

Expression of *Escherichia coli* β -Galactosidase and Rat HPRT in the CNS of *Macaca mulatta* Following Adenoviral Mediated Gene Transfer

BEVERLY L. DAVIDSON, STEPHEN E. DORAN,* DONNA S. SHEWACH,†
JILL M. LATTA, JOHN W. HARTMAN, AND BLAKE J. ROESSLER

Department of Internal Medicine, Division of Rheumatology, *Department of Surgery, Division of Neurosurgery, and † Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0680

Adenoviral-mediated gene transfer to the caudate nucleus of *Macaca mulatta* was accomplished using stereotactic injection of two distinct recombinant Ad5 vectors containing the gene for *Escherichia coli* β -galactosidase and the cDNA for rat hypoxanthine-guanine phosphoribosylphosphotransferase (HPRT), respectively. Multiple analyses (including immunohistochemistry, histochemistry, transmission electron microscopy, RNA *in situ* hybridization, nucleotide pool analysis, and enzyme assay) confirmed efficient expression of β -galactosidase and rat HPRT. Transgene expression was evident in both neurons and glia. Clinically, no evidence of meningitis or cerebritis was observed and no focal neurological deficits were detected in the animal. These preliminary studies indicate that recombinant adenovirus is capable of mediating high level transgene expression to the brains of higher order mammals. © 1994 Academic Press, Inc.

INTRODUCTION

A large percentage of inherited metabolic disorders have as a sequelae of the disease process untreatable neurological abnormalities. Furthermore, the pathophysiology responsible for the neurological manifestations in many of these disorders remains elusive. Complete deficiency of the purine salvage enzyme hypoxanthine-guanine phosphoribosylphosphotransferase (HPRT) is a prototype for inherited enzymopathies with neurological involvement (13, 18). This X-linked disorder, known as the Lesch-Nyhan syndrome (LNS), is characterized clinically by severe neurological abnormalities including mental retardation, choreoathetosis, spasticity, and a tendency toward compulsive self-mutilation. The genetic basis of HPRT deficiency states has been extensively defined and the biochemical basis of the disorder has also been characterized (6, 7, 17). Importantly, however, we do not yet understand the pathophysiology within the central nervous system (CNS) that results in LNS (14).

Although the systemic manifestations of uric acid

overproduction can be controlled with allopurinol, no treatment exists for the neurological manifestations of the LNS. While investigations to decipher the underlying pathophysiology of this devastating disease continue, techniques designed to replace missing gene products to terminally differentiated tissues such as those found in the CNS are continually being developed and refined. The most efficient of these have involved the use of recombinant viral delivery systems, including herpes virus vectors (3, 5, 11, 15, 16, 21), the herpes virus amplicon system (8, 9), and, more recently, replication-defective adenoviruses (1, 2, 4, 12).

The low pathogenicity and high efficiency of adenoviral gene delivery suggests that these vectors may have general applicability toward the therapeutic correction of inherited metabolic diseases with CNS manifestations. Prior to the clinical use of recombinant adenoviruses as adjunctive therapy for the CNS manifestations of the LNS, it is necessary to perform nonhuman primate studies in order to determine the efficiency of gene transfer and the potential side effects associated with administration of these vectors to the brain parenchyma. As an initial step in the development of these vectors for therapeutic use, we describe results using two recombinant adenoviruses to mediate gene transfer of *Escherichia coli* lacZ or rat HPRT to the caudate nucleus of a nonhuman primate (*Macaca mulatta*).

MATERIALS AND METHODS

Construction of recombinant adenoviral vectors. The parent plasmid for these vectors was derived from pAdBglII, a plasmid that contains genomic adenoviral sequences from 0-1 and 9-16 map units of serotype 5 human adenovirus flanking a single *Bgl*II restriction site in addition to pBR322 backbone sequences. This plasmid was modified by the insertion of the Rous sarcoma virus (RSV) promoter to drive transgene transcription, a multiple restriction site polylinker for cloning of genes/cDNAs of interest and an SV40 polyadenylation signal. The resulting plasmid was designated pAdRSV4 and the transgenes of interest were cloned

into the unique *Bgl*II site. As such, pAd.RSVlacZ contains the gene for *E. coli lacZ*; pAd.RSVrHPRT contains the cDNA for rat HPRT (gift of J. Monnat, University of Washington). The cDNA for rat HPRT was chosen because rat and primate HPRT proteins are physically separable from each other using nondenaturing PAGE. This facilitates the identification of transgenic HPRT expression in tissues with endogenous HPRT activity.

Recombinant adenoviral vectors were generated by homologous recombination between pAd.RSVlacZ or pAd.RSVrHPRT and human adenovirus serotype 5 derivative sub360. Sub360 contains a partial deletion in the E3 region. pAd.RSVlacZ and pAd.RSVrHPRT were linearized with *Nhe*I and cotransfected with *Xba*I/*Cla*I-digested sub360 (this results in removal of the E1A and E1B regions) into 293 cells followed by soft agar overlay. Plaques expressing *E. coli lacZ* were identified by X-gal staining. Recombinant adenovirus-expressing rat HPRT sequences were identified by *in situ* gel assays for HPRT activity (Fig. 1). Individual isolates were also subjected to restriction endonuclease mapping.

Recombinant clones were expanded and triple plaque purified to assure that viral suspensions used for *in vivo* experiments were free of wild-type virus. Viral suspensions from large scale preps (10) were used immediately for intracerebral injection. Both the particles/ml (based on OD₂₆₀) and the titers (plaque forming units/ml) were determined.

Animals and surgical procedures. Surgical procedures and the techniques used for the administration of recombinant adenoviral vectors were performed following institutional guidelines and were approved by the University Committee on the Use and Care of Animals. A 3.2-kg juvenile female rhesus monkey (*M. mulatta*) was used for the experiment and housed in the biocontainment facility in the University Laboratory for Animal Medicine throughout the experimental period. Laboratory examination of the animal including CBC, blood chemistries, and urinalysis were normal. Serological examination of the monkey for herpes B antibodies was negative.

