovirus vectors to provide additional antitumor effects. Further studies are necessary to explore this hypothesis.

In conclusion, a baculovirus-based toxin gene therapy vector has been developed that was effective in inhibiting glioma cell growth *in vitro* and *in vivo*. This study is, to the best of our knowledge, the first demonstration of using baculovirus vectors for cancer gene therapy in an animal model. Although our data concerns only glioma therapy, the unique attributes of the vectors are equally appealing for the treatment of other tumors. Looking ahead, further exploration on the use of these vectors would open up new opportunities for virus-mediated gene therapy of other afflictive diseases.

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