

Adenovirus Vector-Mediated Gene Transfer into Human Epileptogenic Brain Slices: Prospects for Gene Therapy in Epilepsy

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As a first step in the development of a gene therapy approach to epilepsy, we evaluated the ability of adenovirus vectors to direct the transfer into and expression of a marker gene in human brain slices obtained from patients undergoing surgery for medically intractable epilepsy. Following injection of adenovirus vectors containing the *Escherichia coli lacZ* gene into hippocampal and cortical brain slices, *lacZ* mRNA, β -galactosidase protein, and enzymatic activity were detected, confirming successful gene transfer, transcription, and translation into a functional protein. Transfected cells were predominantly glial, with some neurons expressing β -galactosidase as well. These results support the potential of adenovirus vectors to transfer genetic information into human epileptogenic brain, resulting in expression of the gene into a functional protein. These findings also have implications for the development of gene therapy approaches to certain seizure disorders. A number of potential therapeutic approaches are discussed, including the elevation of inhibitory neurotransmitter or neuropeptide levels, expression or modulation of postsynaptic receptors, and manipulation of signal transduction systems. © 1997 Academic Press

INTRODUCTION

A number of seizure disorders are characterized by focal onset, including complex partial seizures with mesial temporal lobe origin (4, 30, 61, 65, 101, 107) or seizures with frontal lobe origin (1, 31, 55, 80, 84, 93, 99). Eventually, such forms of epilepsy may be amenable to genetic intervention, based on the introduction

into the seizure focus of transgenes that modulate seizure activity. Included among these genes are those which encode inhibitory neurotransmitters and/or their biosynthetic enzymes, such as glutamic acid decarboxylase (GAD), responsible for the synthesis of γ -aminobutyric acid (GABA), or inhibitory neuropeptides such as neuropeptide Y (NPY) (18, 45, 63, 100, 105). Alternatively, the introduction of transgenes encoding postsynaptic receptors or their coupled intracellular signal transduction systems may prove useful in abating seizure activity. For example, the electrophysiological properties of GABA_A receptor channels vary based on combinations of different receptor subunits (15, 73, 79). Specifically, the presence of the δ -subunit enhanced the inhibitory response to GABA application in distinct GABA_A receptor complexes *in vitro* (85). Therefore, introducing the gene for the δ -subunit might enhance inhibition.

In addition, as the genetic sequences underlying inherited forms of epilepsy are discovered, new targets for gene therapy are certain to emerge. For example, autosomal dominant nocturnal frontal lobe epilepsy has been associated with a mutation in the $\alpha 4$ subunit of the neuronal nicotinic acetylcholine receptor (78, 96). Also, genetic mutations have been identified in progressive myoclonic epilepsy of the Unverricht-Lundborg type (EPM1), ragged red fiber disease, and Batten disease (47, 76, 88).

Several approaches have been used to deliver genetic sequences into the CNS. Broadly, these may be divided into *ex vivo* and *in vivo*, the former characterized by introduction of transgenes into cells which are subsequently grafted into the brain and the latter characterized by introduction of transgenes directly into the brain. Several vectors have been developed for *in vivo* approaches based on attenuated DNA viruses that permit gene transfer and expression in the postmitotic CNS. *In vivo* approaches may have some distinct advantages over *ex vivo* methods, including minimal

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disruption of the normal cytoarchitecture of the brain, absence of introduction of a mass which may be tumorigenic and/or immunogenic, and the ability to directly modulate synaptic physiology.

A number of reports have indicated the potential of adenovirus vectors to deliver genes into cells within the CNS (2, 5, 6, 8, 11, 16, 17, 20, 21, 22, 40, 46, 56, 57, 67, 75, 77, 87). Adenovirus has several attractive features, including the ability to infect a number of cell types, the capacity to accommodate large or multiple genes, minimal cytotoxicity at low multiplicities of infection (m.o.i., the ratio of infectious particles to the number of cells), and the ability to obtain high titers (10^{13} particles/ml). Recently, clinical trials of adenovirus containing the thymidine kinase gene were initiated for therapy of malignant brain neoplasms focusing on the preferential destruction of dividing tumor cells within the postmitotic brain (S. Eck, personal communication). However, current generations of adenovirus vectors have limitations, including the induction of immune responses, intrinsic cytotoxicity, particularly at high m.o.i., and suboptimal stability of gene expression in a variety of tissues (2, 19, 42, 104, 114–117).

This report demonstrates the feasibility of adenovirus-mediated gene transfer into human brain tissue obtained from patients undergoing surgery for medically intractable epilepsy—either an anterior temporal lobectomy or frontal lobectomy (55, 94, 95). Although the transgene used in this study was the nontherapeutic *Escherichia coli* LacZ marker gene, which encodes a readily assayed bacterial β -galactosidase not present in mammalian cells, these results suggest the potential to transduce human brain with vectors expressing products which are therapeutically relevant to epilepsy.

Successful transcription of the lacZ transgene was assessed by reverse transcriptase polymerase chain reaction (rtPCR) and by Northern blot analysis. In turn, cells expressing β -galactosidase were visualized by immunocytochemistry, indicating successful translation of lacZ mRNA into protein. Moreover, colorimetric and fluorometric assays demonstrated functional β -galactosidase expression. Transfected cells were predominantly glial based on morphologic criteria and by colocalization of immunoreactivity to glial fibrillary acidic protein (GFAP) and functional β -galactosidase activity. Some neurons also were transduced.

