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Adenovirus-mediated gene transfer to murine retinal cells in vitro and in vivo

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Abstract

Adenovirus-mediated gene transfer to retinal cells was evaluated using the replication-defective recombinant adenovirus vector Ad2/CMV*lacZ*-1 (coding for β-galactosidase) both in an in vitro murine culture model and in vivo in adult mice. In vitro, no difference in infectability of neuronal and glial cells was observed, and 50% of neurons expressed the exogenous gene at low viral concentration (10 pfu/cell). In vivo, intraocular injection of 3×10^6 pfu Ad2/CMV*lacZ*-1 resulted in expression of the transferred β-galactosidase gene in retinal pigment epithelium and ganglion cells. These results demonstrate that Ad2/CMV*lacZ*-1 is an effective vector for gene transfer into retinal cells.

Key words: Defective recombinant adenovirus; β-Galactosidase; Retina; Gene transfer; Retinal cell culture

1. Introduction

Inherited retinal degenerations comprise a genetically heterogeneous group of diseases responsible for a substantial proportion of human blindness. Numerous causal gene defects for some of these dystrophies have been described (for reviews see [1,2]), and in the majority of cases the mutations reside in genes expressed primarily in the retinal photoreceptor neurons. While investigations to unravel the underlying pathophysiology of these insidious diseases continue, techniques to transfer genes to postmitotic cells such as neurons, with the objective of correcting genetic defects, 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], and replication-defective adenoviruses (for review see [6]). Herpes simplex type 1-derived vectors have the attraction of being naturally neurotropic, but cytotoxicity and uncertainty of long-term safety of these viruses remain important problems [7–9]. Replication-defective recombinant adenovirus vectors have emerged as favourable vehicles for the transfer of exogenous genes owing to their efficient ability to infect a variety of terminally-differentiated cells, including neurons. Further, the low pathogenicity and high efficiency of adenoviral gene delivery into the central nervous system suggest that these vectors may have a general applicability towards the therapeutic correction of inherited retinal diseases. We have therefore investigated transfer of the *E. coli lacZ* gene into murine retinal cells in vitro and in vivo, using the replication-deficient recombinant type 2 adenovirus vector Ad2/CMV*lacZ*-1 [10].

2. Experimental

2.1. Cell culture

Retinas from 1–5 days postnatal C3H mice were dissociated by trypsinization [11]. The dissociated cells were suspended in culture medium and seeded on tissue culture chamber slides at $0.5–1 \times 10^4$ cells/ml and maintained as described [11].

2.2. In vitro adenovirus gene delivery

Adenoviral stocks were prepared, purified and titred as described previously [12]. Ad2/CMV*lacZ*-1 is a replication-deficient recombinant adenovirus deleted in the E1 region and retaining the E3 region [10]. Mouse retinal cell cultures ($1–2 \times 10^5$ cells per well) were infected with virus suspension at different titres and incubated at 35°C. After infection, cultures were maintained for 24–96 h at 35°C, fixed in 4% paraformaldehyde, and assayed in situ for β-galactosidase (β-gal) activity using the chromogenic β-gal substrate, X-gal [13].

2.3. Immunohistochemistry

Polyclonal anti-neurofilament (NF) and anti-glial fibrillary acidic protein (GFAP) primary antibodies (Sigma) were used to discriminate, respectively, neurons and glial cells. Paraformaldehyde-fixed retinal cell cultures, following X-gal staining, were processed using the classical peroxidase-anti-peroxidase technique [14].

2.4. In vivo adenovirus gene delivery

BALB/c mice, 6–8 weeks old, were anaesthetized and Ad2/CMV*lacZ*-1 viral suspension (3×10^6 pfu (plaque-forming units)) or 0.9% saline was injected intraocularly vicinal to the retina. After 24 h the mice were killed, their eyes enucleated and fixed in 4% paraformaldehyde, and stained with X-gal as described above. Frozen sections (10 μm) were then processed for immunohistochemistry.

