Reporter Expression Persists 1 Year After Adeno-Associated Virus-Mediated Gene Transfer to the Optic Nerve

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Objective: To determine the foci and duration of protein expression following virus-mediated gene transfer to the optic nerve.

Methods: A cytomegalovirus (CMV) promoter was linked to a lacZ-SV40 polyA reporter gene or a humanized green fluorescent protein (hgfp) reporter gene, then inserted into a bacterial plasmid containing adeno-associated virus (AAV) terminal repeat sequences. The CMV-lacZ or the CMV-hgfp construct were injected into the vitreous cavity of strain-13 guinea pigs. Controls consisted of eyes injected with AAV without the promoter and reporter elements or eyes that received no injections. The eyes and optic nerves were processed for β-galactosidase immunohistochemistry and hgfp fluorescence analyses. Cellular transduction at the messenger RNA (mRNA) level was evaluated by in situ reverse transcription–polymerase chain reaction.

Results: Weekly fundus photography, done for 1 month, documented the absence of any ocular abnormality due to the viral injections. No in vivo hgfp fluorescence of the retina was visualized. β-Galactosidase histochemical analysis of eye cups that received the lacZ gene construct showed blue lacZ staining of the optic nerve head at 2 weeks. Light microscopy revealed the blue β-galactosidase reaction product in fibers, glial cells, and blood vessels of the optic nerve head and retrobulbar nerve. Histochemistry showed absence of β-galactosidase in the optic nerve at 3 to 12 months, but immunohistochemistry showed the persistence of β-galactosidase in fibers, glial cells, and blood vessels as late as 1 year after a single ocular injection. In the retina, histochemical staining showed evidence of lacZ at 3 months, but not later. In situ reverse transcription–polymerase chain reaction revealed brown lacZ mRNA reaction product in ganglion cells of the retina. Control eyes that received AAV without the promoter and reporter elements and the eyes that received no viral injections and were processed for β-galactosidase showed no reporter gene expression in any ocular tissue or cell type.

Conclusions: Viral-mediated gene transfer can be successfully accomplished in the optic nerve. Further evaluation is needed to determine whether the level of protein expression at 1 year after injection, which is clearly reduced relative to shorter postinjection time, is sufficient for therapeutic purposes.

Clinical Relevance: We have previously shown that gene therapy with catalase suppressed experimental optic neuritis at 1 month after injection. Viral-mediated gene transfer may be a powerful technique for the treatment of optic neuropathies, particularly for recurrences of optic neuritis, if long-term expression of transduced protein can be demonstrated in the optic nerve.


The cellular structure of the optic nerve consists of myelin-forming oligodendroglia, astrocytes, microglia, endothelia, and axons. Oligodendroglia are the cell type most vulnerable to immune-mediated injury in experimental allergic encephalomyelitis, an animal model of multiple sclerosis. However, demyelinated optic nerve fibers are also affected. They exhibit hydroptic degeneration with dissolution of microtubules and neurofilaments. Even endothelial cells that appear ultrastructurally intact have lost their crucial function of maintenance of the blood-brain barrier. Consequently, introduction of a protective gene(s) to treat experimental allergic encephalomyelitis must be able to protect axons, glial cells, and endothelial cells against structural and functional injuries.

The levels of antioxidant enzymes and free radical scavengers in the optic nerve and central nervous system are inadequate to protect these tissues against reactive oxygen species–induced injury in experimental allergic encephalomyelitis and perhaps in other optic neuropathies as well. Therefore, candidate gene(s) for transfer may include reactive oxygen spe-
MATERIALS AND METHODS

PROMOTER AND GENE CONSTRUCTION

The immediate early cytomegalovirus (CMV) promoter was linked to (1) a lacZ-SV40 polyA reporter gene or (2) the reporter Aquaria victoria green fluorescent protein (gfp) complementary DNA (cDNA) using standard protocols. The final constructs contained either the Escherichia coli β-galactosidase gene or a synthetic humanized (hgfp) gene. Each reporter was driven by the CMV immediate early promoter flanked at both sides by wild-type AAV terminal repeats.