These results support the potential of adenovirus vectors to transfer genetic information locally into human epileptogenic brain, resulting in proper gene expression into a functional protein. These findings have significant implications for the development of gene therapy approaches to and models for seizure disorders with focal origins. For more generalized forms of epilepsy, global delivery of viral vectors may be more problematic, although new delivery methods are being developed.

MATERIALS AND METHODS

Viral Vectors

The two adenovirus vectors used in this study have been described previously (20, 52). In brief, the replication defective adenoviral vectors have sequences deleted in the E1A, E1B, and E3 regions, which impair the virus' ability to replicate and transform nonpermissive cells. In the vector, AdCMVlacZ (see Fig. 1), the enhancer/early promoter of cytomegalovirus (CMV) drives lacZ transcription with a SV40 polyadenylation sequence downstream. The vector, AdRSVlacZ, contains the early promoter of the Rous sarcoma virus (RSV). Viral stock solutions of 1.0×10^{12} and 1.6×10^{12} particles/ml were used for AdCMVlacZ and AdRSVlacZ, respectively; 10- or 10²-fold dilutions of these stocks were prepared in phosphate-buffered saline (PBS) immediately prior to injection into brain slices. Control injections were performed with PBS and AdRSVth, an adenovirus vector containing the human tyrosine hydroxylase II gene under control of the RSV promoter.

Patients

All patients were evaluated for surgery according to the protocol of the Graduate Hospital Comprehensive Epilepsy Center (94, 95). Twenty-one patients underwent anterior temporal lobectomy for control of medi-

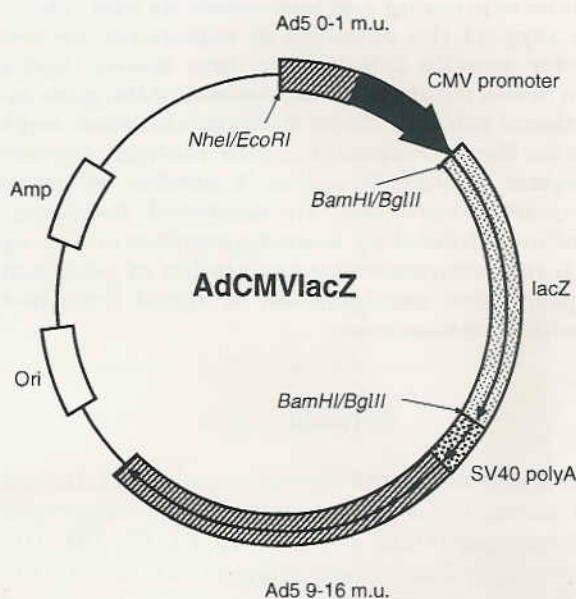


FIG. 1. The vector, AdCMVlacZ, contains a 2.8-kilobase lacZ gene driven by the early/enhancer promoter of cytomegalovirus (CMV) with a SV40 polyadenylation sequence downstream. In the vector, AdRSVlacZ, the CMV promoter is replaced with the early promoter of the rous sarcoma virus. The vector also contains a bacterial origin of replication (Ori), an ampicillin resistance sequence (Amp), and a number of restriction sites. Numbers indicate map units (m.u.) of adenovirus, corresponding to 360 base pairs each.

cally intractable complex partial seizures. Two patients underwent frontal lobectomy with partial corpus callosotomy for control of complex partial seizures or secondarily generalized tonic clonic seizures. The patients (8 men and 15 women) ranged in age from 24 to 83 years with a mean of 39.6 ± 8.4 years (mean \pm SEM).

Brain Slice

Prior to surgery, patients gave informed consent to a protocol approved by the Graduate Hospital Institutional Review Board. Hippocampal and cortical tissue was obtained in the operating room immediately at the time of surgical dissection and placed into ice-cold, low Ca^{2+} , high Mg^{2+} artificial cerebrospinal fluid (ACSF) solution (124.0 mM NaCl, 0.2 mM CaCl_2 , 5.0 mM KCl, 1.25 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 26.0 mM NaHCO_3 , and 5.0 mM $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ saturated with 95% O_2 /5% CO_2). In the laboratory, tissue blocks approximately 2 mm in depth were injected in multiple locations with a total of 5–10 μL of adenovirus stock or control PBS with a 28-gauge Hamilton syringe over 3–5 min and the syringe was gradually removed to prevent reflux of viral particles. Following this, 500- μm slices were cut on a vibratome (Ted Pella, Redding, CA) with the tissue bathing in the same ice-cold low Ca^{2+} , high Mg^{2+} ACSF solution. These sections were incubated at 32–35°C (to increase slice viability) for 5–24 h in biological ACSF (124.0 mM NaCl, 2.0 mM CaCl_2 , 5.0 mM KCl, 1.25 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 26.0 mM NaHCO_3 , 1.3 mM $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ saturated with 95% O_2 /5% CO_2) and then fixed for 24–48 h for immunocytochemistry (ICC) and 45–50 min for X-Gal staining in 4% paraformaldehyde in PBS, followed by immersion for 24–48 h for ICC and 1 h for X-Gal staining in 30% sucrose in PBS as a cryoprotectant (chemicals obtained from VWR, West Chester, PA.) Human brain slices were not viable for more than 24 h. Slices (500 μm) were cut into 30- μm microsections on a freezing microtome and placed in PBS. These floating sections were then stained using solutions appropriate to the particular assay and mounted on glass slides, as described below. For rtPCR, Northern blot, and fluorometric analysis, the 500- μm tissue slices (control and transfected) were snap frozen in Eppendorf tubes using dry ice/methanol or liquid N_2 (without fixation) and maintained at -70°C until further use, as described below.

