Paper III

Widespread Dispersion of Adeno-Associated Virus Serotype 1 and Adeno-Associated Virus Serotype 6 Vectors in the Rat Central Nervous System and in Human Glioblastoma Multiforme Xenografts

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ABSTRACT

The transduction patterns of recombinant adeno-associated virus serotype 1 (AAV1) and serotype 6 (AAV6) vectors were assessed in human glioblastoma multiforme (GBM) cell lines, in human GBM biopsy spheroids, and in tumor xenografts growing in nude rat brains. All the cell lines tested (A172, D37, GaMg, HF66, and U373Mg) were found to be permissive to both AAV1 and AAV6 vectors, and thus displayed a transduction pattern similar to AAV2 vectors. For every cell line tested, the transduction efficiency displayed by AAV2 vectors was better than by isogenic and isopromoter AAV1 vectors. Transduction efficiency was dependent on the viral particle number used, suggesting that the receptors for these vectors are widely distributed in GBM tissues. Interestingly, AAV1, AAV2, and AAV6 vectors were able to infect and transduce the same cells when added simultaneously to monolayer cultures. Infection of human GBM biopsy spheroids with AAV1 and AAV6 vectors resulted in transgene expression both at the surface layers and in the core of the spheroids. Following injection of AAV1 and AAV6 vectors into human GBM biopsy xenografts growing in nude rat brains, reporter gene expression was seen both in the periphery as well as in the central regions of the tumors. When injected into the normal rat brain, both AAV1 and AAV6 vectors were found to transduce several central nervous system (CNS) regions. The presented results suggest a potential therapeutic role for AAV1 and AAV6 vectors in gene therapy for GBM and also for other CNS malignancies.

OVERVIEW SUMMARY

Based on their broad host cell tropism and their ability to penetrate into solid tumor tissue, adeno-associated virus serotype 2 (AAV2) vectors are promising candidates for cancer gene therapy. In theory, the clinical application of different AAV serotypes together may improve transduction diversity, and sequential administration of unique AAV serotypes may limit potential immunological rejection. In this study, recombinant AAV1 and AAV6 serotype vectors were examined for their ability to transduce a panel of human glioblastoma multiforme (GBM) cell lines, for their ability to penetrate human GBM biopsy spheroids and for their ability to transduce intracranial GBM xenografts. For both AAV1 and AAV6 vectors, extensive transgene expression was observed in each model tested, suggesting that AAV1 and AAV6 have similar properties to AAV2, such as broad host cell tropism and efficient tissue penetration. Our data suggest potential for the application of AAV1 and AAV6 vectors as gene delivery vehicles for human brain tumors.

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INTRODUCTION

MALIGNANT CELLS are characterized by the overexpression of oncogenes as well as by defects in key regulators of growth arrest and apoptosis. Gene therapy may be aimed at restoring such defects, but is also explored as a means for sensitizing tumor cells for conventional treatment (Rochlitz, 2001; Hemminki, 2002). While successful gene transfer results have been obtained using viral vectors in experimental systems, suboptimal tissue transduction and penetration *in vivo* together with ineffective systemic delivery underscores the need to develop more efficient vector systems.

Adeno-associated viruses are autonomously replication-deficient parvoviruses containing single-stranded DNA genomes (Smith and Kotin, 2002). Although several different serotypes of AAV have been identified (Chiorini *et al*., 1997, 1999; Rutledge *et al*., 1998; Xiao *et al*., 1999; Gao *et al*., 2002), adeno-associated virus serotype 2 (AAV2) has served as a prototype for genetic and molecular biologic studies of *dependovirinae*. AAV2 vectors have shown efficacy in large animal disease models following local administration (Schimmenti *et al*., 1998; Bankiewicz *et al*., 2000; Rudich *et al*., 2000; Acland *et al*., 2001; Beaty *et al*., 2002; Martin *et al*., 2002; Mount *et al*., 2002; Muramatsu *et al*., 2002). At present, phase I and II trials are underway using AAV2 vectors for monogenic diseases, including cystic fibrosis, hemophilia, and Canavan disease (Kay *et al*., 2000; Flotte *et al*., 2002; Janson *et al*., 2002; Manno *et al*., 2003).

Recently, we showed that AAV2 vectors could transduce a broad range of genetically diverse cancer cell lines and human GBM biopsies (Enger *et al*., 2002). More importantly, we observed that AAV2 serotype vectors were able to penetrate the core of human glioblastoma multiforme (GBM) spheroids *in vitro* and transduce human GBM xenografts growing in the cerebral cortex of nude rats. In contrast, retroviral and adenoviral vectors tested in this stringent model have not been able to penetrate into the deeper layers of the human tumor tissue (Enger *et al*., 1999, 2002).

Vectors derived from AAV serotypes other than AAV2 are interesting novel vehicles that may be of clinical relevance. The different serotype vectors show variations in genomic sequences, and thus have unique viral capsid proteins. AAV serotype vectors therefore utilize different cellular receptors for attachment and entry into host cells (Summerford and Samulski, 1998; Qing *et al*., 1999; Summerford *et al*., 1999; Kaludov *et al*., 2001; Walters *et al*., 2001; Di Pasquale *et al*., 2003) and are neutralized differently by animal sera (Rutledge *et al*., 1998; Xiao *et al*., 1999; Halbert *et al*., 2000). Thus, it is possible that AAV serotype vectors display unique host cell ranges that may be used to overcome barriers to efficient transduction. Additionally, an exposure to different AAV vectors may circumvent the effects of neutralizing antibodies to certain AAV serotypes (Xiao *et al*., 1999).

In the present work, the transduction efficiency of AAV1 and AAV6 in three models of human GBM was examined. In this context, it is important to note that whether or not AAV6 is a genuine serotype is a matter of controversy. Nucleotide and amino acid sequence analysis shows that AAV1 and AAV6 are closely related and are virtually identical with respect to capsid composition (Rutledge *et al*., 1998; Xiao *et al*., 1999; Grimm and Kay, 2003). The most common assumption is that AAV6

is a product of homologous recombination between AAV1 and AAV2, with the immunologic profile of AAV1 (Rutledge *et al*., 1998; Xiao *et al*., 1999). However, because this issue has not yet been conclusively resolved, AAV6 vectors have been handled as a unique AAV serotype in our experimental set-up and will be considered a unique AAV serotype in the text.

AAV1 and AAV6 serotype vectors have shown superior efficiency compared to AAV2 vectors for skeletal muscle transduction (Chao *et al*., 2001; Fraites *et al*., 2002; Hauck and Xiao, 2003) and in animal models of cystic fibrosis (Halbert *et al*., 2001). However, at present little information is available for the potential use of these AAV serotypes in cancer-directed gene therapy. In this study we compared the transduction efficiencies of AAV1 and AAV2 in a panel of human glioma cell lines. In addition, we examined the transduction patterns of AAV1 and AAV6 serotype vectors in multicellular spheroids prepared from human GBM biopsies and in GBM biopsy xenografts growing in nude rat brains. Our results show that AAV1, AAV2, and AAV6 vectors have overlapping transduction ranges in glioma cell lines and in GBM patient biopsies. Combinations of different AAV serotypes could transduce the same tumor cells. Similar to AAV2 vectors (Enger *et al*., 2002), both AAV1 and AAV6 were able to penetrate human GBM biopsy spheroids *in vitro*. Importantly, our data reveal that AAV1 and AAV6 vectors are able to deliver transgenes to GBM xenograft tissue as well as to various CNS structures, suggesting the potential use of these vectors for the treatment of both cancer and neurological disorders.

MATERIALS AND METHODS

Viral vectors

Pseudotyped AAV1 vector harboring the bacterial β-galactosidase cDNA (*lac*Z) was prepared in an adenovirus-free system by calcium phosphate precipitation into HEK 293 cells as described (Xiao *et al*., 1999). AAV1 and AAV2 vectors harboring the enhanced green fluoresecent protein (EGFP) cDNA were prepared in a similar manner. The number of genome-containing paticles (hereafter designated viral particles) was determined by quantitative polymerase chain reaction (PCR) to be 5×10^{12} per milliliter for AAV1lacZ and 1.75×10^{13} per milliliter and 3.4×10^{12} per milliliter for AAV1GFP and AAV2GFP, respectively (Vector Core Facility, University of Pennsylvania, Philadelphia, PA). Pseudotyped vectors harboring the cDNA for nuclear-localizing β-galactosidase (n*lac*Z; Halbert et al., 2000) were generated by triple transfection into HEK 293 cells as described (Halbert et al., 2001). AAV2nlacZ was 1×10^{11} viral particles per milliliter (1.5×10^7 FFU/ml), AAV6nlacZ was 5×10^{11} viral particles per milliliter (8×10^5 FFU/ml). The physical titers (viral particles) were quantified by Southern blotting and the functional titers (foci forming units, FFU) by titration in HT-1080 cells. AAV1GFP and AAV2GFP vectors, (NHLBI, NIH, Bethesda, MD) were prepared in Sf9 cells using three recombinant baculoviruses expressing AAV2 Rep 52 and Rep 78, AAV1, or AAV2 capsid proteins and containing the EGFP transcription casette (Urabe et al., 2002). Physical titers (viral particles) were determined by quantitative PCR to be 1.14×10^{13} per milli-

liter for AAV1GFP and 4.94×10^{12} per milliliter for AAV2GFP. All vectors were purified by CsCl-density gradient centrifugation. The vector constructs are depicted schematically in Figure 1.