rAAV CONSTRUCTION

The test plasmids pTRCMV-lacZ or pTRCMV-hgfp were packaged into rAAV by transfection into 293 cells (human embryonic kidney cells) that were cotransfected with a replication-defective AAV packaging plasmid (pIM45) containing the wild-type AAV genome without the terminal repeats. Cultures were then infected with a temperature-sensitive helper virus—adenovirus ts149 for lacZ or wild-type adenovirus 5 for hgfp at a multiplicity of infection of 10. After 60 hours of incubation, rAAV was harvested by freeze-thawing 3 times. Contaminating helper adenovirus was heat inactivated for 30 minutes at 56°C. The cellular debris was centrifuged and rAAV was purified through a cesium chloride gradient formed in an SW41 rotor for 48 hours at 200,000 g. Fractions with densities of between 1.38 and 1.4 g/cm³ were pooled and dialyzed against Dulbecco modified Eagles medium12,15,23 The final constructs contained either the immediate early cytomegalovirus (CMV) promoter and reporter elements (n = 2). The eyes of 4 animals were processed for immunostaining for lacZ at the light microscope and ultrathin (90 nm) LR-white–embedded sections of the optic nerves of the 6-month and 1-year postinjection animals were processed for immunostaining for lacZ at the light microscope.

INJECTION OF rAAV PROMOTER AND GENE CONSTRUCTS

Strain-13 guinea pigs were sedated with 0.2 mL of a 1:1 mixture of ketamine hydrochloride (100 mg/mL) and xylazine (20 mg/mL) by intramuscular injection. The pupils were dilated with 2.5% phenylephrine and 0.5% tropicamide followed by a topical anesthetic (proparacaine hydrochloride) administered to the cornea. Paracentesis of the anterior chamber with a 23-gauge needle was done to lower the intraocular pressure. A 30-gauge needle attached to a syringe was inserted through the pars plana and positioned over the optic nerve head under visualization with a 28-diopter lens and the indirect ophthalmoscope, then approximately 2 x 10³ to 4 x 10³ infectious particles of the viral-encapsulated cDNA gene constructs of (1) pTRCMV-lacZ (19 animals), (2) pTRCMV-hgfp (5 animals), or (3) AAV without the promoter and reporter elements (2 animals) were injected into the vitreous of 1 eye of each animal. All experimental procedures conformed to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

FUNDUS PHOTOGRAPHY

To test for potential ocular disease due to the viral injections or for ocular infections, we performed in vivo fundus photographs of the retina and optic nerve head of guinea pig eyes with a Zeiss fundus camera. Prior to photography, the pupils were dilated with 2.5% phenylephrine and 0.5% tropicamide. For in vivo visualization of hgfp-induced fluorescence (emission maximum at 509 nm), additional fundus photographs were taken with a blue excitation light and a barrier filter. The contralateral eye was also photographed to serve as a negative control for fluorescence. All fundus photographs were repeated at weekly intervals for 1 month following the intravitreal injections.