Prior to the surgical procedures, the monkey was anesthetized with intramuscular ketamine (7 mg/kg), followed by general inhalational anesthesia with isoflurane. The animal received a single perioperative prophylactic dose of intravenous cefazolin (10 mg/kg). A preoperative lumbar puncture (using a 23-gauge spinal needle) was performed using aseptic technique and 400 μ l of CSF was removed. The animal was placed in a stereotactic frame (Kopf Instruments), and its head was shaved, prepped, and draped in a sterile fashion. Through a 5-mm burr hole, a 25-gauge spinal needle was introduced stereotactically into the head of the right caudate nucleus. Two hundred microliters of purified adenovirus (Ad.RSVlacZ) containing 0.6×10^{12} particles/ml (1.4×10^{11} pfu/ml) resuspended in PBS was infused through a

Hamilton syringe over a 30-min period into the head of the right caudate. The needle was slowly withdrawn over a 5-min period. Two hundred microliters of Ad.RSVrHPRT (1.0×10^{12} particles/ml, 1.6×10^{11} pfu/ml) was injected into the head of the left caudate nucleus. No neurological deficits resulted from the procedure. The animal was then observed and examined daily for 7 days postinfection. No clinical evidence of meningitis or cerebritis was noted during this observation period.

After 7 days, the monkey was anesthetized with intramuscular ketamine followed by intravenous pentobarbital (7.5 mg/kg). A lumbar puncture was aseptically performed and 400 μ l of CSF was withdrawn. The brain was perfused via bilateral internal carotid artery cannulation with 120 cc of ice-cold 0.9% sodium chloride. The monkey was then euthanized with an overdose of pentobarbital (>100 mg/kg). The monkey's scalp was reflected off the calvarium, a Stryker cast saw was used to remove the calvarium, and the brain was removed by sectioning of the dural reflections, cranial nerves, and the cervical-medullary junction.

Analysis of brain tissue for lacZ expression. The entire brain was excised, and the right caudate nucleus was separated from the right cerebral hemisphere by dissection. LacZ expression was detected histochemically using previously described techniques (4). Briefly, portions of brain tissue were obtained from the region surrounding the point of injection and were fixed in 2% paraformaldehyde in Pipes, pH 6.9. Samples were rinsed in PBS/2 mM MgCl₂, reacted in X-gal in PBS for 2 h, and photographed using a dissecting microscope. Samples were postfixed, and cryopreserved in 20% sucrose, and 10- μ m sections were placed onto slides. Sections were counterstained with neutral red and examined by light microscopy.

Analysis of additional organ systems for transgenic lacZ expression. In order to determine if significant amounts of recombinant adenovirus had escaped from the CNS into the systemic circulation, samples of liver, lung, and spleen were obtained from the animal at the time of sacrifice. Representative samples of liver (6.5 g), lung (3.0 g), and spleen (1.5 gm) were dissected into 0.5-cm sections and immersion fixed for 1 h using 2% paraformaldehyde in 100 mM Pipes, pH 6.9. Tissue samples were then rinsed twice in PBS/2 mM MgCl₂ and stained overnight with X-gal solution as described. Samples reacted with X-gal were also postfixed in 4% glutaraldehyde, cryopreserved using 20% sucrose, and frozen in OCT. Sections (10 μ m) were placed onto slides, counterstained lightly with hematoxylin and eosin, and examined by light microscopy.

Electron microscopy. Samples stained with X-gal were processed for transmission electron microscopy (TEM) by fixation in 2% glutaraldehyde, 1.5% paraformaldehyde, and 0.0015% CaCl₂ in 0.1M Na cacodylate,

followed by 1% osmium tetroxide in 0.1M Na cacodylate containing 5% sucrose. Samples were then dehydrated in ethanol and embedded. Ultrathin sections were obtained and analyzed using a Philips CM 10 transmission electron microscope.

Immunohistochemistry. Immunohistochemical staining was performed on fresh frozen samples of brain tissue. Ten-micrometer cryostat sections were placed onto poly-L-lysine coated slides, fixed in methanol at -20°C , and blocked by incubation with 20% goat serum and 20% donkey serum in PBS. Slides were then incubated for 1 h with rabbit anti-gial fibrillary acidic protein (GFAP, Sigma) and murine (monoclonal) anti- β -galactosidase. Following several rinses in PBS, sections were incubated with FITC-labeled goat-anti rabbit and Texas red-labeled donkey anti-mouse (Jackson Immuno Research) for 1 h at 37°C . Additional slides were blocked by incubation with 10% BSA and then incubated for 1 h with murine anti- β -galactosidase conjugated to biotin (Sigma). Following several rinses with PBS, sections were incubated with AMCA-avidin (Vector Labs) for 1 h at 37°C . Slides were mounted under an anti-fadent and examined using light and fluorescent microscopy.

In vitro and in vivo rat HPRT enzyme activity. *In vitro* rHPRT activity was analyzed by nondenaturing PAGE. Plaques containing putative AdrHPRT were amplified and cell lysates prepared. Lysates were then fractionated by 8% PAGE at 30 V for 18 h, and gels were reacted with 300 μM [$8-^{14}\text{C}$]hypoxanthine (New England Nuclear, 57 mCi/mmol), 100 mM Tris, pH 7.4, 2.5 mM PP-ribose-P, and 14 mM MgCl_2 for 30 min at 37°C . Reaction products were absorbed onto polyethyleneimine (PEI) paper, dried, and exposed to Kodak XAR-5 film. rHPRT activity was detected by autoradiography.

For *in vivo* rHPRT activity analysis of infected rhesus caudate, tissues immediate to and including the point of injection were frozen, and serial 500- μm sections were obtained. Tissues were homogenized in 60 λ of 10 mM Tris, pH 7.4, and lysed by three cycles of freeze/thawing, and cell debris were removed by centrifugation. The protein concentrations of each sample were determined using the method of Lowry. Tissue lysates were then analyzed using the radiochemical *in situ* gel assay described above. Total HPRT activity present in the tissue samples was then determined by analysis of PEI transfers using phased array phosphoimaging densitometry (Molecular Dynamics).