Reverse Transcriptase Polymerase Chain Reaction

RNA was extracted from snap-frozen tissue samples using RNazol B (Cinna/Biotech, Houston, TX), a commercial mixture containing phenol and guanidinium isothiocyanate. Following chloroform extraction and ethanol precipitation, RNA was resuspended in diethylpyrocarbonate-treated water and OD₂₆₀ readings were taken to determine concentration. Reverse transcrip-

tion (RT) was then performed on each sample using 1 μg RNA, 1 \times PCR buffer, 1 mM each of dCTP, dGTP, dTTP, dATP (Cetus, Emeryville, CA), 5 mM downstream PCR primer, and 200 U Mo-MuLV reverse transcriptase (Gibco BRL, Grand Island, NY) in a final volume of 20 μL . A negative control was added containing all of the reaction components without RNA. A positive control contained AdlacZ plasmid and all other reaction components without RNA. RT reaction was allowed to proceed at 42°C for 60 min, followed by heat inactivation of RT at 65°C for 15 min. Samples were then brought to 100 μL by addition of 5 mM of both upstream and downstream primers, additional PCR buffer for final concentration of 1 \times and 2.5 U of *Taq* polymerase (Cetus). Primers directed against the lacZ gene were lacZ 182 (5'-CCGACTGATGCCTTCTGAA-CAA-3') and lacZ 560 (5'-GACGACAGTATCGGCCT-CAGGA-3') which have been described previously (50). Thirty cycles of PCR were performed with each cycle consisting of 45 s at 94°C, 45 s at 60°C, and 45 s at 72°C. Ten microliters of each PCR reaction was then analyzed on a 3% NuSieve/1% agarose gel (FMC, Chicago, IL) in 1 \times TAE and products were visualized by staining for 15 min in 1 mg/ml ethidium bromide (Sigma, St. Louis, MO). The expected length of the amplified lacZ sequence was 380 base pairs (bp). Reference markers ($\Phi\text{X174}/\text{HaeIII}$ digest) were obtained from New England Biolabs (Beverly, MA).

Northern Analysis

Following isolation of total RNA as described above, approximately 10 μg RNA was incubated at 55°C for 15 min in 10 μL denaturation buffer consisting of 26 mM Mops (Sigma), 6.6 mM CH_3COONa , 0.66 mM EDTA, 8.6% (w/v) formaldehyde, and 63.7% deionized formamide (Clontech, Palo Alto, CA) and immediately cooled on ice. One microliter of loading buffer consisting of 30% glycerol and 0.25% bromophenol blue (Sigma) was added to this mixture. RNA was separated by formaldehyde-agarose gel electrophoresis in 1.4% agarose, 40 mM Mops, 10 mM CH_3COONa , 1 mM EDTA, 0.66% formaldehyde, and 10 mg/mL ethidium bromide, transferred to Nytran Plus membrane (Schleicher & Schuell, Keene, NH) by capillary action in 10 \times SSC buffer consisting of 1.5 M NaCl, 0.15 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, pH 7.0 (Clontech), and fixed to the membrane in a UV crosslinker (Stratagene, La Jolla, CA). The locations 18S and 28S were marked under UV light. To probe the blot, a lacZ cDNA probe was random labeled by the NEBlot method (New England Biolabs). Ten nanograms of probe were boiled in 16 μL H_2O for 5 min. This mixture was incubated at 37°C for 1 h in labeling solution [2.5 μL 10 \times labeling buffer, 3.0 μL 500 mM dXTP mix (1:1:1 of dATP, dGTP, and dTTP), 2.5 μL [α -³²P]dCTP (Amersham, Arlington Heights, IL), and 0.5 μL Klenow fragment of DNA polymerase I] and

purified on a G75 Sephadex column in TNE buffer consisting of 10 mM Tris HCl, 140 mM NaCl, 0.1 mM EDTA (Sigma). Concentration of radiolabeled probe was measured in a scintillation counter. The blot was prehybridized at 42°C for 1 h in 2× prehybridization buffer (5' → 3', Inc., Boulder, CO), 50% formamide, 0.1 mg/mL sonicated salmon sperm DNA, and 0.05 mg/ml denatured yeast RNA (Sigma). For hybridization, the lacZ probe was added to the prehybridization solution to a final concentration of 10⁶ cpm/mL at 42°C for 24 h. The blot was washed 3 × 10 min in 2× SSC at room temperature and 1 × 10 min in 0.1× SSC at 50°C and exposed to Kodak XAR film using an intensifier screen at -70°C for 72 h. Adequate loading of RNA was subsequently confirmed by hybridization of the blot with a α -³²P-labeled 18S probe.