Quantification of transduction efficiency in monolayer cultures

The percentage of viral transduction in monolayer cultures was determined by flow cytometric analysis of glioma cells infected by GFP encoding vectors. Briefly, 100,000 cells of the GBM cell lines A172, D37, HF66, GaMg, and U373Mg were seeded separately into the wells of 6-well tissue culture plates (Nunc, Roskilde, Denmark). The cells were cultured overnight prior to exposure to 5000 or 50,000 viral particles per cell. The isogenic and isopromoter AAV1GFP and AAV2GFP vectors prepared at the University of Pennsylvania Vector Core Facility were used for these experiments, because these vectors have identical backbones and have undergone the same purification procedures. The transduction abilities of these vectors may thus be compared head-to-head with regard to differences in capsid composition only. AAV6nlacZ vectors were not included in this comparison due their different genetic make-up compared to AAV1GFP and AAV2GFP vectors (Fig. 1). Four days postinfection, the cells were processed for flow cytometric analysis. The cells were detached using 1 ml of 0.025% Trypsin-ethylenediaminetetraacetic acid (EDTA; BioWhittaker Molecular Applications, Rockland, ME). After addition of 1 ml complete growth medium supplemented with 10% fetal calf serum (FCS; BioWhittaker), the samples were centrifuged at $300g$ for 4 min at 4°C. The supernatant was carefully discarded and each sam-

ple was resuspended in 1 ml of phosphate-buffered saline (PBS) supplemented with 1% D-glucose (Sigma, St. Louis, MO). The samples were kept on ice prior to analysis. The transduction efficiencies were determined using a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The GFP fluorescence intensities were obtained by gating a two-parameter forward- and side-scatter cytogram into a one-parameter green fluorescence intensity plot (1024-channel [10-bit] data). A total of 2000 gated cells were collected for each sample. MilliQ water and PBS supplemented with 1% D- glucose served as negative controls, while fluorescein isothiocyanate-conjugated (FITC) fluorescent CaliBRITE beads (Becton Dickinson) were used as positive controls and to calibrate the flow cytometer. Experiments were performed in duplicate (AAV1 samples) and triplicate (AAV2 samples), with two parallel measurements within each experiment. The transduction efficiency for each cell line was determined by histogram subtraction. Negative control histograms of the uninfected glioma cell lines were subtracted from the fluorescence intensity histograms of the respective infected cells. The number of transduced cells in the fluoresence intensity histogram was counted and the percent transduction calculated.

Monolayer and spheroid cultures

The GBM cell lines A172, D37, HF66, GaMg, and U373Mg were cultured at 37°C (100% relative humidity, 5% CO_2) in a standard tissue-culture incubator (Binder, Tuttlingen, Germany) and maintained in complete medium as described (Enger *et al*., 2002). Tumor biopsy tissue was obtained from three different patients diagnosed with GBM. The tumor samples were con-
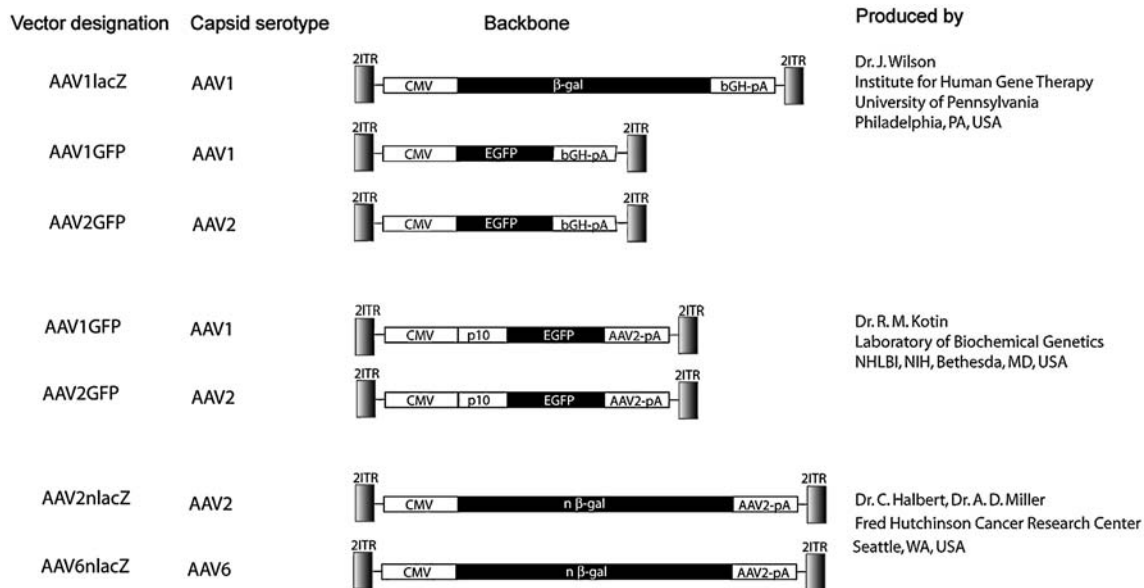

FIG. 1. Adeno-associated virus (AAV) vectors. Each vector contained a cytomegalovirus (CMV) promoter, a reporter gene (enhanced green fluorescent protein [EGFP], LacZ, or nuclear LacZ) followed by a polyadenylation signal sequence derived either from AAV2 (AAV2-pA) or the human growth hormone (hGH-pA). The expression cassettes were flanked by AAV2 inverted terminal repeat (ITR) sequences. AAV1 and AAV6 vectors were prepared by using serotype specific capsid sequences for packaging. The p10 sequence is an insect sequence used for packaging as described (Urabe *et al*., 2002). Schematics are not drawn to scale.

firmed as glioblastomas by histological evaluation. Multicellular biopsy spheroids were prepared and maintained as described previously (Bjerkvig *et al*., 1990). All patients gave their verbal informed consent, and the study was approved by the Regional Ethical Committee.

Generation of xenograft models

Intracranial tumor xenografts were generated in nude rats as previously described (Engebraaten *et al*., 1999; Enger *et al*., 2002). Briefly, a burrhole was drilled 1 mm posterior to the bregma suture and 3 mm to the right of the midline suture, and 20 GBM spheroids (diameter, 300–500 μm) were implanted into the cerebral cortex at 2.5 mm depth using a Hamilton type 7125 syringe (Hamilton, Bonaduz, Switzerland). After surgery, the animals were allowed to recover in an incubator set at 35°C before being returned to their cages. Tumor growth was validated 3 weeks postimplantation by magnetic resonance imaging as described previously (Enger *et al*., 2002; Thorsen *et al*., 2003). The handling of the animals and the experimental protocol were approved by the Norwegian Animal Research Authority.

Vector infection and delivery

GBM cell lines grown in monolayer cultures were seeded at a density of 8000 cells per well of 8-well chamber slides (Nunc, Roskilde, Denmark). The cells were allowed to attach overnight prior to infection with AAV serotype vectors. Combinations of two AAV serotype vectors containing different reporter genes (EGFP and *lacZ*, respectively) were added to the monolayer cultures simultaneously. Combinations of AAV1GFP and AAV6nlacZ, AAV2GFP and AAV6nlacZ, and AAV1GFP and AAV2nlacZ were tested.

Glioblastoma biopsy spheroids (500–800 μm in diameter) from three patients were kept individually in agar-coated 96-well dishes (Nunc). Eight spheroids from each tumor sample were infected with AAV1lacZ or AAV6nlacZ vectors (4×10^9 viral particles per spheroid). An identical number of noninfected spheroids served as negative controls.