Determination of purine pools in brain tissue. Samples of brain tissue (approximately 0.5 cm^3) were obtained from the region of the right (control) and left caudate nuclei. Brain tissues from both hemispheres were processed for nucleotide pool determination by extraction in 0.4 N perchloric acid and neutralization with KOH. Mono-, di-, and triphosphate concentrations were quantitated using HPLC column chromatography.

Samples were separated and detected on a gradient HPLC system equipped with a strong anion-exchange column using a linear gradient of 100.0% 0.005 M NH_4PO_4 , pH 2.8, to 100% 0.75 M NH_4PO_4 , pH 3.9, over 40 min.

In situ RNA hybridization. Antisense and sense RNA probes specific for β -galactosidase were prepared *in vitro* using T7 polymerase (Promega), ^{35}S -UTP, and ^{35}S -CTP, to a specific activity of 1×10^7 cpm/ μl . *In situ* hybridizations were done essentially as described (19). Tissue sections were fixed in 4% paraformaldehyde in PBS, dehydrated, placed in proteinase K at 30°C for 30 min, washed, dehydrated again, and then prehybridized in a prehybridization solution containing 50% formamide, 2.5 \times Denhardt's, 0.6 M NaCl, 10 mM Tris (pH 8.0), 0.1% SDS, 1 mM EDTA, 10 mM DTT, and 500 $\mu\text{g}/\text{ml}$ *E. coli* tRNA for 4 hours at 55°C . Hybridizations were done at 58°C for 17 h using sense or antisense probes (1×10^6 cpm/section) in a hybridization solution consisting of prehybridization solution plus 10% dextran sulfate. Posthybridization, slides were washed extensively, treated with RNase A (20 $\mu\text{g}/\text{ml}$) for 30 min at 37°C , washed four times in 2.0 \times SSC for 5 min at RT, and then washed three times in 0.5 \times SSC for 20 min at 58°C . Dehydrated and dried sections were then coated with photographic emulsion (Kodak) and exposed for 4 days in lightproof containers. Slides were developed, counterstained with hematoxylin/eosin, and examined using a combination of light- and dark-field microscopy to identify cells containing areas of specific antisense probe hybridization. Sections hybridized to sense probe were used as a control.

RESULTS

In vitro analysis of rHPRT recombinants. Seven of eight recombinant adenoviruses screened for rHPRT activity were positive as shown in Fig. 1. The recombinant rat HPRT activity is readily detectable over endogenous human HPRT using this gel assay system. Although two discernible bands of activity are detected, there is evidence for human/rat heterodimer formation as noted by the smearing between the major zones of enzyme activity.

Analysis of tissues for lacZ expression. *En bloc* X-gal staining of sections obtained from the right caudate nucleus showed extensive areas of *E. coli lacZ* activity within and extending approximately 2 cm from the point of injection (Fig. 2A). X-gal-stained cells consisted of approximately 70–80% of total cells at the site of injection and declined to 30–40% 1.0 cm distal to the site of injection. Within these regions both white and gray matter were transduced, as well as cortical cells surrounding the needle tract. Light microscopic examination of sections from the right caudate nucleus stained with X-gal and counterstained with neutral red demon-

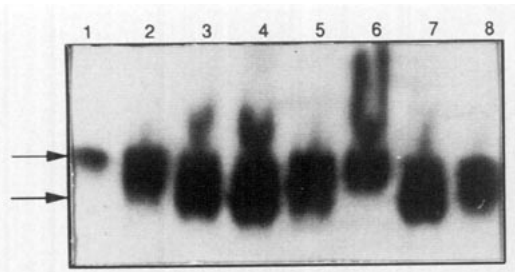


FIG. 1. Identification of two electrophoretically distinct and enzymatically active HPRT proteins present in Ad.RSVrHPRT-infected 293 cells. An *in situ* gel assay for HPRT activity was used to screen candidate plaques for rat HPRT activity. Membrane-free lysates were fractionated on nondenaturing polyacrylamide gels, transferred to PEI paper, and reacted with [14 C]Hx containing enzyme reaction mixture as described under Materials and Methods. Lane 1, human HPRT activity from uninfected 293 cells (upper arrow); lanes 2–8, LE293 cells infected with seven different isolates. Note that six of seven isolates express recombinant rat HPRT (denoted by lower arrow).

strated *E. coli lacZ* activity in multiple cell types, including radial astrocytes (Fig. 2B), ependymal cells of the adjacent lateral ventricle (Fig. 2C), and neurons (Fig. 2D). Samples of liver, lung, and spleen were processed identically. Multiple sections from these tissues were screened microscopically with no evidence of lacZ activity.

Transgene expression in brain was confirmed using anti-sense cRNA probes specific for *E. coli* β -galactosidase in *in situ* RNA hybridization experiments. LacZ mRNA was detected within multiple cells within the right caudate nucleus (Fig. 3A). Sections from the left caudate nucleus (infected with Ad.RSVrHPRT) were used as a control. Although only background levels of signal were present in the parenchyma, the ependymal cells lining the lateral ventricle were strongly positive (Fig. 3C) indicating delivery of the vector via diffusion through CSF. Expression of transgenic lacZ protein in the ependymal cells of the left hemisphere was confirmed using immunohistochemistry (Fig. 3E).

Immunohistochemistry was also used to confirm that the β -gal activity detected by X-gal staining was due to transgenic expression rather than endogenous (monocyte/macrophage or microglial) galactosidase activity. This is an important distinction as a focal inflammatory

response was evident in the region of the injection as noted by neuropathologic examination of H & E sections. Antibodies specific for *E. coli lacZ* and type II astrocytes were used to colocalize transgene activity in the glial population, and cells positive for both *E. coli lacZ* and GFAP were readily identified using fluorescent microscopy (Fig. 4).