Immunocytochemical Techniques

For immunocytochemical analysis, microtome-generated, 30- μ m sections were placed into 0.1% H₂O₂ in PBS solution for 40–60 min, followed by a blocking solution containing 2% normal goat serum (NGS), 2% avidin D, and 0.2% Triton in PBS for 1 h, followed by exposure for 24–48 h to solution with or without primary antibody (5' → 3') to β -galactosidase (rabbit monoclonal, 1:2000 dilution in 1.5% NGS and 2% biotin in PBS). The addition of blocking solution, secondary antibody, and the visualization step were carried out by the Vectastain ABC-DAB method (Vector Laboratories, Burlingame, CA). Secondary antibody (50 μ L anti-rabbit, 150 μ L NGS, and 10 mL PBS) and ABC solution (50 μ L A, 50 μ L B, and 10 mL PBS) were added for 1 h each. The DAB solution consisted of 50 μ L DAB, 50 μ L H₂O₂, 200 μ L buffer, and 10 mL H₂O with an exposure time of 2–4 min. Each step was followed by two washes in PBS. Sections were dehydrated according to standard histological procedure and coverslipped in Permount (Fisher, Pittsburgh, PA).

Colorimetric X-gal Assay for β -Galactosidase Activity

Microtome sections were immediately immersed in detergent solution (2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in PBS) for 20 min, transferred into a colorimetric X-gal substrate solution consisting of 0.1% 5-bromo-4-chloro-3-indolyl- β -D-galactoside (5' → 3'), with 5 mM K₃Fe(CN)₆ and K₄Fe(CN)₆ · 3H₂O (VWR) for 1–4 h and mounted on slides in a bath of PBS and coverslipped in glycerol/PBS (Sigma). Only cells with β -galactosidase activity turn a dark blue color.

Chemiluminescent Assay for β -Galactosidase

An alternate assay was used to quantitate β -galactosidase activity following transfection based on lumiGAL chemiluminescence (Clontech). This assay

appears to be more sensitive than standard colorimetric and fluorometric assays (3). For application to our experiments, cell lysates were prepared by first pulverizing adeno-transfected and control frozen brain specimens on a liquid N₂-cooled mortar and pestle. Approximately 100 mg pulverized specimens was added to 200 μ L of cell lysis buffer/DTT (1 mM), vigorously vortexed, and then centrifuged at 14,000 rpm. Thereafter, 20- μ L aliquots of these lysates were added to 200 μ L of cell reaction buffer and 2 μ L Galacton chemiluminescent substrate; this reaction proceeded at room temperature for 30 min. Following this incubation period, 300 μ L of reaction accelerant was added to each sample prior to prompt measurement of relative light units (RLU) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Double Labeling of β -Galactosidase and GFAP

Brain sections (500 μ m) were fixed in 4% paraformaldehyde in PBS for 2 h, immersed in 30% sucrose in PBS for 1 h, cut into 30- μ m sections on a microtome and stained by X-gal histochemistry at room temperature in the dark for 12–24 h. For immunostaining of GFAP, the above immunocytochemical procedure was modified as follows: prior to H₂O₂ treatment, sections were placed in 0.025% trypsin-EDTA solution (Mediatech, Herndon, VA) for 8 min followed by two 10-min washes in PBS; a primary antibody to human GFAP (mouse monoclonal, 1:250 dilution; DAKO, Carpinteria, CA) replaced anti- β -galactosidase, normal horse serum replaced NGS, and the DAB exposure time was 18 min. Mounted sections were dehydrated and coverslipped in Permount.

RESULTS

We used rtPCR to evaluate the presence of lacZ mRNA in transfected tissue. As shown in Fig. 2, a band corresponding to a lacZ fragment was detected between the 310- and 603-bp markers in AdCMVlac-transfected tissue which was not seen in the PBS control. This finding confirms transfer of the lacZ gene with subsequent transcription into its messenger RNA.

Northern blot analysis corroborated the presence of lacZ mRNA in AdCMVlac- and AdRSVlac-transfected tissue which was not seen in PBS or vector (AdRSVth, containing the tyrosine hydroxylase gene) controls, confirming successful translation of the lacZ gene into its message. As shown in Fig. 3, a lacZ probe hybridized to a band with an approximate size of 3 kilobases (kb), closely corresponding to the 2.8-kb lacZ DNA vector insert. The 18S and 28S RNA bands, corresponding to 4718 and 1874 bp, respectively, were used as internal size markers.

Cryostat sections of human frontal cortex, temporal cortex, and hippocampus injected with AdCMVlac or

AdRSVlac demonstrated significant immunoreactivity for β -galactosidase in cells surrounding the injection tract extending up to several millimeters from the site of injection (Figs. 4A, 4B, and 4E). Immunoreactive cells demonstrated predominantly glial morphology (Fig. 4C) with some neurons present (Fig. 4D). Staining was seen in both nuclear and perikaryonal regions. In summed cryostat sections, each 2- to 3-mm tissue block contained approximately 1000–3000 cells with β -galactosidase immunoreactivity. In contrast, tissue injected with buffer (Fig. 4F) or a control vector, AdRSVth, containing a human tyrosine hydroxylase gene (results not shown), did not demonstrate any β -galactosidase-immunoreactive cells.

To ascertain the function of the transgene-directed protein expression, two assays for β -galactosidase activity were employed. The X-gal colorimetric assay revealed numerous blue cells surrounding the injection tract in hippocampal and temporal lobe slices transfected with AdCMVlac (Figs. 5A and 5B). Summed cryostat sections for each tissue block contained several hundred to thousands of blue cells. Tissue injected with control vector (AdRSVth, Fig. 5C) or buffer (results not shown) did not show any evidence of β -galactosidase histochemical staining. In addition to morphological criteria, glial cells expressing β -galactosidase were identified by double labeling with the X-gal reaction and immunocytochemistry for GFAP. As shown in Fig. 5D, GFAP-immunoreactive cells also demonstrated functional β -galactosidase activity. Using the chemiluminescent assay, AdCMVlac-transfected tissue showed marked increases in β -galactosidase activity in RLU compared to controls (Fig. 6), with a mean of 1.4×10^6 RLU for experimental samples and 3.2×10^3 RLU for control samples ($P < 0.01$, Student *t* test). These results are consistent with the synthesis of functionally active enzyme in the transfected human tissue, which was not present in control tissue.