HISTOCHEMISTRY

For evaluation of transgene expression, the animals were overdosed with pentobarbital as follows: (1) at 1 week (n = 1), 2 weeks (n = 8), 4 weeks (n = 5), 5 weeks (n = 1), 3 months (n = 2), 6 months (n = 1), and 1 year (n = 5) for the reporter gene lacZ; (2) at 4 weeks for the reporter gene hgfp (n = 5); or (3) at 2 weeks for the AAV without the promoter and reporter elements (n = 2). The eyes of 4 animals that received no viral injections were also used. After deep surgical anesthesia was obtained with the pentobarbital overdose, the chest cavity was opened and the animals were perfused by intracardiac injection with 4% paraformaldehyde (London Resin Co, Ltd, Basingstoke, Hampshire, England) after dehydration in a graded series of 7.5%, 15%, and 30% sucrose buffers in 0.1-mol/L PBS (pH 7.4). The eye cups were immersion fixed in 4% paraformaldehyde and 0.1-mol/L PBS for 15 minutes and washed in 0.1-mol/L PBS (pH 7.4). The eye cups were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (1 mg/mL) plus 5-mmol/L potassium ferricyanide, 5-mmol/L potassium ferrocyanide, and 2-mmol/L magnesium chloride in a gently shaking water bath at 35°C overnight.12-14 The reaction was terminated by washes in 0.1-mol/L PBS, then the eye cups were cryoprotected in a graded series of 7.5%, 15%, and 30% sucrose buffers in 0.1-mol/L PBS (pH 7.4). The optic nerves were trephined from the eye cups, then the specimens were embedded in OCT medium and snap frozen in liquid nitrogen or embedded in LR-white resin (London Resin Co, Ltd, Basingstoke, Hampshire, England) after dehydration in a graded series of ethanol buffers. Cryosections 10-µm to 30-µm thick were mounted on gelatin-subbed glass slides for immunostaining and “silanized” glass slides for in situ reverse transcription–polymerase chain reaction (RT-PCR). Semithin (0.5 µm) and ultrathin (90 nm) LR-white–embedded sections of the optic nerves of the 6-month and 1-year postinjection animals were processed for immunostaining for lacZ at the light and ultrastructural levels. Photographs of specimens were taken with a Zeiss Axioscop microscope. The specimens were then semi-thin sectioned, stained with toluidine blue, and photographed with a Zeiss Axioscop microscope for electron microscopy.

Enzymes Scavengers

Superoxide dismutase dismutates superoxide to hydrogen peroxide (H₂O₂) and catalase detoxifies the H₂O₂ to H₂O and O₂. We have previously shown that exogenous administration of catalase scavenged H₂O₂ and reduced blood-brain barrier disruption and demyelination in animals with experimental allergic encephalomyelitis.5-6 However, optic neuritis recurred in these conditions.
made with bright-field and differential interference contrast optics with a microscope (Axioskop; Carl Zeiss, Inc, Thornwood, NY). Ultrastructural examination of selected tissue was made with a transmission electron microscope (model H 7000; Hitachi, Inc, Tokyo, Japan). FlgG fluorescent images were collected on a scanning laser confocal microscope (BioRad Lab, Inc, Hercules, Calif). The BioRad A1-A2 cubes were used with argon laser excitation at 514 nm and emission collected at 520 to 560 nm.

**IMMUNOHISTOCHEMISTRY**

After quenching of endogenous peroxidase activity by incubation in 0.5% H2O2 for 30 minutes, specimens that had been reacted with X-Gal were washed in 0.1-mol/L PBS, then incubated in 5% normal goat serum for 30 minutes. Sections were incubated in rabbit polyclonal (1) anti-glial fibrillary acidic protein (GFAP) (Sigma-Aldrich Corporation, St Louis, Mo) to label astrocytes, (2) anti-carbonic anhydrase (CA) II (Sigma-Aldrich) to label oligodendroglial cells, or (3) anti-lacZ (5-Prime-3-Prime, Inc, Boulder, Colo) overnight at 4°C. After washings, sections were incubated in goat anti-rabbit IgG peroxidase (for GFAP and CA II) or goat anti-rabbit IgG conjugated to alkaline phosphatase (for lacZ) overnight at 4°C. Sections were washed, developed in diaminobenzidine (DAB)-H2O2 substrate (for IMF) or 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (for alkaline phosphatase), and then washed in tap water. The above protocols were also used on control specimens with the exception that the primary antibody was omitted.

For ultrastructural analysis of lacZ-transduced cells, ultra-thin sections were placed on nickel grids, then washed in deionized water. Grids were floated on 0.01-mol/L PBS, 0.1-mol/L sodium chloride, and 5% bovine serum albumin (pH 7.2), and then reacted with rabbit anti-lacZ antibody in the same buffer for 2 hours at room temperature. After washes in 0.01-mol/L Tris-hydrochloride–buffered saline, the grids were reacted with 5-nm gold-labeled goat anti-rabbit IgG antibody that had been reacted with X-Gal, as described above, were washed in 0.1-mol/L PBS, then the horseradish peroxidase mixture and the edges were glued with rubber cement until dry. Then the slides were turned at 94°C for 15 seconds, 60°C for 15 seconds, 72°C for 60 seconds, for 20 cycles. Reagents for PCR were obtained from the GeneAmp PCR Core Kit (Perkin-Elmer, Norwalk, Conn). After completion of PCR, the coverslips were removed and the slides were washed in 0.1 × standard saline citrate at 45°C for 20 minutes, followed by a wash in 0.1% bovine serum albumin at 20°C for 15 minutes.