TEM was done to specifically identify transduced cells within the caudate nucleus based upon ultrastructural criteria (Fig. 5). Electron-dense granules and spicules (consistent with X-gal reaction product) are evident in neurons, astrocytes, and oligodendrocytes of the right caudate nucleus. No electron-dense particles were observed in samples obtained from the contralateral caudate nucleus. These data, in combination with the histochemical, *in situ*, and immunohistochemical studies, confirm efficient transgenic lacZ expression within multiple cell types in the CNS.

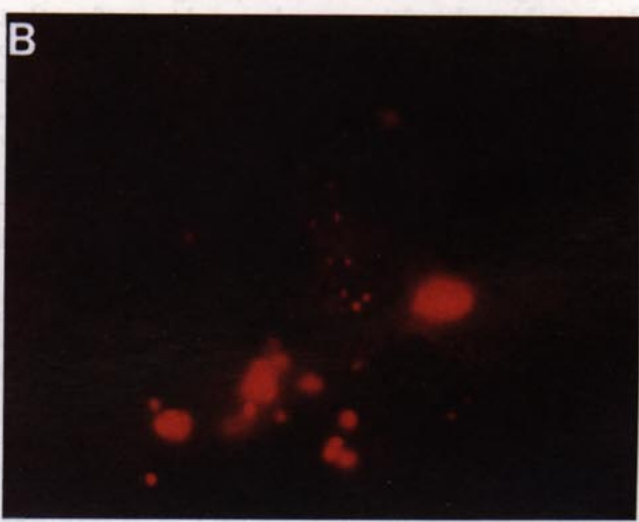
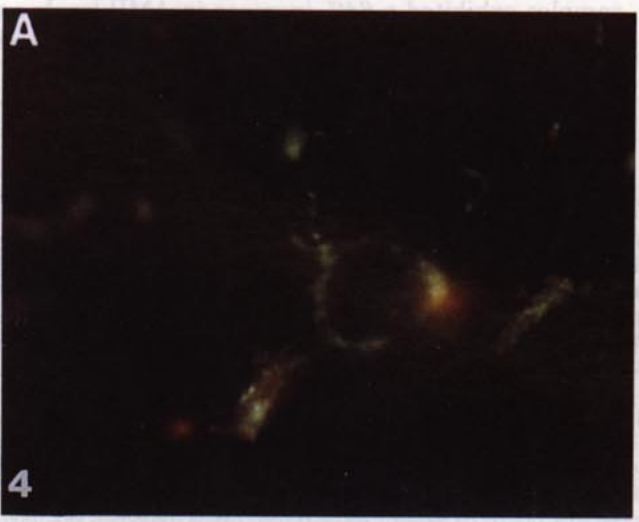
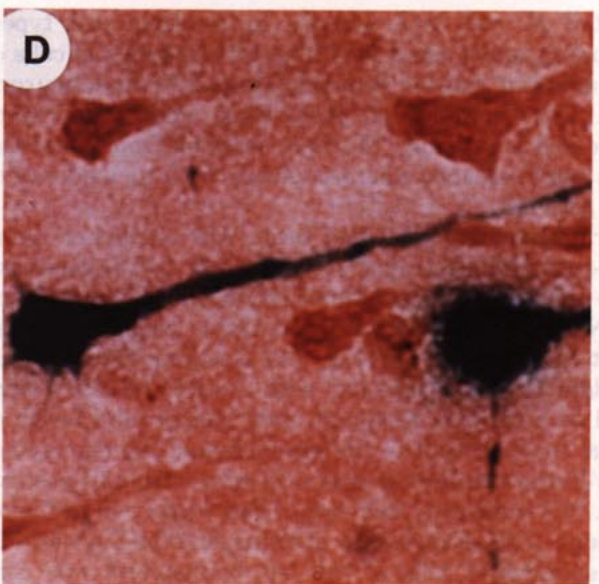
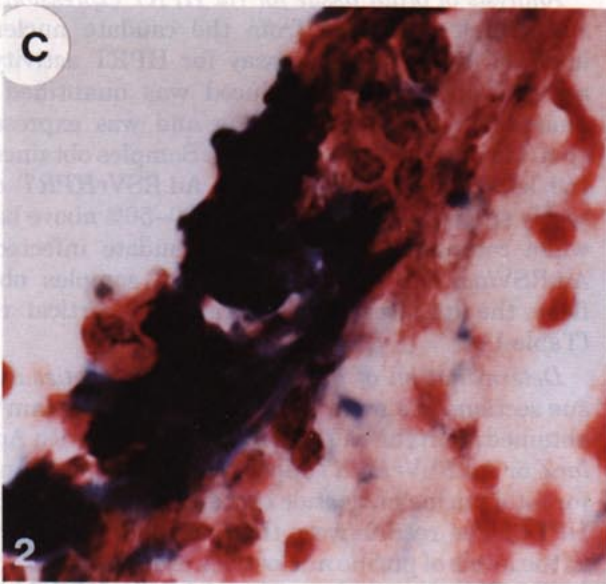
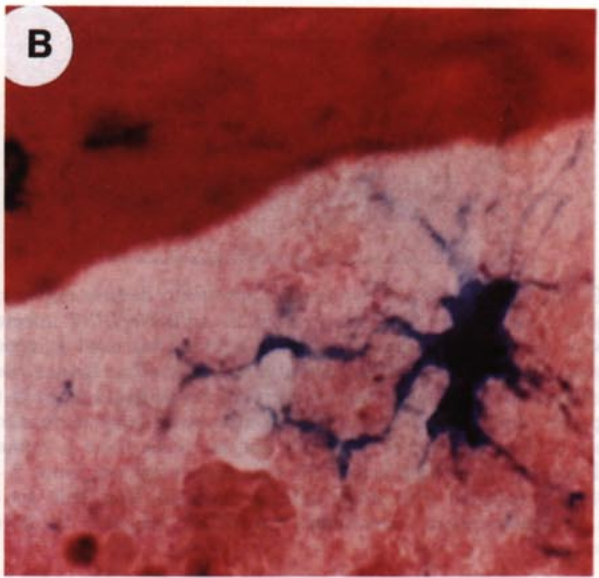
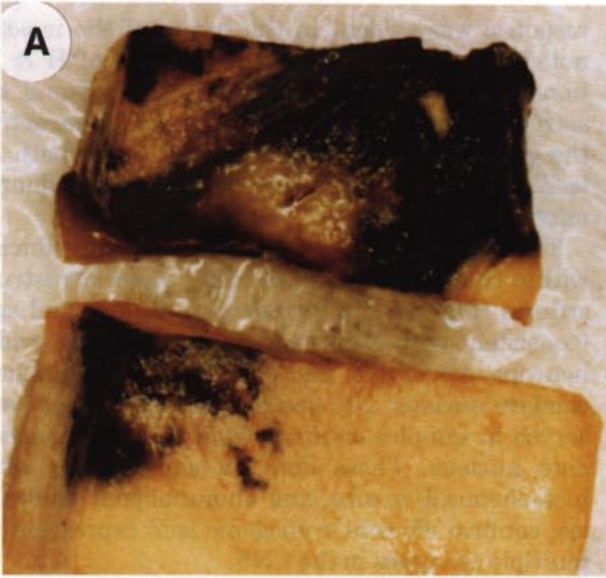
Analysis of brain tissue for rat HPRT expression. Tissue samples obtained from the caudate nuclei were analyzed by *in situ* gel assay for HPRT activity. The amount of [14 C]IMP produced was quantified using phased array phosphoimaging and was expressed as cpm/mg of total cellular protein. Samples obtained from the left caudate infected with Ad.RSVrHPRT consistently showed levels of [14 C]IMP 20–50% above baseline when compared to samples of caudate infected with Ad.RSVlacZ or uninfected control samples obtained from the ipsilateral or contralateral cortical regions (Table 1).