Three weeks after intracranial implantation of biopsy spheroids, the animals received an injection of AAV1lacZ (range, $1.5–2.5 \times 10^{10}$ viral particles) or AAV6lacZ vector (range, $5 \times 10^8–5 \times 10^9$ viral particles) into the growing tumors as described (Enger *et al*., 2002). The flow rate was varied according to the volume of the vector delivered (2–8 μl), keeping the duration of injections constant at 1 hr. The animals were sacrificed when tumor-related neurologic symptoms were evident. Typically, the animals remained symptom-free for 2–3 months, depending on the aggressive nature of the tumor biopsy. Non-xenografted animals received intracerebral vector injections administered at the same stereotaxic location as used for tumor-bearing animals. These animals were sacrificed 2 months postinjection and served as controls.

Tissue processing

After 4 days in monolayer culture, the infected cells were analyzed for transgene expression. The transduction efficiencies of EGFP-containing vectors were visualized by fluorescence microscopy and quantified by flow cytometric analysis, while the transduction efficiencies of β-galactosidase–encoding vectors were determined by light microscopy after β-gal histochemistry. The results obtained for every AAV serotype tested were validated by performing equivalent experiments using the same AAV serotype harboring a different transgene.

Detection of transgene expression and tissue penetration of β-galactosidase–encoding vectors in the biopsy spheroids was performed by light microscopic analysis after β-gal histochemistry of cryosectioned specimens. Upon sacrifice of the animals, the brains were dissected out, embedded in Tissue Tek OCT medium (Sakura Finetek, Tokyo, Japan), and quickly frozen in a chamber cooled by liquid nitrogen. Axial brain cryosections (12–18 μm) were cut using a cryotome and collected on coated glass slides (SuperFrost Plus, Menzel, Germany).

β-gal histochemistry

Before processing for β-gal histochemistry, the sections were dried for 30 min at room temperature (20°C). Cryosections, cell monolayers, and spheroids were fixed for 15 min in a mixture of 2% formaldehyde and 0.2% glutaraldehyde in PBS containing 2 mM Mg^{2+}. Specimens were rinsed in PBS buffer containing 1 mM Mg^{2+} thrice for 10 min each. X-gal reaction buffer (1 mg/ml X-gal, 5 mM K$_4$Fe(CN)$_6$, 5 mM K$_3$Fe(CN)$_6$, 2 mM MgCl$_2$ in PBS) was added to the sections. To reduce endogenous β-galactosidase activity, the pH value of the reaction buffer was kept at 7.4. The solution was filtered through a 0.45-μm syringe filter (Pall Gelman Laboratory, Lane Cove, UK) to avoid excessive crystal formation. Cells and cryosections were stained either at room temperature (22°C) for 4 hr or at 4°C overnight. Routine Harris hematoxylin and eosin counterstaining was performed to visualize tissue structure and cell nuclei.

Immunohistochemistry

Frozen cryosections were equilibrated to room temperature for 45 min and fixed in acetone for 10 min at room temperature. After rinsing, the sections were blocked for 6 min against endogenous peroxidase activity (DAKO peroxidase block, DAKO Cytomation, Glostrup, Denmark). After additional rinsing, the sections were blocked for 10 min against nonspecific protein binding (DAKO Protein Block, DAKO Cytomation). The sections were probed with monoclonal mouse antivimentin primary antibody (DAKO Cytomation) at a dilution of 1:250 in TBS/1% bovine serum albumin. Purified nonspecific mouse immunoglobulin G (IgG; Sigma) diluted to the same protein concentration was added to negative control sections. Incubations were performed overnight at 4°C. Antibody-probed sections and controls were then incubated with horseradish peroxidase coupled goat anti-mouse secondary antibodies and developed using 3′3′-diaminobenzidine as chromogenic substrate, according to the manufacturer's protocol (EnVision™+ system, DAKO Cytomation). Routine Harris hematoxylin and eosin counterstaining was performed to visualize tissue structure and nuclear morphology.

RESULTS

AAV1, AAV2, and AAV6 serotype vectors have similar cell tropisms

Based on the evidence that AAV serotypes utilize distinct cellular receptors, we postulated that each AAV serotype could

infect and transduce various cell types with different efficiencies. In order to test this hypothesis, we performed flow cytometric analysis of monolayer cultures infected with isogenic and isopromoter AAV1 and AAV2 serotype vectors (Fig. 1). The human GBM cell lines A-172, D37, HF66, GaMg, and U373Mg were infected with AAV1-GFP and AAV2-GFP vectors at 5000 and 50,000 viral particles per cell. The cells were processed for flow cytometry 4 days postinfection. AAV2 vec-

tors displayed superior transduction efficiency compared to AAV1 vectors in every GBM cell line examined (Fig. 2A and 2B). For both AAV serotypes, an increase in the number of viral particles per cell from 5000 to 50,000 yielded an increase in percent transduction (Fig. 2B). These findings were validated by microscopic analysis of monolayer cultures infected with AAV vectors expressing the *lacZ* and EGFP transgenes (data not shown).
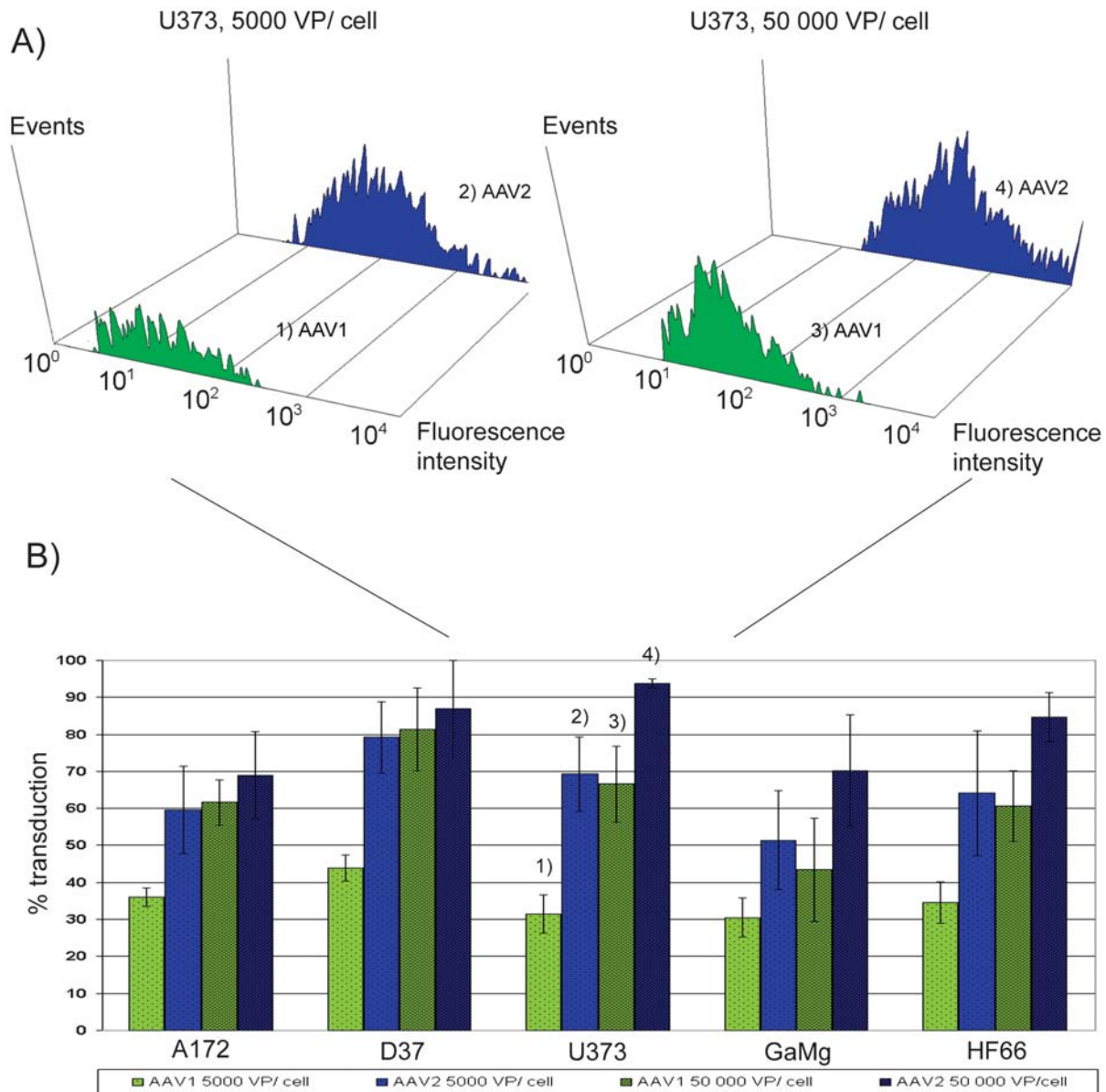

FIG. 2. Adeno-associated virus serotype 2 (AAV2) vectors transduce glioblastoma multifore (GBM) cell lines more efficiently than AAV1 vectors. GFP expression of transduced U373 cells lines monitored by FLOW cytometry (**A**). Left panel show the fluorescence intensity histograms of U373 cells after exposure to: 1) 5000 AAV1, and 2) 5000 AAV2 genome-containing particles per cell. Right panel show the fluoresence intensity histograms after infection with: 3) 50,000 AAV1 and 4) 50,000 AAV2 genome-containing particles per cell. The transduction efficiencies of AAV1 and AAV2 vectors in A172, D37, GaMg, HF66, and U373 cells is shown as the mean percentage of green fluorescent protein (GFP) expressing cells ± standard deviation (SD; **B**). For U373 the numbers 1), 2), 3), and 4) depicts the bars in (**B**) corresponding to the fluorescence intensity histograms shown in (**A**). Interestingly, AAV2 vectors transduced every GBM cell line better than AAV1 vectors, and the transduction efficiency of both vectors increased with a 10-fold increase in the number of viral particles added.