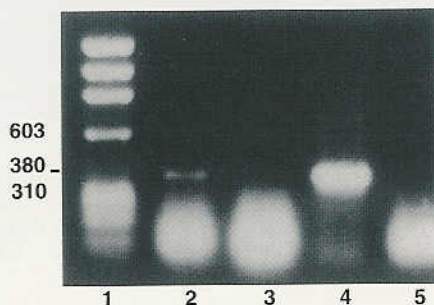


FIG. 2. LacZ mRNA was detected in human brain slices transfected with AdCMVlacZ. Reverse transcriptase polymerase chain reaction analysis revealed a band corresponding to a partial lacZ sequence between the 310- and 603-bp markers, which was not present in PBS-transfected tissue. Lanes correspond to: 1, markers; 2, AdCMVlacZ; 3, PBS control; 4, positive control containing lacZ plasmid; 5, control without RNA. Positive bands were at the expected 380-bp position.

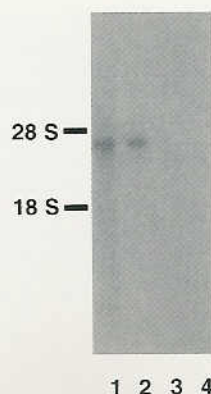


FIG. 3. The presence of lacZ mRNA was confirmed by Northern blot analysis of RNA from temporal cortex slices transfected with AdCMVlacZ and AdRSVlacZ. Hybridization with a radiolabeled probe for lacZ mRNA revealed a band of approximately 3 kb in adeno-transfected tissue, which was not seen in controls. Lanes 1 to 4 correspond to AdCMVlacZ, AdRSVlacZ, AdRSVth, and PBS, respectively. 18S (4.7 kb) and 28S (1.9 kb) ribosomal RNA bands, visualized under UV light and with a ^{32}P -labeled 18S probe (results not shown), were used as internal size markers.

The presence of β -galactosidase was demonstrated by at least one of the above methods in 15 of 15 experiments for AdCMVlacZ and 5 of 14 experiments for AdRSVlacZ.

DISCUSSION

Over the past decade, rapid advances in molecular biology have permitted the development of novel virus-based vector systems for the transfer of genetic information into mammalian cells. Based on such systems, gene therapy clinical trials for human diseases, such as cystic fibrosis, malignant brain tumors and inherited neurometabolic disorders and preclinical evaluations for familial hypercholesterolemia have already begun (10, 44, 53, 62, 109, 117). Furthermore, a number of preclinical trials have been initiated for gene therapy approaches to other neurological diseases with defined pathologic and biochemical abnormalities, such as Parkinson's disease (35, 98). We and other groups have used HSV-1, adenovirus, and adeno-associated virus vectors containing genes encoding dopamine biosynthetic enzymes and/or neurotrophic factors in rodent and primate models of Parkinson's disease (25, 27, 28, 33, 35, 36, 46, 51, 82). In such studies, significant biochemical and behavioral recovery was observed.

Based on these encouraging results, as well as other experimental applications of viral vector gene transfer to the CNS, we believe that a gene therapy approach to epilepsy is feasible, although the precise gene targets have not yet been identified. By introducing into a seizure focus transgenes encoding enzymes responsible for the synthesis of inhibitory neurotransmitters or