Determination of purine pools in brain tissue. Tissue sections of similar size (approximately 4 mm³) were obtained from the caudate nuclei infected with Ad.RSVlacZ or Ad.RSVrHPRT, and were immediately analyzed for total purine nucleotide content using anion exchange HPLC. The results were then normalized with respect to the levels of purine nucleotides present in the Ad.RSVlacZ-infected samples. The Ad.RSVrHPRT-infected samples exhibited a 64% increase in AMP levels and a more than fourfold increase in GMP levels compared to the Ad.RSVlacZ-infected control samples (Table 2).

Analysis of host immune response. Western blots of purified adenoviral proteins and rat neuroblastoma cell

FIG. 2. Histochemical stain for β -galactosidase activity in *Macaca mulatta*. One week poststereotactic injection of Ad.RSVlacZ into the caudate nucleus, the animal was anesthetized, the brain was perfused with 120 ml of 0.9% sodium chloride into each internal carotid, and blocks of caudate were removed and fixed in 2% paraformaldehyde for 8 h. Following a 2-h rinse in PBS, 2 mM MgCl₂, blocks were soaked in X-gal staining solution. A, *en fosse* photograph taken with a dissecting microscope showing extensive distribution of lacZ-expressing cells. B–D, blocks were further fixed in 2% paraformaldehyde for 48 h, cryoprotected in 20% sucrose, and sectioned. Sections were placed on poly-L-lysine coated slides and counterstained in neutral red. Light microscopic examination allowed identification of astrocytes (B), ependymal cells (C), and neurons (D), all expressing transgenic lacZ (336 \times).

FIG. 4. Immunohistochemical staining of brains infected with Ad.RSVlacZ for phenotypic identification of β -galactosidase expressing cells. A representative GFAP-positive astrocyte is also stained positive for β -galactosidase. Control sections stained only with secondary antibodies exhibited no fluorescence (data not shown). Sections (10 μ m) were stained with monoclonal antibodies to GFAP (secondary antibody conjugated to FITC) and *E. coli* β -galactosidase (secondary antibody conjugated to Texas red) and analyzed using fluorescent microscopy. A representative field photographed following excitation of FITC (A) and Texas red (B) allowed identification of a GFAP-positive astrocyte expressing transgenic lacZ (336 \times).