AAV2, AAV4, and AAV5 have been shown to utilize different cell-surface receptors for intracellular entry (Summerford and Samulski, 1998; Qing *et al.*, 1999; Summerford *et al.*, 1999; Walters *et al.*, 2001, 2002; Di Pasquale *et al.*, 2003). The serotype-specific receptors for AAV1 and AAV6 have not yet been identified. Our findings imply that the receptors for AAV1 and AAV2 are widely expressed by the glioma cell lines, and do not suggest the lack of receptors to be a major limitation to viral infection. To characterize the transduction profiles of AAV1, AAV2, and AAV6 vectors further, we simultaneously infected glioma monolayer cultures with two AAV serotypes, each expressing a different reporter gene. For each cell line examined, individual cells could be simultaneously transduced by combinations of two different AAV serotypes (Fig. 3).

AAV1 and 6 penetrate deeper layers of solid human biopsy spheroids

In contrast to adenoviral or retroviral vectors, AAV2 vectors have been shown to penetrate solid tumor tissue (Enger *et al.*, 1999, 2002). To examine whether the AAV1 and AAV6 serotype vectors also displayed this trait, multicellular GBM biopsy spheroids (500–800 μm) were exposed to AAV1-LacZ and AAV6-nLacZ vectors and cultured for 3 weeks. Upon sectioning and histochemical processing, transgene positive cells were observed both in peripheral as well as in central areas of the spheroids, in a pattern similar to that previously described for AAV2 vectors (Fig. 4A and 4B). Endogenous β-galactosidase activity was not detected in uninfected spheroids (Fig. 4D).

AAV1 and AAV6 show effective transduction in vivo

The ability of AAV1 and AAV6 vectors to transduce solid tumor tissue *in vivo* was evaluated using an intracranial xenograft model prepared from human GBM tumor spheroids. Importantly, these spheroids were generated from patient biopsy material and not from immortalized cell lines, and therefore represent a favorable model for studying the growth of human glioblastoma multiforme *in situ*. Within the xenografts,
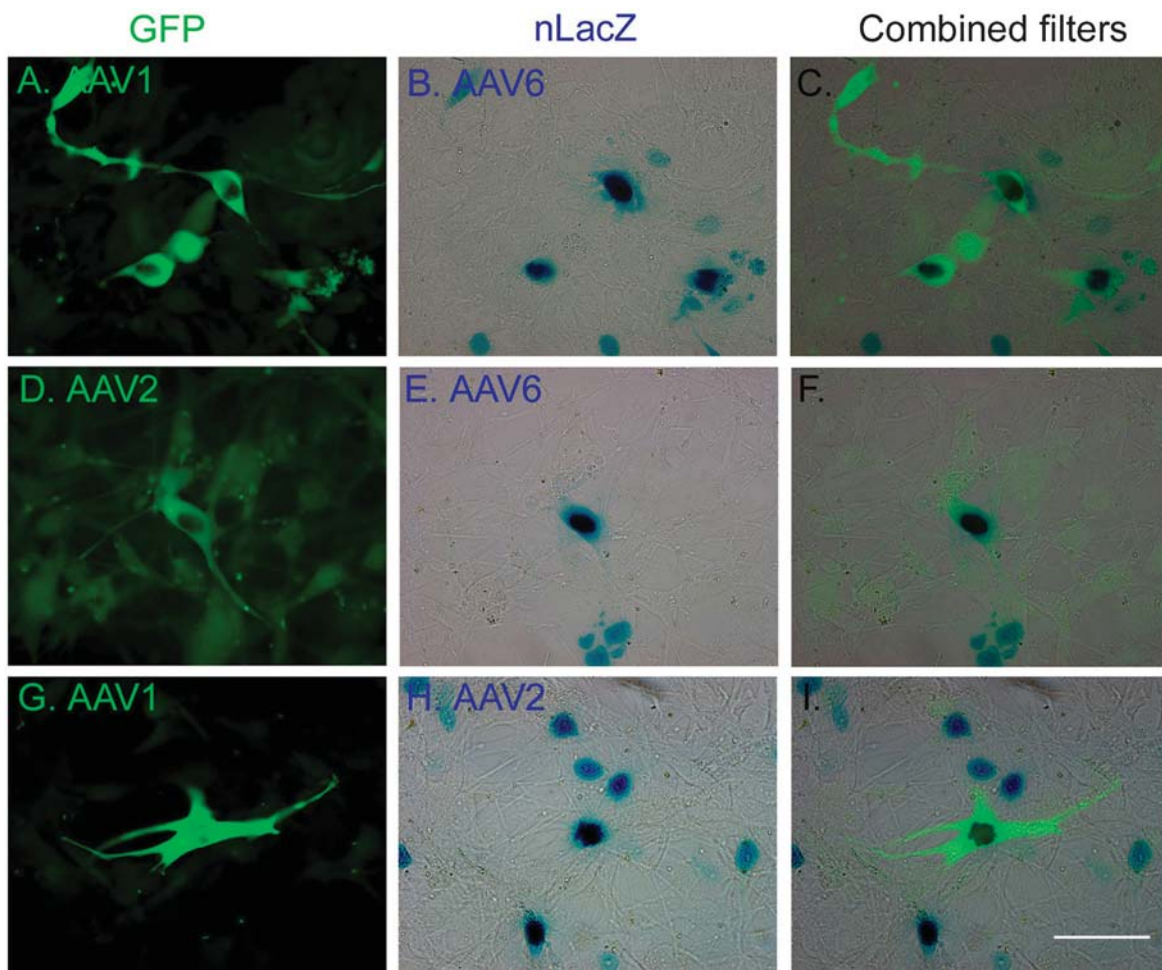