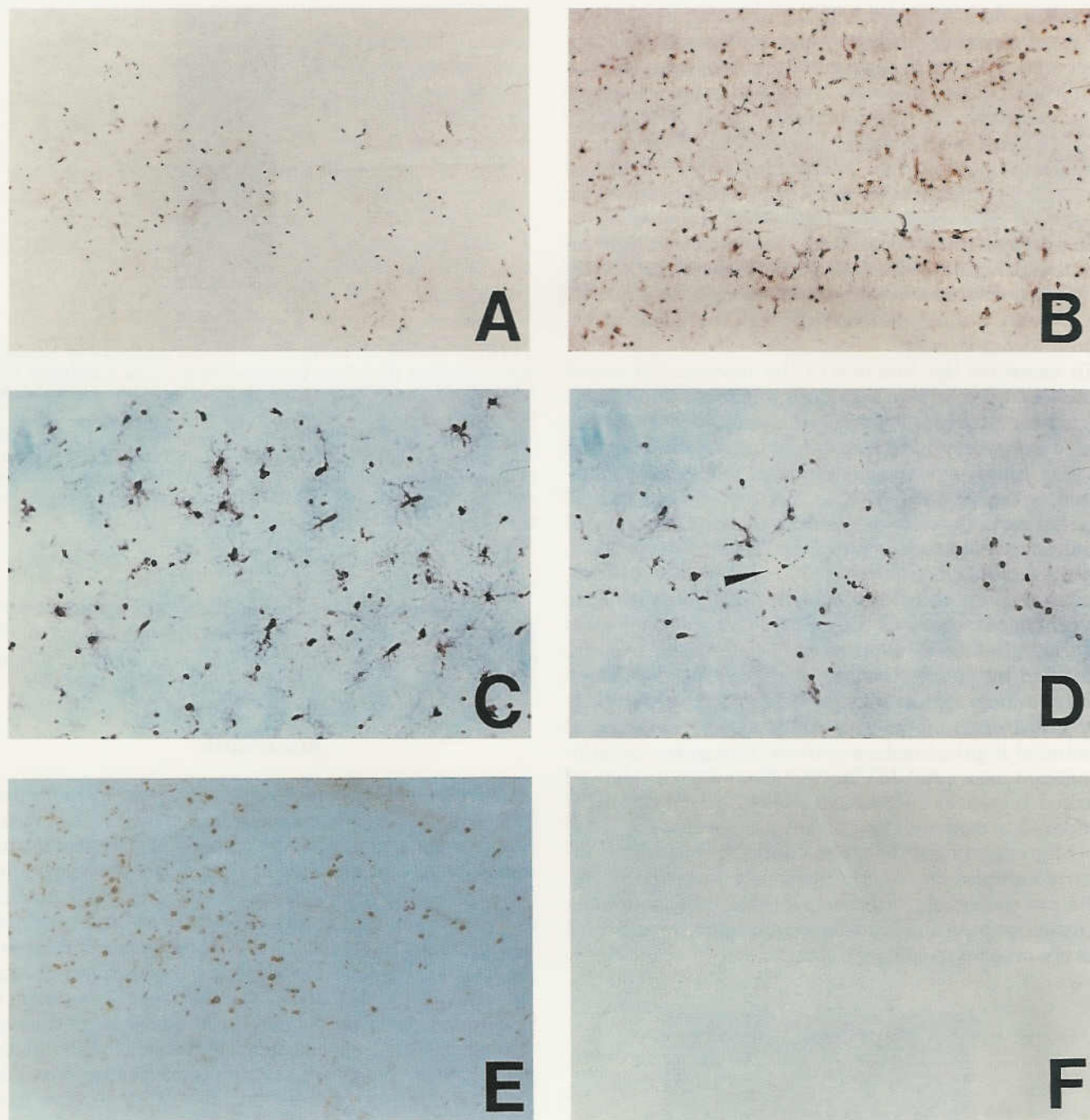


FIG. 4. Immunoreactivity for β -galactosidase was demonstrated in human brain sections following transfection with AdCMVlacZ and AdRSVlacZ. Control tissue sections in which the primary antibody was omitted did not show any immunoreactivity for β -galactosidase (results not shown). (A) Frontal cortex, AdCMVlacZ. (B) Temporal cortex, AdCMVlacZ. (C) Temporal cortex, AdCMVlacZ. (D) Temporal cortex, AdCMVlacZ (arrow indicates neuron). (E) Hippocampus, AdRSVlacZ. (F) Temporal cortex, control PBS. Magnification: 25 \times for A, B, E, and F; 50 \times for C and D.

their membrane transporters, inhibitory neuropeptides, selected subunits of postsynaptic receptor complexes, antisense sequences to other receptor subunits, or signal transduction systems, it may be possible to inhibit seizure onset or spread.

A number of effective anti-seizure pharmacological

agents appear to increase GABAergic activity in the CNS. γ -Vinyl-GABA, which increases GABA concentrations by irreversibly inhibiting the catabolic enzyme, GABA transaminase, can have a benefit in some epilepsies (66, 68, 81, 83, 89, 91). Similarly, the introduction of a GAD-encoding transgene into the CNS may in-