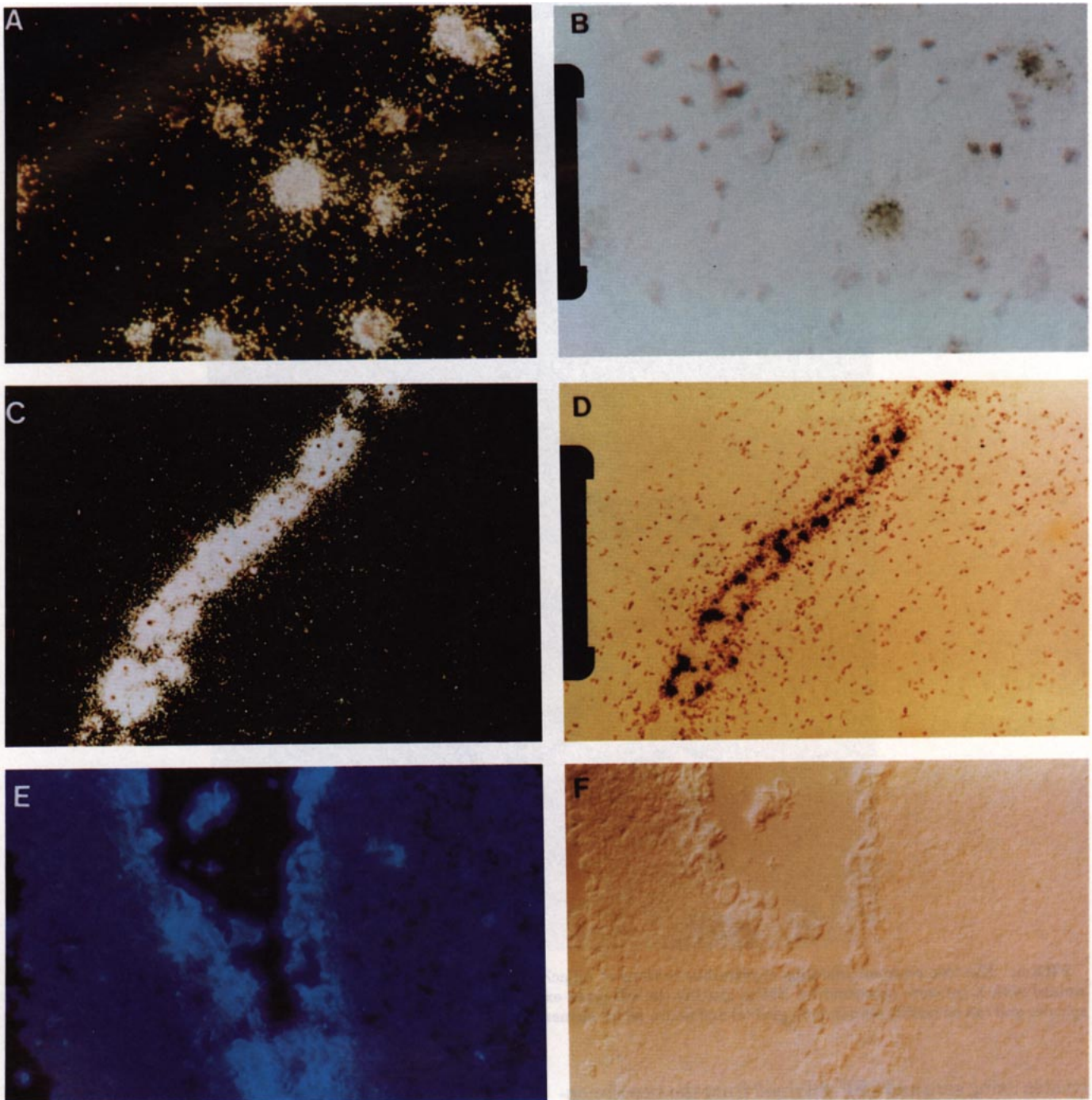


FIG. 3. *In situ* RNA staining for lacZ following Ad.RSVlacZ-mediated gene transfer to the caudate nucleus of rhesus monkey. *Escherichia coli* lacZ was cloned into pSP72 and radiolabeled antisense message was made by transcription with T7 polymerase in the presence of ^{35}S -CTP and ^{35}S -UTP. Radiolabeled probes were then hybridized to 10 μm fresh-frozen sections of caudate nucleus from both hemispheres isolated from rhesus 1 week postinjection of Ad.RSVlacZ. Following hybridization, sections were treated with RNase A and dipped for autoradiography. After 4 days the slides were developed. A (dark field) and B (lightfield) are photomicrographs of a section taken from the caudate nucleus injected with Ad.RSVlacZ. Note positive staining throughout the parenchyma. C (dark field) and D (light) are photomicrographs of a section isolated from the caudate nucleus of the contralateral hemisphere (100 \times). Note the periventricular staining (positive ependymal cells) and lack of deep parenchymal staining. The specificity of this signal was confirmed using immunohistochemistry. Staining was done using a monoclonal antibody to *E. coli* β -galactosidase conjugated to biotin, followed by incubation with AMCA-avidin. The fluorescence photomicrograph (E) demonstrates a similar staining pattern to that seen in C. F is a light photomicrograph of the same field as that in E using Nomarski optics (100 \times).



FIG. 5. Electron photomicrographs of ultrathin sections of AdlacZ-infected caudate nucleus of *M. mulatta*. Sections of tissue previously stained with X-gal were processed for EM to identify the cell types expressing transgene. The X-gal precipitate appears as dark granules or spicules and can be readily seen in both glial (a) and neuronal (n) elements (4180 \times).

lysates using sera and CSF obtained from the experimental animal showed that the animal was able to mount a humoral immune response to intracerebral challenge with adenoviral vectors. The formation of cross-reactive antibodies directed against adenoviral structural proteins occurred within 1 week of infection and was present in both the sera and CSF. Preimmune titers of cross reactive antibodies were present in preimmune sera and were specifically directed against polypeptide IIIa (vertex region protein) or polypeptide IV (fiber). No preimmune antibodies were detected in the preimmune

CSF. One week postinjection, antibodies to 72K and increased titers to vertex region and fiber proteins were noted. No antibodies to rat HPRT or β -galactosidase were identified in the postimmune sera or CSF of the infected animal (Fig. 6).

DISCUSSION

The expression of the purine salvage enzyme HPRT in normal and deficient human brain has been defined on both a neuroanatomical and a functional level (13, 14,

TABLE 1

HPRT Activity Present in the Caudate Nuclei of *Macaca mulatta* Infected with lacZ or HPRT Adenovirus Vectors^a

Sample (infected with)	Counts/mg protein × 10 ⁻⁵ (% lacZ)
AdrHPRT	2.93 (139%)
AdlacZ	2.11 (100%)

^aTissue sections from the left (Ad.RSVrHPRT infected) and right (Ad.RSVlacZ infected) caudate nucleus and liver were prepared as described under Materials and Methods and counts determined and expressed as %HPRT activity in lacZ-infected hemisphere.

18). Postmortem examination of LNS patients has shown significantly decreased indices of dopaminergic function within the caudate nucleus, putamen, pallidum, and nucleus accumbens (14). [¹⁸F]Fluorodeoxyglucose positron emission tomography studies of brains from LNS patients indicate that functional abnormalities can also be defined using neuroanatomical criteria (T. D. Palella, unpublished observations). This information is consistent with the hypothesis that the neurological manifestations of the LNS are in part mediated via alterations in the function of dopaminergic neurons within anatomically defined regions of the human brain. This suggests that regional, transgenic expression of HPRT activity may have therapeutic effects by ameliorating or reducing the severity of neurological manifestations associated with the LNS. Currently, no treatment exists to correct this aspect of complete HPRT deficiency. In this report we have demonstrated that recombinant adenovirus can mediate the short-term expression of the transgenic HPRT to the caudate nucleus of the rhesus monkey. The high efficiency of regional gene transfer achieved by using recombinant adenoviral vectors represents a potential method for the augmentation of HPRT expression in the CNS of LNS patients.