FIG. 3. Adeno-associated virus serotype 1 (AAV1), AAV2, and AAV6 vectors can target the same cell. Two AAV serotypes expressing different reporter genes were simultaneously added to glioblastoma multifore (GBM) monolayer cultures. Four days postinfection the cultures were processed for β-galactosidase histochemistry. The cells were scored for coexpression of β-galactosidase and green fluorescent protein (GFP) by combining light and fluorescence microscopy. Shown here are single cells of the GBM cell line A172 transduced simultaneously by AAV1-GFP and AAV6-nLacZ (**A**, **B**, **C**), AAV2-GFP and AAV6nLacZ (**D**, **E**, **F**), and AAV1-GFP and AAV2nLacZ (**G**, **H**, **I**). Scale bar = 50 μm.
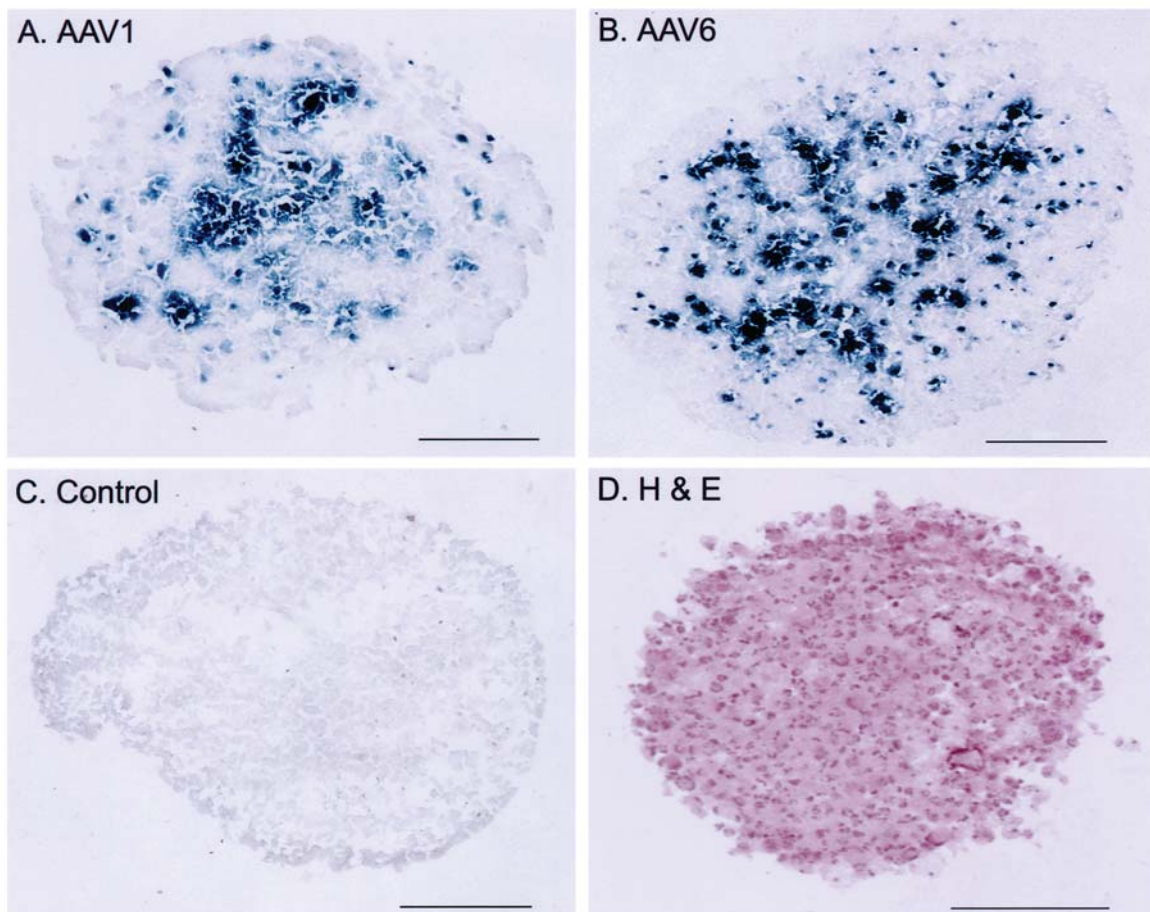
FIG. 4. Adeno-associated virus serotype 1 (AAV1) and AAV6 penetrate multicellular glioblastoma biopsy spheroids. Glioblastoma multiforme (GBM) biopsy spheroids were infected separately with AAV1-nLacZ and AAV6-nLacz vectors. After 2 weeks in culture, the tumor spheroids were cryosectioned and processed for β-galactosidase histochemistry. Transgene positive cells were detected both peripherally and centrally in the spheroids infected with AAV1 (**A**) and AAV6 (**B**) vectors. No endogenous β-galactosidase activity was detected in uninfected controls (**C**). Hematoxylin and eosin-stained spheroid section, showing the cellular density of a multicellular biopsy spheroid (**D**). Scale bars = 200 μm.

necrotic areas, vascular hyperproliferation and in some cases, pseudopallisading of tumor cells were evident. AAV1 ($n = 7$ xenografts) or AAV6 vectors ($n = 11$ xenografts) containing *lac*Z or nuclear *lac*Z transgenes, respectively, were stereotaxically injected into the tumors. After histochemical processing, AAV1 and AAV6 transgene expression was detected over large distances in the axial (x-y) plane (Fig. 5). Furthermore, in the dorsoventral (z) plane, transgene-positive areas encompassing several millimeters were detected (AAV1 animals: range, 25–7000 μm; mean 1920 μm; AAV6 animals: range, 518–5404 μm; mean, 2894 μm). The transduction profiles within the tumors injected with AAV1 and AAV6 were similar. For both serotypes, transgene positive cells were most often observed at the tumor periphery, but in some cases also within central regions of the tumors (Figs. 5A and 6B).

To examine the potential divergence of AAV1 and AAV6 transduction within the normal brain, we injected AAV vectors into the same intracerebral coordinates as used for tumor-bearing animals ($n = 2$ for AAV1, $n = 3$ for AAV6). Two months postinjection, normal brain tissue was processed for β-galac-

tosidase histochemistry. AAV1- and AAV6-mediated transgene expression was seen in white matter regions with a high abundance of neurons, such as the cererbral cortex, caudate putamen, corpus callosum, and hippocampus (Figs. 5 and 6).

DISCUSSION

To achieve efficient transduction of solid tumor tissue, both cellular targeting and sufficient tissue penetration is required. Insufficient penetration ability has precluded the use of several candidate vectors with large viral capsids, including adenoviral-, retroviral-, and herpes simplex viral vectors. Although AAV vectors have not been tested in clinical trials for cancer, they possess specific biologic features that may be of advantage in cancer gene therapy (Ponnazhagan *et al.*, 2001). First, AAVs are packaged into significantly smaller capsids compared to other viral vectors. Second, the receptor molecules for several AAV serotypes appear widely expressed in tumor tissue (Isaka *et al.*, 1994; Sonmez *et al.*, 1995; Shee *et al.*, 1998;
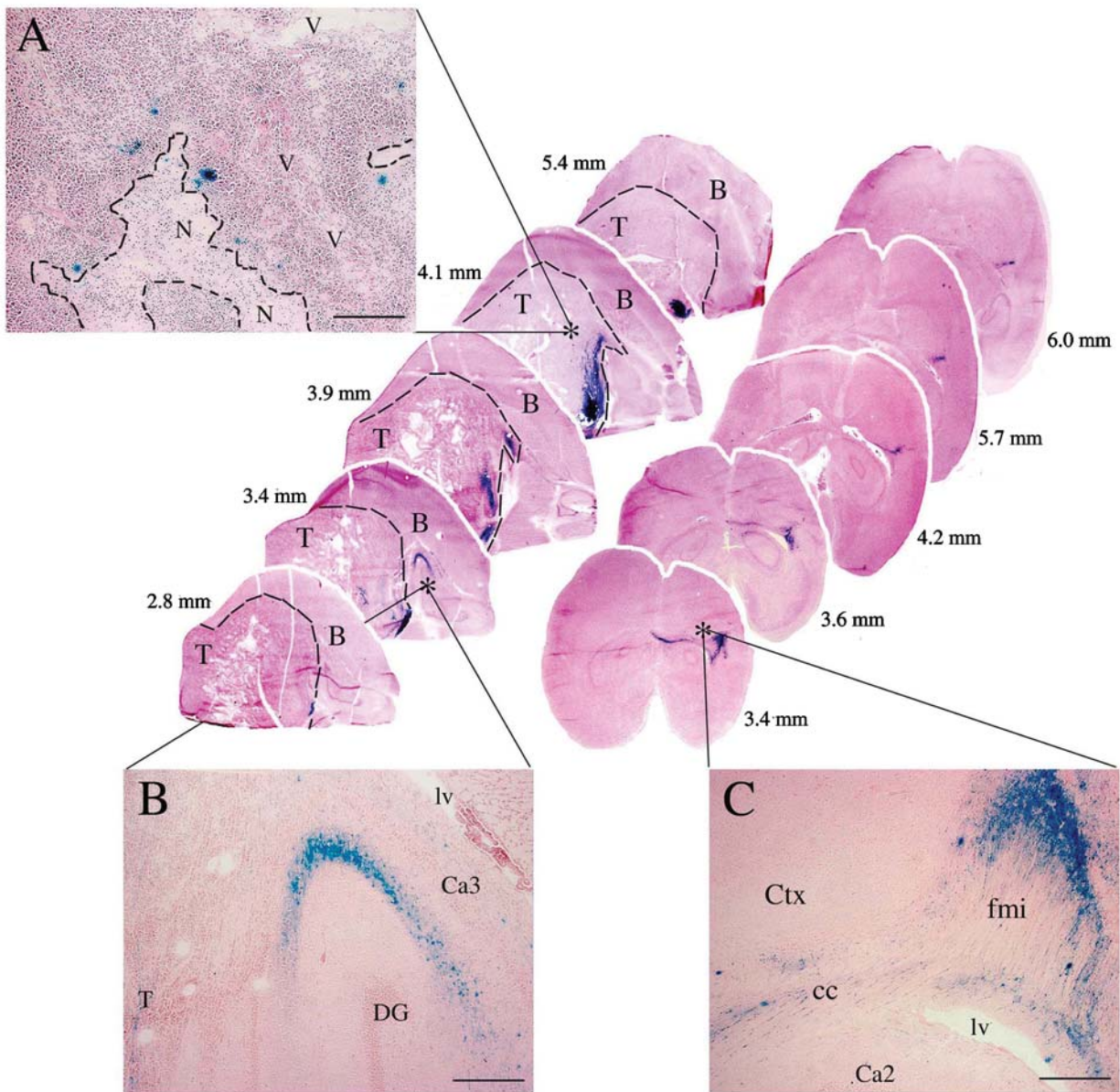
FIG. 5. Adeno-associated virus serotype 1 (AAV1) transduces regions in the rodent brain as well as human glioma xenografts derived from patient biopsies. Series of β-galactosidase-stained axial cryosections revealed AAV1-mediated transgene expression after injection of 1.5×10^{10} genome-containing particles into xenografted (left row) or 2×10^{10} genome-containing particles into normal (right row) rat brain. Approximate distances measured from the cerebral surface are indicated. Asterisks mark the enlarged regions (**A**), (**B**), and (**C**). Tumor-host tissue border is marked by broken lines. Brain tissue is indicated by B and tumor tissue by T. Light microscopic image of the central area of a glioblastoma multiforme (GBM) xenograft injected with AAV1. Blue-stained cells represent AAV-infected cells expressing the *lacZ* transgene. Areas of necrosis are outlined (**A**). AAV infection of the hippocampus (**B**). AAV1 diffused over the corpus callosum toward the contralateral hemisphere in the nonxenografted rat brain, infecting hippocampal nuclei and regions of the corpus callosum (**C**). Ca2, Ca2 region of the Ammon`s horn; Ca3, Ca3 region of the Ammon`s horn; DG, dentate gyrus; fmi, forceps minor corpus callosum; lv, lateral ventricle; ctx, cortex; N, necrotic areas; V, blood vessels. Scale bars: **A** = 200 μm; **B** and **C** = 400 μm.