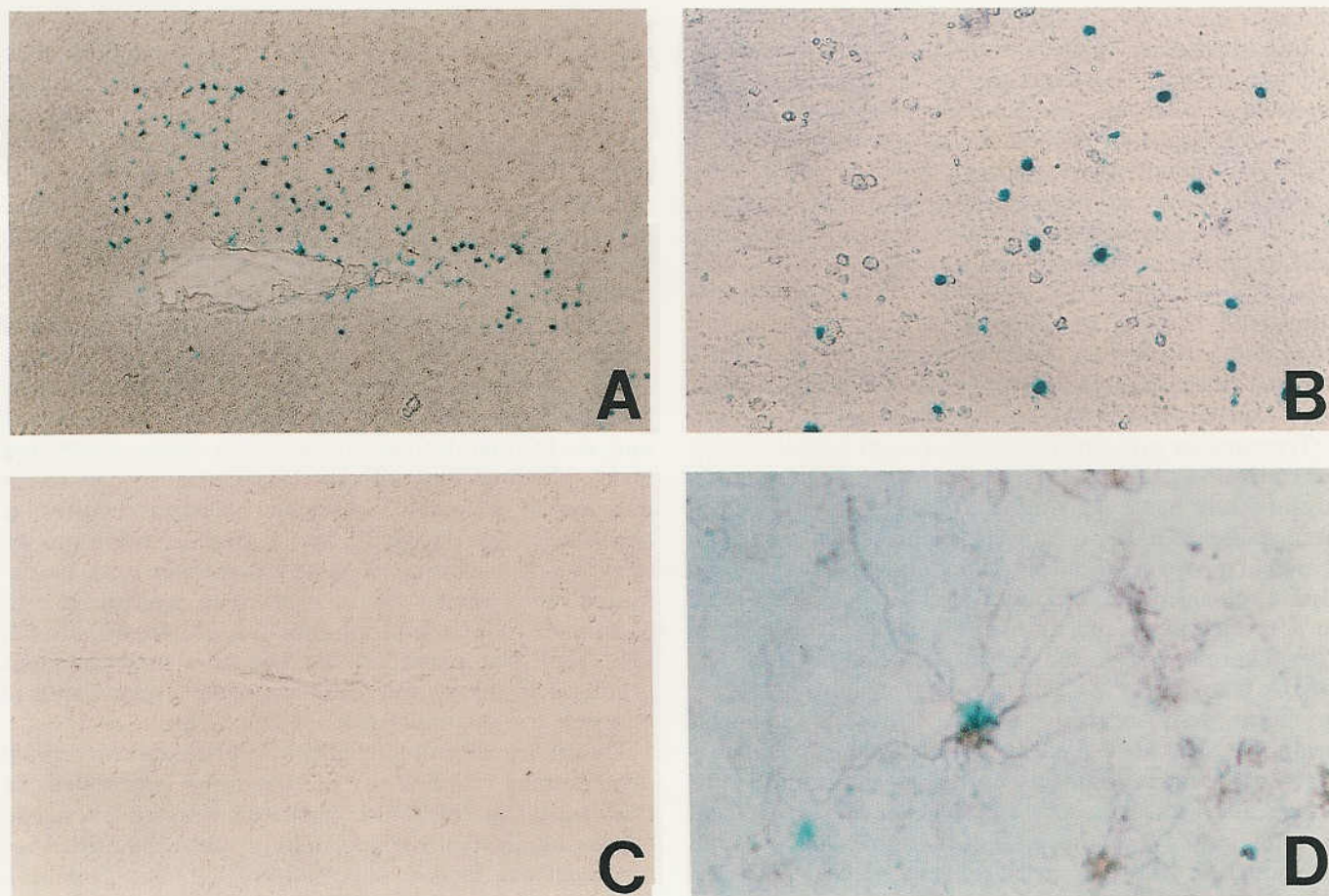


FIG. 5. Using the X-gal technique, β -galactosidase enzymatic activity was demonstrated in tissue sections transfected with AdCMVlacZ (A, B) which was not seen in controls (C). Treatment of AdCMVlacZ-transfected tissue with immunocytochemistry for GFAP showed that glial cells had functional β -galactosidase activity (D). Immunocytochemical controls included tissue transfected with buffer and omission of primary antibody for GFAP (results not shown). (A) Temporal cortex, AdCMVlacZ. (B) Hippocampus, AdCMVlacZ. (C) Temporal cortex, control AdRSVth. (D) Hippocampus, AdCMVlacZ, anti-GFAP. Magnification: 25 \times for A and C; 50 \times for B; 200 \times for D.

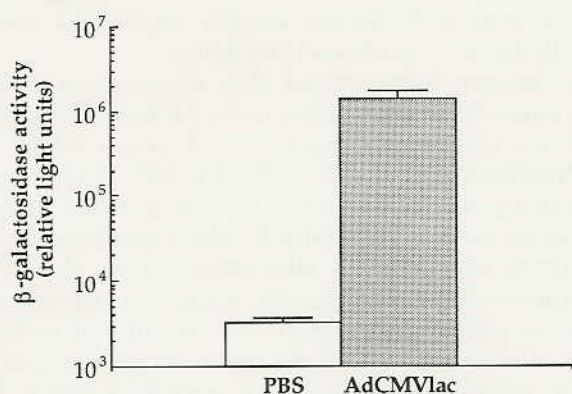


FIG. 6. β -Galactosidase activity was demonstrated by the LumiGal chemiluminescent assay in human brain slices transfected with AdCMVlacZ, which was not seen in controls. Results represent means \pm SEM for six experimental and six control tissue slices derived from two patients. Results from the two patients were normalized to background fluorescence. Enzymatic activity is expressed in relative light units.

crease GABAergic activity and prevent seizures in some partial epilepsies. However, one potential drawback of this approach could be the activation of GABA_B receptors with a possible increase in excitability (12, 91).

Postsynaptic mechanisms may be targeted as well. Benzodiazepines and barbituates may increase inhibition by enhancing the response of ligand-gated chloride channels to GABA application (60, 73, 74). Similarly, a number of studies suggest that enhancing the function of postsynaptic receptors may augment inhibition within the seizure focus. For example, decreased function of receptors for adenosine, which has been suggested to modulate seizure activity in human epileptic patients (26), may play a role in loss of inhibition in epileptic hippocampus. Therefore, introduction of a vector encoding the adenosine A1 receptor may restore the inhibitory response to adenosine. Also, in certain GABA_A receptor complexes *in vitro*, the presence of the δ -subunit slowed the rate of acute desensitization of GABA-evoked current during GABA application and

the rate of recovery of GABA-evoked current following GABA application relative to receptor complexes which did not contain the δ -subunit (85). Thus, it may be possible to augment the function of existing GABA_A receptor complexes by introducing a transgene encoding the δ -subunit. The specificity of this single subunit approach is particularly attractive: since the δ -subunit cannot form functional homomeric receptor complexes (85), its presence may only have an effect in cells which already contain GABA receptors. This approach is supported further by Bergold *et al.*'s virus vector-mediated introduction of the gene encoding the GluR6 subunit of the kainate receptor into rat hippocampus, resulting in selective loss of CA3 neurons (7).