For immunodetection of digoxigenin–deoxyuridine triphosphate, an unconjugated monoclonal anti-digoxigenin antibody raised in the mouse (Boehringer Mannheim) was diluted 1:500. The diluted antibody (100 µL) was applied to the slides that were then incubated overnight at 0°C. After incubation, the slides were washed in PBS, then a rabbit anti-mouse antibody conjugated to horseradish peroxidase (100 µL) (Sigma-Aldrich) was applied to the slides that were then incubated overnight at 0°C. After incubation, the slides were washed in PBS, then the horseradish peroxidase was developed in DAB-H2O2 substrate (100 µL) for 10 to 20 minutes. The slides were then washed in tap water.

We included 2 controls that (1) omitted the RT step to detect nonspecific binding to nuclear or mitochondrial DNA and (2) omitted the primers in the PCR mix to detect nonspecific staining that was unrelated to cDNA amplification.

**RT-PCR ANALYSIS**

Cryostat sections (stored at −70°C) of the eye cups and optic nerves that had previously been reacted with X-Gal, as described above, were washed in 0.1-mol/L PBS. Endogenous peroxidase activity was quenched by incubation in 0.5% H2O2 for 30 minutes. For detection of lacZ mRNA, the specimens were digested in 100 µL of proteinase K (10 µg/mL) (Sigma-Aldrich) at 37°C for 15 minutes. Heating cycles were performed in a thermal cycler (PTC-100-12MS; MJ Research, Woburn, Mass). To prevent evaporation during heating in the thermal cycler, the slides were covered with a 22 × 22-mm piece of paraffilm. The digestion was stopped by immersing the slides containing the tissue sections in 0.1-mol/L glycine-PBS buffer for 5 minutes, then they were washed in PBS for 15 minutes.

For reverse transcription (RT), the slides were incubated in 70 µL of the RT reaction mixture (65-µL deionized water + 5-µL oligo[dT]) at 70°C for 10 minutes, then they were incubated in 30 µL of the RT reaction mixture (10 × RT buffer [10 µL] + 10-mmol/L deoxyribonucleoside triphosphates [dNTPs] [5 µL] + 1.0-mol/L dithiothreitol [10 µL] + reverse transcriptase [5 µL]) at 20°C for 10 minutes, then at 42°C for 30 minutes. The RT was inactivated by heating to 70°C for 15 minutes and the slides were cooled to 20°C for 10 minutes. All reagents for RT were stored in the SuperScript Premi-Plification System (Life Technologies, Gaithersburg, Md). For PCR, the slides were incubated in 70 µL of the RT reaction mixture consisting of 10 × PCR buffer (10 µL), 1.0-mmol/L of each of the 4 dNTPs (deoxyadenosine triphosphate, deoxyctydine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate) (8 µL), 10-µmol/L digoxigenin–deoxyuridine triphosphate (Boehringer Mannheim, Indianapolis, Ind) (1 µL), 1-mmol/L forward primer 5’CTG GCG GTA ATA GCC AAG AGG3’ at nucleotide position 105 (1 µL), 1-mmol/L reverse primer 5’GGT GTA GAT GGG CGC ATC3’ at nucleotide position 303 (1 µL), 25-mmol/L magnesium chloride (10 µL), Taq DNA polymerase (0.5 µL), TaqStart antibody (Clontech, Palo Alto, Calif) (0.5 µL), and dh2O (68 µL). A glass coverslip was placed over the slide-PCR mixture and the edges were glued with rubber cement until dry. Then the slides were cycled at 94°C for 15 seconds, 60°C for 15 seconds, 72°C for 60 seconds, for 20 cycles. Reagents for PCR were obtained from the GeneAmp PCR Core Kit (Perkin-Elmer, Norwalk, Conn). After completion of PCR, the coverslips were removed and the slides were washed in 0.1 × standard saline citrate at 45°C for 20 minutes, followed by a wash in 0.1% bovine serum albumin at 20°C for 15 minutes.