Tysnes *et al.*, 2002). Finally, AAV2 vectors have been shown to be effective in delivering transgenes to solid tumors in stringent preclinical models (Enger *et al.*, 2002).

In this study, we evaluated the abilities of AAV1 and AAV6 serotype vectors to transduce human glioma cell lines and to penetrate solid human GBM tissues in preclinical models of GBM.

Cellular tropism of AAV serotypes

We found that both AAV1 and AAV6 vectors were able to infect a wide range of glioma cell lines and biopsy spheroids, and thus displayed a similar transduction profile to AAV2 vectors. The receptors for AAV1 and AAV6 have not yet been
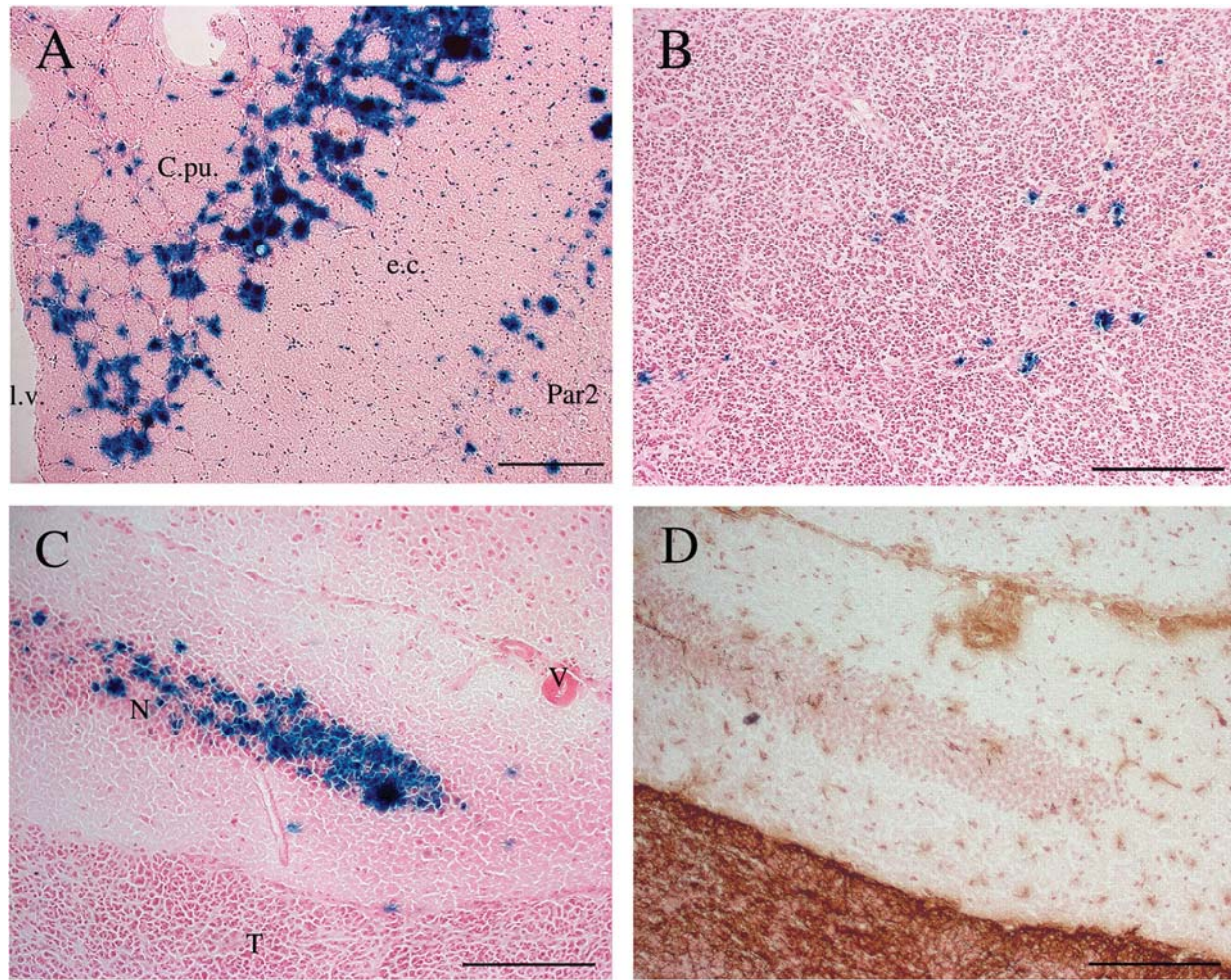
FIG. 6.   Efficient transduction of GBM tumor xenografts and cerebral regions by adeno-associated virus serotype 6 (AAV6). Normal rat brains as well as xenografted animals were injected with AAV6 vectors carrying the nuclear β-galactosidase gene and analyzed for reporter gene expression. Widespread AAV6-mediated transgene expression was observed in both normal (**A**, 5×10^9 VP genome-containing particles injected) and tumor harboring animals (**B**, 4×10^9 VP genome-containing particles injected). Infection of host neuronal nuclei after injection of 2×10^9 genome-containing particles AAV6 (**C**). The infected cells were negative for human-specific vimentin, whereas the tumor was positive (**D**). Panel (**C**) and (**D**) are parallel cryosections. C.pu., caudate putamen; e.c., external capsule; Par2, parietal cortex. Scale bars: **A** = 200 μm; **B**, **C**, and **D** = 100 μm.

identified. However, based on our findings, it is likely that the receptors responsible for AAV1 and AAV6 uptake are widely expressed by glioma cell lines. Quantitative comparison of the transduction efficiencies of isogenic and isopromoter AAV1 and AAV2 vectors revealed that AAV2 displayed superior transduction efficiency to AAV1 in every GBM cell line studied. This may suggest that receptors for AAV2 are more widely expressed by these cells than the receptors involved in AAV1 binding and uptake. However, the transduction rate increased in response to a 10-fold increase in the number of viral particles added per cell for both AAV1 and AAV2 vectors (Fig. 2), indicating that receptor expression may not be a limiting factor for transduction at the concentrations studied. However, it is important to note, that differences in transduction efficiency may also be the result of differential intracellular trafficking and uncoating of the AAV serotypes. This issue needs further

investigation. The finding that different AAV serotypes are able to transduce a variety of glioma cells and tissues may have clinical implications, because this justifies repeated injections using different serotype AAV vectors. Such an approach may circumvent neutralizing antibodies that can develop against the capsids of particular serotype. The observation that individual cells could be transduced simultaneously with different AAV serotypes suggests that different AAV serotype vectors may be administered in concert or in sequence to enhance transduction efficiency.