Whether global correction of LNS can result from regional administration remains to be determined. *In vitro* evidence obtained from several models of HPRT deficiency indicates that metabolic cooperation can occur between neuronal and glial elements of the CNS (20,

TABLE 2

Comparison of Nucleotide Pool Levels in Tissue from the Caudate Nucleus of *M. mulatta* Infected with lacZ or HPRT Adenovirus Vectors

Transgene	Nmol/tissue section	
	AMP (% lacZ)	GMP (% lacZ)
LacZ	7.5 (100)	0.39 (100)
HPRT	12.3 (164)	1.78 (444)

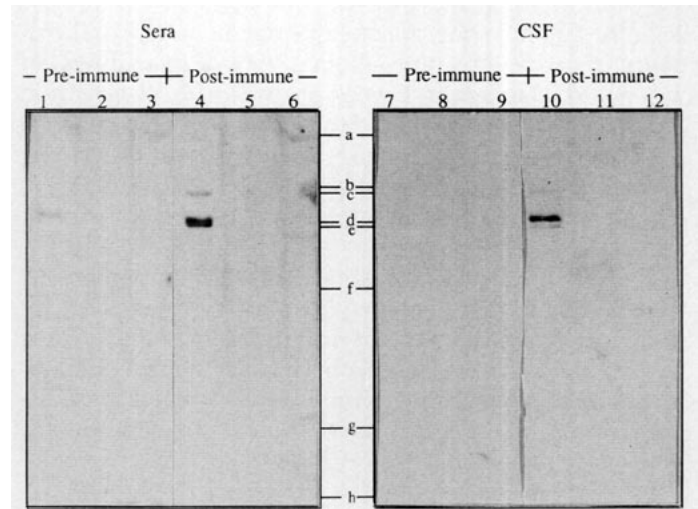


FIG. 6. Analysis of pre- and postinfection sera and CSF for immune response to transgenic proteins and adenoviral structural and associated proteins. Sera (left) and CSF (right) were used in a Western blot assay to determine if titers against adenoviral, adenoviral-associated, and transgenic proteins developed as a result of intraparenchymal administration of high titer recombinant adenovirus. Purified adenoviral proteins (lanes 1, 4, 7, and 10), purified *E. coli* β -galactosidase (lanes 2, 5, 8, and 11), and 200 μ g of total cellular protein from a rat neuroblastoma cell line (source of rat HPRT; lanes 3, 6, 9, and 12) were fractionated by 10% PAGE, transferred to PVDF membranes, and probed with sera or CSF as indicated above the panels. Secondary antibody hybridizations and visualization are described under Materials and Methods. A preexisting titer to the vertex region protein (polypeptide IIIa) is evident in sera. One week postinfection titers against 72K nonstructural and fiber proteins (polypeptide III and IV) are also present in both the sera and the CSF. The lowercase letters located between the panels correspond to the migration pattern of adenoviral structural and associated proteins and their corresponding size (MW in kDa): a, hexon or polypeptide II (100); b, penton base or polypeptide III (85); c, 72K nonstructural or 72K (72); d, vertex region or polypeptide IIIa (66); e, fiber or polypeptide IV (62); f, core (180) or polypeptide V (48); g, hexon-associated or polypeptide VI (24); h, core (1070) or polypeptide VII (18.5). Note the absence of cross-reactive antibodies 1 week postinjection to either *E. coli* β -galactosidase (subunit MW 120) or rat HPRT (subunit MW 24).

22). Additionally heterozygote female carriers of LNS are asymptomatic, suggesting metabolic cooperation between normal and deficient brain cells that characterize the mosaicism of the carrier state (22). Finally, axonal transport can result in the exchange of metabolic products throughout functionally related but neuroanatomically distinct brain regions. Thus, regional expression of transgenic HPRT via Ad.RSVrHPRT may result in significant metabolic cooperation leading to the correction of purine pools toward normal levels in areas physically removed from the area of injection.

Prior to the application of recombinant adenoviral vectors for brain-directed gene therapy in humans, issues related to safety and efficacy need to be determined. This study suggests that recombinant adenoviral vectors can be used to mediate efficient transgene expression in both neurons and glia of specific brain

regions. In this short-term experiment, no clinical evidence of meningitis, encephalitis, or systemic infection was noted. During the 1-week postinfection observation period the animal continued to feed, groom, and perform complex physical activities, all indicative of grossly normal motor and cognitive function. At the time of sacrifice, no gross evidence of cerebral necrosis or inflammation was present in the caudate nuclei or adjacent brain regions, although histological examination of sections stained with hematoxylin/eosin showed focal inflammation at the site of injection that diminished significantly with distance from the injection site. Experiments in progress using animals that received the same dose of Ad.RSVrHPRT have shown no evidence for neurologic deficits in these long-term animals.

In addition to the potential for acute toxicity secondary to the administration of highly concentrated virus, we were specifically interested in the humoral immune response to the intracerebral administration of recombinant adenoviral vectors. Within 1 week of intracerebral injection the animal was able to produce antibodies against several adenoviral structural proteins both in sera and cerebral spinal fluid. These included the 72K, fiber, and vertex region proteins. Antibodies against fiber or vertex region proteins were also identified in preimmune sera but not preimmune CSF. No antibodies against rat HPRT or β -galactosidase could be detected in sera or CSF over the period of 1 week. The presence of antibodies in CSF after 1 week may have been a result of local production of Ig or perhaps local alteration of the blood-brain barrier and transfer of serum Ig to the CSF.