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We included 2 controls that (1) omitted the RT step to detect nonspecific binding to nuclear or mitochondrial DNA and (2) omitted the primers in the PCR mix to detect nonspecific staining that was unrelated to cDNA amplification.

**RESULTS**

Animals due to the inability of catalase to cross the blood-brain barrier after integrity was restored by the catalase-mediated detoxification of H2O2. Transfer of genes encoding reactive oxygen species scavengers may help surmount this problem by increasing the cellular defenses against reactive oxygen species in the optic nerve.

Currently, gene delivery and expression have been demonstrated in many mammalian tissues, including...
retina, neural tissues, and endothelial cells, but only one report has described gene transfer to the optic nerve. In that report, only axons of the optic nerve and their cell bodies in the retina were labeled by the transferred reporter gene. However, in experimental allergic encephalomyelitis and multiple sclerosis, oligodendroglia and endothelia are the targets of mediators of inflammation; thus, for neuroprotection of the optic nerve, the genes that encode for defenses against reactive oxygen species must be transferred to these cell types. In this study we investigated the feasibility and duration of foreign gene expression in glial cells, blood vessels, and axons of the optic nerve with a single injection of recombinant adenovirus (rAAV) in strain-13 guinea pigs that are susceptible to induction of experimental allergic encephalomyelitis.

RESULTS

FUNDUS PHOTOGRAPHY

Weekly fundus photography, done for 1 month, documented the absence of any ocular abnormality due to the viral injections. No inflammatory response or toxic reaction was detected in the cornea, anterior chamber, crystalline lens, vitreous body, or retina. However, no in vivo hgp-induced fluorescence of the retina was visualized either.

OPTIC NERVE HISTOPATHOLOGY

All eyes injected with the CMV-lacZ constructs exhibited transduction of cells of the optic nerve at each of the postinjection time points. The optic nerve head and adjacent retrobulbar nerve were the tissues that were most heavily stained by the X-Gal. Two weeks after the CMV-lacZ injection, intense blue X-Gal staining of the optic nerve head was evident in the eye cups processed for β-galactosidase expression (Figure 1, A). β-Galactosidase tissue staining was absent in eye cups from control animals that received (1) no viral cDNA complexes, (2) AAV tissue staining was absent in eye cups from control animals that received no viral cDNA complexes, or (3) the CMV-hgp gene constructs that were also processed for lacZ histochemistry.

Light microscopic examination of the eyes that received the CMV-lacZ test virus and evaluated at the 2-week injection time point showed X-Gal heavily labeled the optic nerve head and adjacent retrobulbar optic nerve (Figure 1, C). However, no histochemical labeling of X-Gal was detected in the optic nerve at 3 months (Figure 1, D), 6 months, or 1 year. X-Gal labeling of nerve fiber bundles was best seen between foci heavily labeled by X-Gal and unlabeled foci of the retrobulbar nerve (Figure 1, E). In addition, blood vessels of the optic nerve (Figure 1, F) and glial cells (Figure 1, G and I) expressed blue β-galactosidase reaction product.

Glia cells that were immunolabeled for CA II (Figure 1, H) had similar morphologic features as those expressing lacZ (shown in the lower right of Figure 1, G), thus suggesting that these lacZ-positive cells were oligodendrocytes. Similarly, the morphologic characteristics of cells that were immunolabeled for GFAP (Figure 1, J) were similar to some of those expressing lacZ (Figure 1, G, at left, and I), suggesting that these lacZ-positive cells were astrocytes. While double labeling of lacZ and GFAP or CA II was difficult to visualize, the double labeling of GFAP and lacZ in an astrocyte may be appreciated somewhat in a cell (Figure 1, J, at left) that appeared blue-black rather than the blue seen for lacZ (Figure 1, I) and the brown seen with GFAP immunoperoxidase (Figure 1, J, at right).