AAV1 and AAV6 gene transfer to GBM biopsy spheroids

AAV1 and AAV6 vectors, similar to AAV2, were able to penetrate and transduce deep layers of multicellular tumor

spheroids. This may be accounted to the relatively small size of the AAV capsid and to the widespread distribution of AAV receptors throughout the tumor tissue. In the biopsy spheroids, only a small fraction of the cells are dividing (Bjerkvig *et al.*, 1992). Therefore, extensive transgene expression caused by cell proliferation and passing the transgene to daughter cells can not explain the high AAV transduction efficiency. These findings suggests that AAV1 and AAV6 vectors possess significant advantages compared to nonreplicating adenoviral and retroviral vector systems with respect to tissue penetration (Benedetti *et al.*, 1997; Puumalainen *et al.*, 1998; Enger *et al.*, 1999, 2000; Sandmair *et al.*, 1999).

AAV1 and AAV6 gene transfer in vivo

To examine AAV1 and AAV6 delivery to solid tumors further, we generated animal models in which the GBM biopsy spheroids were transplanted into the cerebral cortex of nude rats. Upon injection with AAV vectors, transgene expression was detected in each tumor xenograft. AAV-mediated β-galactosidase activity was seen in areas covering several millimeters throughout central and peripheral tumor regions. The area of transduction often did not coincide with the location of vector injection, suggesting that cerebrospinal fluid flow may have distributed the vector to various brain areas. As mentioned above, extensive transduction was observed at the tumor periphery (Fig. 5 for AAV1, not shown for AAV6). This may be explained by an elevated interstitial fluid pressure within the tumors (Boucher *et al.*, 1997), which would lead to flow of the viral particles to more peripheral areas, where transduction would occur. In addition, transgene-positive cells were found scattered throughout the central areas of viable tumor tissue (Figs. 5A and 6B). This may be caused by the extensive migration of transduced cells within the tumor, or by the retention of pockets of viral vector in isolated tumor areas.

As seen in Figures 5 and 6, these vectors were able to transduce various brain regions such as the cerebral cortex, caudate putamen, corpus callosum, and the hippocampus. Both AAV1 and AAV6 showed transgene distribution to large areas within the rat brain. Our data thus support previous findings that have shown superior transduction efficiency of AAV1 vectors in the mammalian brain (Passini *et al.*, 2003; Vite *et al.*, 2003; Wang *et al.*, 2003). In addition, our data demonstrate efficient transgene delivery to the central nervous system (CNS) by AAV6 vectors. The ability of AAV vectors to target several CNS structures suggests a possible role for AAV1 and AAV6 in several gene therapy protocols. Interestingly, as in the tumor models, several millimeters of transduced tissue were detected, likely explained by cerebrospinal fluid (CSF)-mediated transport. In the context of cancer gene therapy, our data emphasize the importance of designing AAV vectors that are transcriptionally targeted to cancer cells in order to avoid the expression of potentially toxic transgenes in normal tissues. In summary, we report for the first time that AAV1 and AAV6 vectors were able to transduce and penetrate human GBM models *in vitro* and *in vivo*. Our findings provide a proof of principle for the development of AAV1 and AAV6 vectors as delivery vehi-

cles for cancer gene therapy and also suggest use of other AAV serotypes in complementary approaches of cancer gene therapy.

REFERENCES

ACLAND, G.M., AGUIRRE, G.D., RAY, J., ZHANG, Q., ALEMAN, T.S., CIDECIYAN, A.V., PEARCE-KELLING, S.E., ANAND, V., ZENG, Y., MAGUIRE, A.M., JACOBSON, S.G., HAUSWIRTH, W.W., and BENNETT, J. (2001). Gene therapy restores vision in a canine model of childhood blindness. Nat. Genet. **28,** 92–95.

BANKIEWICZ, K.S., EBERLING, J.L., KOHUTNICKA, M., JAGUST, W., PIVIROTTO, P., BRINGAS, J., CUNNINGHAM, J., BUDINGER, T.F., and HARVEY-WHITE, J. (2000). Convection-enhanced delivery of AAV vector in parkinsonian monkeys: In vivo detection of gene expression and restoration of dopaminergic function using pro-drug approach. Exp. Neurol. **164,** 2–14.

BEATY, R.M., JACKSON, M., PETERSON, D., BIRD, A., BROWN, T., BENJAMIN, D.K., JR., JUOPPERI, T., KISHNANI, P., BONEY, A., CHEN, Y.T., and KOEBERL, D.D. (2002). Delivery of glucose-6-phosphatase in a canine model for glycogen storage disease, type Ia, with adeno-associated virus (AAV) vectors. Gene Ther. **9,** 1015–1022.

BENEDETTI, S., DIMECO, F., POLLO, B., CIRENEI, N., COLOMBO, B.M., BRUZZONE, M.G., CATTANEO, E., VESCOVI, A., DIDONATO, S., COLOMBO, M.P., and FINOCCHIARO, G. (1997). Limited efficacy of the HSV-TK/GCV system for gene therapy of malignant gliomas and perspectives for the combined transduction of the interleukin-4 gene. Hum. Gene Ther. **8,** 1345–1353.

BJERKVIG, R., TØNNESEN, A., LAERUM, O.D., and BACKLUND, E.O. (1990). Multicellular tumor spheroids derived from human brain tumors. J. Neurosurg. **72,** 463.

BJERKVIG, R., HØSTMARK, J., PEDERSEN, P.H., and LíRUM, O.D. (1992). Tumor spheroids from biopsy specimens. In *Spheroid culture in cancer research*. R. Bjerkvig, ed. (CRC Press, Boca Raton, FL) *pp.* 41–56.

BOUCHER, Y., SALEHI, H., WITWER, B., HARSH, G.R.T., and JAIN, R.K. (1997). Interstitial fluid pressure in intracranial tumours in patients and in rodents. Br. J. Cancer **75,** 829–836.

CHAO, H., MONAHAN, P.E., LIU, Y., SAMULSKI, R.J., and WALSH, C.E. (2001). Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. Mol. Ther. **4,** 217–222.

CHIORINI, J.A., YANG, L., LIU, Y., SAFER, B., and KOTIN, R.M.

(1997). Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J. Virol. **71,** 6823–6833.

CHIORINI, J.A., KIM, F., YANG, L., and KOTIN, R.M. (1999). Cloning and characterization of adeno-associated virus type 5. J. Virol. **73,** 1309–1319.

DI PASQUALE, G., DAVIDSON, B.L., STEIN, C.S., MARTINS, I., SCUDIERO, D., MONKS, A., and CHIORINI, J.A. (2003). Identification of PDGFR as a receptor for AAV-5 transduction. Nat. Med. **9,** 1306–1312.

ENGEBRAATEN, O., HJORTLAND, G.O., HIRSCHBERG, H., and FODSTAD, O. (1999). Growth of precultured human glioma specimens in nude rat brain. J. Neurosurg. **90,** 125–132.

ENGER, P.O., VISTED, T., THORSEN, F., BJERKVIG, R., and LUND JOHANSEN, M. (1999). Retroviral transfection of the lacZ gene from Liz-9 packaging cells to glioma spheroids. Int. J. Dev. Neurosci. **17,** 665–672.

ENGER, P.O., THORSEN, F., LONNING, P.E., BJERKVIG, R., and HOOVER, F. (2002). Adeno-associated viral vectors penetrate human solid tumor tissue in vivo more effectively than adenoviral vectors. Hum. Gene Ther. **13,** 1115–1125.

FLOTTE, T.R., VIRELLA-LOWELL, I., and CHESNUT, K.A. (2002). Adeno-associated viral vectors for CF gene therapy. Methods Mol. Med. **70,** 599–608.

FRAITES, T.J., JR., SCHLEISSING, M.R., SHANELY, R.A., WALTER, G.A., CLOUTIER, D.A., ZOLOTUKHIN, I., PAULY, D.F., RABEN, N., PLOTZ, P.H., POWERS, S.K., KESSLER, P.D., and BYRNE, B.J. (2002). Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. Mol. Ther. **5,** 571–578.

GAO, G.P., ALVIRA, M.R., WANG, L., CALCEDO, R., JOHNSTON, J., and WILSON, J.M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc. Natl. Acad. Sci. U.S.A. **99,** 11854–11859.

GRIMM, D., and KAY, MA. (2003). From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. Curr. Gene Ther. **3,** 281–304.

HALBERT, C.L., RUTLEDGE, E.A., ALLEN, J.M., RUSSELL, D.W., and MILLER, A.D. (2000). Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. J. Virol. **74,** 1524–1532.

HALBERT, C.L., ALLEN, J.M., and MILLER, A.D. (2001). Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. J. Virol. **75,** 6615–6624.

HAUCK, B., and XIAO, W. (2003). Characterization of tissue tropism determinants of adeno-associated virus type 1. J. Virol. **77,** 2768–2774.