Transporter proteins may also present useful targets for controlling seizure activity. For example, a microdialysis study has suggested that GABA release in human epileptic patients may be mediated in part by a transporter-reversal mechanism and that decreased GABA release in amygdala-kindled rats may be related to a reduction in the number of GABA transporters (29). Therefore, a vector containing the cDNA for the GABA transporter, under control of a neuron-specific promoter, may augment GABA release in epileptic brain. Finally, since signal transduction pathways mediate the extracellular-intracellular coupling of inhibitory neurotransmission, application of vectors containing genes encoding mediators of these signal transduction systems may be considered. GABA_B receptors, for example, are coupled via GTP-binding proteins to a variety of effector systems which may have subtle neuromodulatory functions (110). We and others have developed viral vectors that contain genes encoding the catalytic domain of such enzymes, including calcium/calmodulin-dependent protein kinase II (24), adenylate cyclase (39), and protein kinase C (92).

Prior to initiating studies aimed at developing specific virus vectors for application to epilepsy, however, it must be shown that such vectors will, in fact, permit transgene expression into a functional protein in human CNS tissue. To date, many studies using viral vector-mediated gene transfer into the human CNS have focused on dividing malignant brain tumors, the cellular physiology of which is markedly different than the underlying postmitotic CNS. Therefore, our study was performed to evaluate the potential of adenovirus vectors to direct the expression of a bacterial marker gene (*LacZ*) in human hippocampal and cortical tissue derived from patients undergoing epilepsy surgery for medically intractable seizure disorders.

In this study, immunohistochemical staining of adenovirus vector-transfected tissue demonstrated β -galactosidase expression predominantly in glial cells, with some neurons showing β -galactosidase immunoreactivity. This finding is consistent with results obtained in rat mixed neuronal-glial cultures, which showed prefer-

ential expression of β -galactosidase in glial cells versus neurons (71) and with results from other laboratories (16, 46, 56). Since glia support neuronal function through a variety of processes, including supply of neurotrophic factors and maintenance of the neuronal extracellular milieu, tonic supply of a chemical or protein messenger may suffice to elicit a functional response from receptor cell populations. For example, a local population of glial cells producing GABA may provide tonic seizure inhibition.

Postsynaptic approaches based on the introduction of GABA receptor complexes, transporter proteins, or signal transduction pathways would depend primarily on delivery of transgenes to neurons. This requirement may be fulfilled by vectors based on adeno-associated virus which readily directs transgene expression in neurons (51) or HSV-1, which has a natural tropism for neurons (13, 32, 33, 34, 37, 38, 41, 49, 111). Additionally, cell-specific targeting of gene expression may be obtained using recent vector constructs containing cell-specific or inducible promoters (14, 33, 48, 50, 59, 92, 112, 113). For example, the use of a neurofilament promoter sequence may limit transgene expression to neurons, even using an adenovirus vector.

The choice of promoter may also prove to be a crucial determinant of efficiency of transgene expression. In this study, the AdCMVlac construct directed transgene expression in 15 of 15 experiments, while the AdRSVlac construct was far less successful (5 of 14 experiments). In contrast, *in vitro* transfection studies in rat demonstrated that both promoters had equal efficiency (71). A similar adenovirus vector with the RSV promoter driving TH expression achieved robust expression in rat brain *in vivo* (46), providing further evidence that RSV promoter activity may be high in rat brain. These differences in efficiency suggest that virus vector systems may display species specificity. Therefore, animal models may not always predict successful vector-mediated gene expression in humans.

The human hippocampal slice preparation derived from patients undergoing anterior temporal lobectomy has been extensively characterized in our laboratory and by others (23, 64, 72, 86, 90, 97, 102, 103) and may provide a powerful method of screening vector systems and gene targets. Specifically, the introduction of a transgene into a brain slice may permit the direct electrophysiological assessment of that transgene's functional or therapeutic significance in human epileptic tissue. Conversely, vector-mediated introduction of antisense sequences may create a "knockout brain slice" which is capable of elucidating the function of native proteins. These experimental approaches are currently under investigation in our laboratory.

This study has limitations, including the short timespan of incubation (limited by the duration of survival of human CNS tissue in a slice chamber) and

the introduction of a nontherapeutic transgene. However, this study does demonstrate the significant ability of adenovirus vectors to introduce a transgene into human brain tissue, resulting in its proper transcription and translation into a functional protein. These findings provide support for the further development of viral vectors which contain therapeutic transgenes relevant to human epilepsy disorders. Recently, one group has reported the development of a HSV-1 vector containing a human GAD gene; transfection of cultured rat cells with this vector resulted in functional human GAD activity (70). We have also recently developed an AAV vector with a human GAD gene (unpublished results).

For seizures with focal pathology, delivery of an appropriate virus vector may be achieved with a stereotactic neurosurgical approach. Targeting of the correct cells may be augmented by the use of cell-specific or inducible promoters. For generalized seizure disorders without an identifiable focal origin, virus vector-mediated gene delivery is more problematic. However, recent progress in developing protocols for endovascular delivery with osmotic disruption of the blood-brain barrier (22, 67), endovascular delivery by catheters (43, 44, 69, 106), intraventricular or intraparenchymal delivery by stereotactic injection (75), or convection delivery by interstitial infusion (9, 54, 58, 75) may permit global CNS delivery of vectors, with potential for modulating overall CNS inhibitory neurotransmission.

Although further developments in particular vectors and the evaluation of the stability of vector-mediated gene expression will be important in any long-term gene therapy strategy for epilepsy, these results support the aggressive development of a gene therapy approach to epilepsy based on the introduction of therapeutic genetic information into the human brain.

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