Histochemical labeling for lacZ was negative in all optic nerves examined 3 months to 1 year after injection of the viral reporter gene constructs. However, immunohistochemical staining for lacZ detected β-galactosidase in all 5 of the optic nerves studied as long as 1 year after a single intraocular injection of the viral cDNA complexes, although the number of positive cells at this late time period was clearly reduced relative to that seen at 2 to 4 weeks after injection. At 1 year, lacZ-positive cells, immunolabeled dark blue by alkaline phosphatase, were seen in the perivascular space (Figure 1, K) and included glial cells (Figure 1, L) of the interstitial optic nerve. The 1-year optic nerve control specimens with the primary antibody omitted from the alkaline phosphatase immunostaining protocol showed no immunolabeling (Figure 1, M).

Since the number of positive cells was clearly reduced at 1 year, we used transmission electron microscopy coupled with immunogold to morphologically identify β-galactosidase—positive cells rather than use dual labeling at the light microscopic level, as done for the earlier time points. At this 1-year time point, transmission electron microscopy revealed heavy immunogold labeling for lacZ in oligodendrocytes (Figure 2, A), astrocytes (Figure 2, B), fibers (Figure 2, C), and endothelial cells (Figure 2, D) of the optic nerve head and adjacent retrobulbar optic nerve. Histopathologic analysis of the eyes of control animals that received no viral cDNA complexes, or AAV without the promoter and reporter gene elements, showed no β-galactosidase staining in any fibers or cell types of the optic nerve. Demyelination, reactive gliosis, and inflammatory cells were absent at any time point, including 1 year after injection, thereby suggesting the absence of previous immune-mediated tissue damage from the recombinant viral injections. Controls with the primary antibody omitted from the immunogold protocol had scant background particles in the optic nerve (Figure 2, E). Additional controls with the β-galactosidase antibody preabsorbed with the purified β-galactosidase protein showed no immunogold staining (Figure 2, F), thereby proving that immunogold labeling of the β-galactosidase in the optic nerve was due to the viral gene inoculation of lacZ.

RETINA HISTOPATHOLOGY

While X-Gal staining of the optic nerve was apparent in eye cups, X-Gal labeling of the retina was not visible in any of our animals by visual inspection of the eye cups with the naked eye or with the X10 magnification of the dissecting microscope as shown in Figure 1, A. For orientation purposes, the guinea pig retina was stained with toluidine blue (Figure 3, A). LacZ histochemical analysis of unstained retina from eyes that received the lacZ...
viral constructs demonstrated that most cells of the ganglion cell layer were positive for β-galactosidase at 2 weeks (Figure 3, B). Diffuse X-Gal labeling of the outer retina was also evident at this time point. Retinas injected with the CMV-hgf viral constructs that served as controls for the histochemical labeling of CMV-lacZ–injected eyes showed some very mild endogenous histochemical staining for β-galactosidase in the outer retina (Figure 3, C), supporting detection of endogenous outer nuclear layer activity. Confocal fluorescent microscopic examination for hgf revealed hgf fluorescence in cells of the ganglion cell layer (Figure 3, D) 4 weeks after injection of the hgf viral constructs. No signal in this cell layer was detected in eyes that received no viral cDNA complexes, the AAV without the promoter and reporter gene elements (Figure 3, E), or the lacZ gene construct. However, autofluorescence of the outer retina was seen in both hgf-injected and control eyes. At 3 months after injection, lacZ histochemical analysis revealed some positively labeled cells in the outer nuclear layer (Figure 3, F), while ganglion cell labeling was much weaker than earlier. At 6 months to 1 year after injection, the retina was negative for lacZ histochemical staining; however, lacZ immunogold staining was seen in ganglion cells of the retina at 1 year.

IN SITU RT-PCR

Indicative of transcription of the transferred gene, cellular mRNA expression of the reporter lacZ was readily detected in cells of the ganglion cell layer. Two weeks after intravitreal injections with the CMV-lacZ viral constructs, the brown mRNA-derived reaction product labeled cells of the ganglion cell layer (Figure 4, A). Co-labeling of blue lacZ protein and brown mRNA was seen in ganglion cells (Figure 4, B). In addition, cells that ap-
peared to contain only the brown mRNA reaction product were seen adjacent to cells that appeared to contain only the blue β-galactosidase reaction product in the ganglion cell layer (Figure 4, C). The controls, with omission of the RT (Figure 4, D) or PCR primers, showed only the blue lacZ staining.