The presence of antibodies directed against the 72K protein in preimmune sera and the time course of postinfection antibody production in both the sera and CSF suggest that this was an amnestic humoral response. This is consistent with the known ubiquitous distribution of adenoviruses throughout the animal kingdom and the frequent infection of laboratory animals with species specific and/or xenogeneic (human) adenoviruses. Because humans are subject at an early age to multiple infections with adenoviruses via the respiratory system, the use of rhesus monkeys with preexisting cross-reactive immunity to human adenoviruses will allow us to model the dynamics of the host immune response associated with the use of recombinant adenoviral vectors for gene transfer in humans.

In summary, this report suggests that recombinant adenoviral vectors are effective in mediating expression of transgenic proteins in the brains of primates. Additional studies to more specifically examine the temporal aspects of transgene expression and the host immune response to the intracerebral administration of recombinant adenoviral vectors are ongoing.

ACKNOWLEDGMENTS

The authors thank James M. Wilson for many helpful discussions and Jeanne-Marie Quevedo for expert secretarial assistance. B.L.D. is

a fellow of the National Arthritis Foundation. This work was supported in part by NIH R01 DK47968 and the University of Michigan Multipurpose Arthritis Center.

REFERENCES

- AKLI, S., C. CAILLAUD, E. VIGNE, L. D. STRATFORD-PERRICAUDET, M. PERRICAUDET, AND M. R. PESCHANKSKI. 1993. Transfer of a foreign gene into the brain using adenovirus vectors. *Nature Genet.* **3**: 224-228.
- BAJOCCHI, G., S. H. FELDMAN, R. G. CRYSTAL, AND A. MASTRANGELI. 1993. Direct *in vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. *Nature Genet.* **3**: 229-234.
- BREAKEFIELD, X. O., AND N. A. DELUCA. 1991. Herpes simplex virus for gene delivery to neurons. *New Biol.* **3**: 203-218.
- DAVIDSON, B. L., E. D. ALLEN, K. F. KOZARSKY, J. M. WILSON, AND B. J. ROESSLER. 1993. A model system for *in vivo* gene transfer into the CNS using an adenoviral vector. *Nature Genet.* **3**: 219-223.
- DAVIDSON, B. L., M. PASHMFORUSH, W. N. KELLEY, AND T. D. PALELLA. 1989. Human hypoxanthine-guanine phosphoribosyltransferase deficiency: The molecular defect in a patient with gout (HPRT^{Ashville}). *J. Biol. Chem.* **264**: 520-525.
- DAVIDSON, B. L., S. A. TARLE, T. D. PALELLA, AND W. N. KELLEY. 1989. Molecular basis of hypoxanthine-guanine phosphoribosyltransferase deficiency in ten subjects determined by direct sequencing of amplified transcripts. *J. Clin. Invest.* **84**: 342-346.
- DAVIDSON, B. L., S. A. TARLE, M. VAN ANTWERP, D. A. GIBBS, R. W. WATTS, W. N. KELLEY, AND T. D. PALELLA. 1991. Identification of 17 independent mutations responsible for human hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency. *Am. J. Hum. Genet.* **48**: 951-958.
- FEDEROFF, H. J., M. D. GESCHWIND, A. I. GELLER, AND J. A. KESSLER. 1992. Expression of nerve growth factor *in vivo* from a defective herpes simplex virus 1 vector prevents effects of axotomy on sympathetic ganglia. *Proc. Natl. Acad. Sci. USA* **89**: 1636-1640.
- GELLER, A. I., AND X. O. BREAKEFIELD. 1988. A defective HSV-1 vector expresses *Escherichia coli* β -galactosidase in cultured peripheral neurons. *Science* **241**: 1667-1669.
- GRAHAM, F. L., AND A. J. VAN DER EB. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**: 456-467.
- KUEHN, M. R., A. BRADLEY, E. J. ROBERTSON, AND M. J. EVANS. 1987. A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* **326**: 295-298.
- LE GAL LA SALLE, G., J. J. ROBERT, S. BERRARD, V. RIDOUX, L. D. STRATFORD-PERRICAUDET, M. PERRICAUDET, AND J. MALLET. 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* **259**: 988-990.
- LESCH, M., AND W. L. NYHAN. 1964. A familial disorder of uric acid metabolism and central nervous system function. *Am. J. Med.* **36**: 561-570.
- LLOYD, K. G., O. HORNYKIEWICZ, L. DAVIDSON, K. SHANNAK, I. FARLEY, M. GOLDSTEIN, M. SHIBUYA, W. N. KELLEY, AND I. H. FOX. 1981. Biochemical evidence of dysfunction of brain neurotransmitters in the Lesch-Nyhan syndrome. *N. Engl. J. Med.* **305**: 1106-1111.
- PALELLA, T. D., Y. HIDAKA, L. J. SILVERMAN, M. LEVINE, J. GLORIOSO, AND W. N. KELLEY. 1989. Expression of human HPRT mRNA in brains of mice infected with a recombinant herpes simplex virus-1 vector. *Gene* **80**: 137-144.

16. PALELLA, T. D., L. J. SILVERMAN, C. T. SCHROLL, F. L. HOMA, M. LEVINE, AND W. N. KELLEY. 1988. Herpes simplex virus-mediated human hypoxanthine-guanine phosphoribosyltransferase gene transfer into neuronal cells. *Mol. Cell. Biol.* **8**: 457-460.
17. ROSSITER, B. J. F., A. EDWARDS, AND C. T. CASKEY. 1991. HPRT mutation and the Lesch-Nyhan syndrome. In *Molecular Genetic Approaches to Neuropsychiatric Disease* (J. Brosius and R. Freneau, Eds.), pp. 97-124. Academic Press, San Diego.
18. SEEGMILLER, J. E., F. M. ROSENBLOOM, AND W. N. KELLEY. 1967. Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. *Science* **155**: 1682-1684.
19. SIMMONS, D. M., J. L. ARRIZA, AND L. W. SWANSON. 1989. A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. *J. Histotechnol.* **12**: 169-181.
20. WOLFE, J. H., S. L. DESHMANE, AND N. W. FRASER. 1992. Herpes virus vector gene transfer and expression of β -glucuronidase in the central nervous system of MPS VII mice. *Nature Genet.* **1**: 379-384.