HEMMINKI, A. (2002). From molecular changes to customised therapy. Eur. J. Cancer **38,** 333–338.

ISAKA, T., YOSHIMINE, T., MARUNO, M., KURODA, R., ISHII, H., and HAYAKAWA, T. (1994). Altered expression of antithrombotic molecules in human glioma vessels. Acta. Neuropathol. (Berl) **87,** 81–85.

JANSON, C., MCPHEE, S., BILANIUK, L., HASELGROVE, J., TESTAIUTI, M., FREESE, A., WANG, D.J., SHERA, D., HURH, P., RUPIN, J., SASLOW, E., GOLDFARB, O., GOLDBERG, M., LARIJANI, G., SHARRAR, W., LIOUTERMAN, L., CAMP, A., KOLODNY, E., SAMULSKI, J., and LEONE, P. (2002). Clinical protocol. Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. Hum. Gene Ther. **13,** 1391–1412.

KALUDOV, N., BROWN, K.E., WALTERS, R.W., ZABNER, J., and CHIORINI, J.A. (2001). Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. J. Virol. **75,** 6884–6893.

KAY, M.A., MANNO, C.S., RAGNI, M.V., LARSON, P.J., COUTO, L.B., MCCLELLAND, A., GLADER, B., CHEW, A.J., TAI, S.J., HERZOG, R.W., ARRUDA, V., JOHNSON, F., SCALLAN, C., SKARSGARD, E., FLAKE, A.W., and HIGH, K.A. (2000). Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat. Genet. **24,** 257–261.

MANNO, C.S., CHEW, A.J., HUTCHISON, S., LARSON, P.J., HERZOG, R.W., ARRUDA, V.R., TAI, S.J., RAGNI, M.V., THOMPSON, A., OZELO, M., COUTO, L.B., LEONARD, D.G., JOHNSON, F.A., MCCLELLAND, A., SCALLAN, C., SKARSGARD, E., FLAKE, A.W., KAY, M.A., HIGH, K.A., and GLADER, B. (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood **101**: 2963–2972.

MARTIN, K.R., KLEIN, R.L., and QUIGLEY, H.A. (2002). Gene delivery to the eye using adeno-associated viral vectors. Methods **28,** 267–275.

MOUNT, J.D., HERZOG, R.W., TILLSON, D.M., GOODMAN, S.A., ROBINSON, N., MCCLELAND, M.L., BELLINGER, D., NICHOLS, T.C., ARRUDA, V.R., LOTHROP, C.D., Jr., and HIGH, K.A. (2002). Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. Blood **99,** 2670–2676.

MURAMATSU, S., FUJIMOTO, K., IKEGUCHI, K., SHIZUMA, N., KAWASAKI, K., ONO, F., SHEN, Y., WANG, L., MIZUKAMI, H., KUME, A., MATSUMURA, M., NAGATSU, I., URANO, F., ICHINOSE, H., NAGATSU, T., TERAO, K., NAKANO, I., and OZAWA, K. (2002). Behavioral recovery in a primate model of Parkinson's disease by triple transduction of striatal cells with adeno-associated viral vectors expressing dopamine-synthesizing enzymes. Hum. Gene Ther. **13,** 345–354.

PASSINI, M.A., WATSON, D.J., VITE, C.H., LANDSBURG, D.J., FEIGENBAUM, A.L., and WOLFE, J.H. (2003). Intraventricular brain injection of adeno-associated virus type 1 (AAV1) in neonatal mice results in complementary patterns of neuronal transduction to AAV2 and total long-term correction of storage lesions in the brains of beta-glucuronidase-deficient mice. J. Virol. **77,** 7034–7040.

PONNAZHAGAN, S., CURIEL, D.T., SHAW, D.R., ALVAREZ, R.D., and SIEGAL, G.P. (2001). Adeno-associated virus for cancer gene therapy. Cancer Res. **61,** 6313–6321.

PUUMALAINEN, A.M., VAPALAHTI, M., AGRAWAL, R.S., KOSSILA, M., LAUKKANEN, J., LEHTOLAINEN, P., VIITA, H., PALARVI, L., VANNINEN, R., and YLA HERTTUALA, S. (1998). Beta-galactosidase gene transfer to human malignant glioma *in vivo* using replication-deficient retroviruses and adenoviruses. Hum. Gene Ther. **9,** 1769–1774.

QING, K., MAH, C., HANSEN, J., ZHOU, S., DWARKI, V., and SRIVASTAVA, A. (1999). Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat. Med. **5,** 71–77.

ROCHLITZ, C.F. (2001). Gene therapy of cancer. Swiss Med. Wkly. **131,** 4–9.

RUDICH, S.M., ZHOU, S., SRIVASTAVA, R., ESCOBEDO, J.A., PEREZ, R.V., and MANNING, W.C. (2000). Dose response to a single intramuscular injection of recombinant adeno-associated virus-erythropoietin in monkeys. J. Surg. Res. **90,** 102–108.

RUTLEDGE, E.A., HALBERT, C.L., and RUSSELL, D.W. (1998). Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J. Virol. **72,** 309–319.

SANDMAIR, A.M., LOIMAS, S., POPTANI, H., VAINIO, P., VANNINEN, R., TURUNEN, M., TYYNELA, K., VAPALAHTI, M., and YLA HERTTUALA, S. (1999). Low efficacy of gene therapy for rat BT4C malignant glioma using intra-tumoral transduction with thymidine kinase retrovirus packaging cell injections and ganciclovir treatment. Acta Neurochir. Suppl. **141,** 867–873.

SCHIMMENTI, S., BOESEN, J., CLAASSEN, E.A., VALERIO, D., and EINERHAND, M.P. (1998). Long-term genetic modification of rhesus monkey hematopoietic cells following transplantation of ade-

noassociated virus vector-transduced CD34+ cells. Hum. Gene Ther. **9,** 2727–2734.

SHEE, W.L., ONG, W.Y., and LIM, T.M. (1998). Distribution of perlecan in mouse hippocampus following intracerebroventricular kainate injections. Brain Res. **799,** 292–300.

SMITH, R.H., and KOTIN, R.M. (2002). In *Mobile DNA II*. N.L. Craig, ed. (ASM Press, Washington, D.C.) pp. 905–923.

SONMEZ, H., KOKOGLU, E., SUER, S., and OZYURT, E. (1995). Fibronectin and sialic acid levels in human meningiomas and gliomas. Cancer Lett. **90,** 119–122.

SUMMERFORD, C., and SAMULSKI, R.J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. **72,** 1438–1445.

SUMMERFORD, C., BARTLETT, J.S., and SAMULSKI, R.J. (1999). AlphaVbeta5 integrin: A co-receptor for adeno-associated virus type 2 infection. Nat. Med. **5,** 78–82.

THORSEN, F., ERSLAND, L., NORDLI, H., ENGER, P.O., HUSZTHY, P.C., LUNDERVOLD, A., STANDNES, T., BJERKVIG, R., and LUND-JOHANSEN, M. (2003). Imaging of experimental rat gliomas using a clinical MR scanner. J. Neurooncol. **63,** 225–231.

TYSNES, B.B., THORSEN, F., AFIONE, S., KOTIN, R.M., BJERKVIG, R., LØNNING, P.E., and HOOVER, F. (2002). 5th Annual Meeting for the American Society of Gene Therapy, June 5–9, 2002, Boston.

URABE, M., DING, C., and KOTIN, R.M. (2002). Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum. Gene Ther. **13,** 1935–1943.

VITE, C.H., PASSINI, M.A., HASKINS, M.E., and WOLFE, J.H. (2003). Adeno-associated virus vector-mediated transduction in the cat brain. Gene Ther. **10,** 1874–1881.

WALTERS, R.W., YI, S.M., KESHAVJEE, S., BROWN, K.E., WELSH, M.J., CHIORINI, J.A., and ZABNER, J. (2001). Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. J. Biol. Chem. **276,** 20610–20616.

WALTERS, R.W., PILEWSKI, J.M., CHIORINI, J.A., and ZABNER, J. (2002). Secreted and transmembrane mucins inhibit gene transfer with AAV4 more efficiently than AAV5. J. Biol. Chem. **277,** 23709–23713.

WANG, C., WANG, C.M., CLARK, K.R., and SFERRA, T.J. (2003). Recombinant AAV serotype 1 transduction efficiency and tropism in the murine brain. Gene Ther. **10,** 1528–1534.

XIAO, W., CHIRMULE, N., BERTA, S.C., MCCULLOUGH, B., GAO, G., and WILSON, J.M. (1999). Gene therapy vectors based on adeno-associated virus type 1. J. Virol. **73,** 3994–4003.

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