Our results, and those of Cayouette and Gravel, 22 showing expression of lacZ in retinal ganglion cells and axons of the optic nerve, prove the feasibility of viral-mediated gene transfer to the optic nerve in 2 different

Figure 2. At 1 year after injection, transmission electron microscopy revealed immunogold labeling (electron-dense particles) for lacZ in an oligodendrocyte (Ol) (A) (unstained, ×3600) and in astrocytes (As) (B) (unstained, ×3500). C, Some axons (indicated by A) of the optic nerve were labeled by immunogold, while others were not (unstained, ×3000). D, A blood vessel of the perineural nerve was labeled by lacZ immunogold (Lum indicates lumen) (unstained, ×5000). E, A control optic nerve head, with the primary antibody omitted from the immunogold protocol, shows little background (unstained, ×4000). F, A control with the β-galactosidase antibody preabsorbed with the purified β-galactosidase protein shows no immunogold staining (unstained, ×4500).
mammalian species, thereby suggesting viral-mediated gene transfer may be useful for transfer of potentially therapeutic proteins to humans. The 2 different approaches used, injections into the eye vs the brain, suggest that the introduction of transduced proteins into the optic nerve presents somewhat of a challenge. Unlike glial cells, the cell bodies of axons that comprise the nerve do not reside within the nerve itself, but in the retina. Since axons of the optic nerve do not have the organelles necessary for protein synthesis, transcription, and translation of introduced DNA must occur in retinal ganglion cells. We used RT-PCR to show that ganglion cells were directly infected by the recombinant virus. While most cells exhibited both the brown mRNA reaction product and blue lacZ protein, thereby indicating transcription of the transgene and translation of the transgene protein, respectively, it was apparent that some cells expressed only the mRNA, thus indicating that they were recently transduced, but as yet not translating detectable amounts of the lacZ protein. More important, the brown mRNA reaction product within transduced ganglion cells makes it highly unlikely that substantial amounts of the blue β-galactosidase protein were transferred to ganglion cells from other cell types. Therefore, the blue labeling of the optic nerve head was most likely due to the orthograde axonal transport of β-galactosidase from retinal ganglion cells that also suggested most blue cells in the ganglion cell layer were retinal ganglion cells and not displaced amacrine cells. Our findings of reporter gene mRNA in ganglion cells with transport of the resultant protein to the optic nerve head and retrobulbar nerve—the foci most frequently affected by the demyelinating inflammation of both experimental allergic encephalomyelitis and multiple sclerosis, the disc edema of anterior ischemic optic neuropathy, and Leber hereditary optic neuropathy—suggest the feasibility of therapeutic gene transfer to the optic nerve.

Transduction of the cell type(s) affected by the disease process is a common prerequisite for potentially therapeutic gene transfers. For this reason we chose the CMV promoter since it is well known for its potential to drive gene expression in heterogeneous cell types. It supported cellular expression of β-galactosidase in axons, glia, and endothelia of the optic nerve. Adenoviral promoters have also been successfully used to drive expression in various cell types and tissues. While we chose a promoter for transgene expression in different cell types, promoters may be designed for cell-specific expression. Cell-specific promoters may have an advantage over the CMV promoter by inducing a higher efficiency of transduction in targeted cells. Several examples of cell-specific promoters include the opsin promoter to drive hgfp expression exclusively in photoreceptors of the retina with a high rate of efficiency. In comparison, a CMV promoter had a much lower rate of transduction efficiency in these same cells. Another example in a different neural tissue includes the use of neuronal promoters (neuron-specific enolase or platelet-derived growth factor) to drive expression of hgfp in spinal neurons, but not in astroglial cells. However, for now, a CMV promoter may be the best option for transduction of the heterogeneous optic nerve cell population that

![Image](http://archopht.jamanetwork.com/pdfaccess.ashx?url=/data/journals/ophth/9859/ on 06/16/2017)
is affected by the demyelinating inflammation of experimental allergic encephalomyelitis.

The choice of vector for gene delivery is of paramount importance, particularly if gene therapy is to be applied to the treatment of human optic neuropathies. For transduction of the mammalian optic nerve, the vector must be capable either of incorporating the designated cDNA into the host genome without the need for cell replication, as cell division is limited in the mammalian optic nerve, or of creating a stable episomal state. While AAV is capable of meeting both these requirements, adenovirus meets only the latter. Thus far they are the only 2 vectors that have been described for gene delivery to the optic nerve. The next requirement is that the vector must be nonpathogenic. The host inflammatory response generated by adenovirus in ocular tissues is particularly well recognized. Unlike adenovirus, AAV is nonpathogenic and it has not incited an ocular inflammatory response. This is an important consideration for future investigations designed to study the effects of gene therapy on an inflammatory model of optic nerve demyelination, namely, experimental allergic encephalomyelitis.

For therapeutic efficacy, cellular expression of transgene protein must persist for the duration of the disease process and perhaps longer to reduce recurrences that occur in optic neuritis and multiple sclerosis. Our results and those of Cayouette and Gravel suggest that either rAAV or adenovirus may be used to drive short-term gene transfer in the optic nerve that persists for 1 month. Cayouette and Gravel showed by immunostaining that β-galactosidase was present 1 month after injection, and whereas histochemical staining for β-galactosidase was negative at this time period, it was present earlier at 2 weeks after injection. Similarly, our results showing the detection of β-galactosidase at 1 year by immunostaining, while histochemistry results were negative, also illustrate that histochemical detection was not as sensitive as immunochemical detection. Our control experiments with the β-galactosidase antibody preabsorbed with the β-galactosidase protein confirm that the immunogold labeling detected 1 year after injection was specific for β-galactosidase, thereby proving continued transduction of the transferred lacZ gene even though transcription of mRNA was not confirmed by RT-PCR at this later time point. On the other hand, long-term studies with adenoviral vectors have shown that lacZ was undetectable 2 months after injection into skeletal muscle, but may persist longer in the eyes of animals with defective immune systems. In contrast, long-term expression of rAAV-transferred genes has been demonstrated in various tissues such as brain (3-4 months) and muscle (40 weeks to 1½ years). In these studies, protein expression began to diminish after approximately 3 months. Similarly, we found transduction in the optic nerve with rAAV persisted 1 year after a single injection of the viral cDNA complexes. The persistent expression of the transgene means that the gene is still being transcribed and protein product is still being translated from the resulting transcript. Our RT-PCR results showing the absence of the brown mRNA reaction product in some of the cells that contained only the blue lacZ reaction product suggest that the transgene gene in these cells was somehow turned off, and is no longer producing mRNA. This

![Figure 4. A. In situ reverse transcription–polymerase chain reaction showed the brown messenger RNA (mRNA)–derived reaction product in a cell (arrowhead) in the ganglion cell layer (original magnification ×200). B, Colabeling of blue lacZ protein and brown mRNA is shown in a ganglion cell (arrowhead) (original magnification ×400). C, A cell with the brown mRNA reaction product (arrowhead) is seen adjacent to a cell with the blue β-galactosidase reaction product in the ganglion cell layer (original magnification ×400). D, A control with omission of the reverse transcription showed only the blue lacZ staining (original magnification ×400).](http://archoph.tamanetwork.com/pdfaccess.ashx?url=/data/journals/ophth/9859/ on 06/16/2017)
finding at the earlier time point helps to explain the reductions in the levels of tissue expression of lacZ seen 1 year after injection, as β-galactosidase has a short half-life and would not be expected to persist for long periods in cells that are no longer transcribing lacZ mRNA. We have previously shown that gene therapy with catalase suppressed experimental optic neuritis at 1 month after injection. Whether the level of expression at 1 year—clearly reduced relative to shorter postinjection time—is sufficient for therapeutic purposes remains to be evaluated.

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REFERENCES