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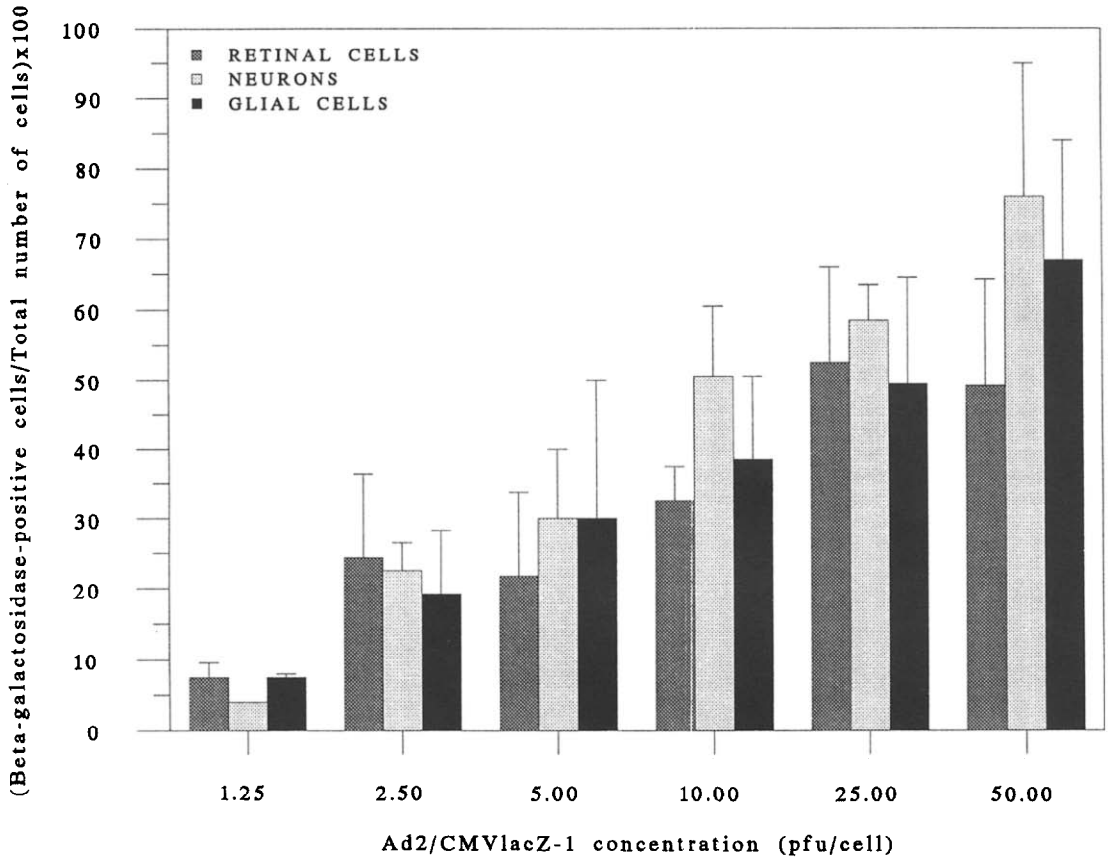

Fig. 1. Effect of Ad2/CMV*lacZ*-1 concentration on transferred β-galactosidase gene expression in retinal cell culture. Retinal cells were infected with different concentrations of Ad2/CMV*lacZ*-1, and β-gal activity was assessed after 24 h by scoring the percentage of blue-stained cells. Neurons and glial cells are defined as those giving positive immunoreactivity with antibodies to NF and GFAP respectively. Values are the mean ± S.D. from three independent experiments.

3. Results and discussion

The culture of dissociated murine retinas provides a model system to assess the effectiveness of adenoviral infection of specific cell types in the absence of in vivo three-dimensional structural impediments. The expression of *lacZ* reporter gene, coding for β-gal, permits the detection of successfully infected cells. No X-gal staining was detectable in non-infected cultures.

3.1. Quantification of reporter gene transfer in vitro

Murine retinal cell cultures consisted of approximately equal populations of neuronal and glial cells as determined by immunoreactivity with antibodies to the corresponding marker proteins. Cultures infected with Ad2/CMV*lacZ*-1 at multiplicities from 1.25 to 50 pfu/cell yielded detectable expression of the reporter gene in ~10 to 65% of the total cells at 24 h post-infection (Fig. 1). Over the same range of moi (multiplicity of infection), the percentage of β-gal-positive neurons increased from ~5 to 95%. At 10 pfu/cell, 50% of neurons were β-gal positive, an efficiency 2.5 times greater than that reported with an adenovirus type 5 vector used to infect neuronal cultures from rat brain [15]. This difference may relate to the particular vector promoters used (CMV vs. RSV LTR) and most probably also to the cell culture origin and preparation. Infectability of retinal cells was tested as a function of time in culture at 1.25 pfu/cell Ad2/CMV*lacZ*-1 (Fig. 2). Expression of β-gal reached a plateau at 48 h post-infection with ~30% of cells positive in agreement with data previously reported on neuronal brain cell cultures [15]. Analysis of the long-term expression of the reporter gene in vitro is in progress. Throughout this experimental series, the percentage of β-gal-positive neurons and glial cells was similar, suggesting that the infectivity of Ad2/CMV*lacZ*-1 was independent of retinal cell type. This contrasts with a previous report in which the infectivity of an adenovirus type 5 vector was four times lower for glial cells than for neurons in brain cell cultures [15]. This divergence could similarly arise from differences in the culture origin and preparation or in the promoter driving the *lacZ* gene.

3.2. Characterization of infected cells in vitro

Since cultures of cells dissociated from early postnatal mouse retinas lack the in vivo three-dimensional struc-

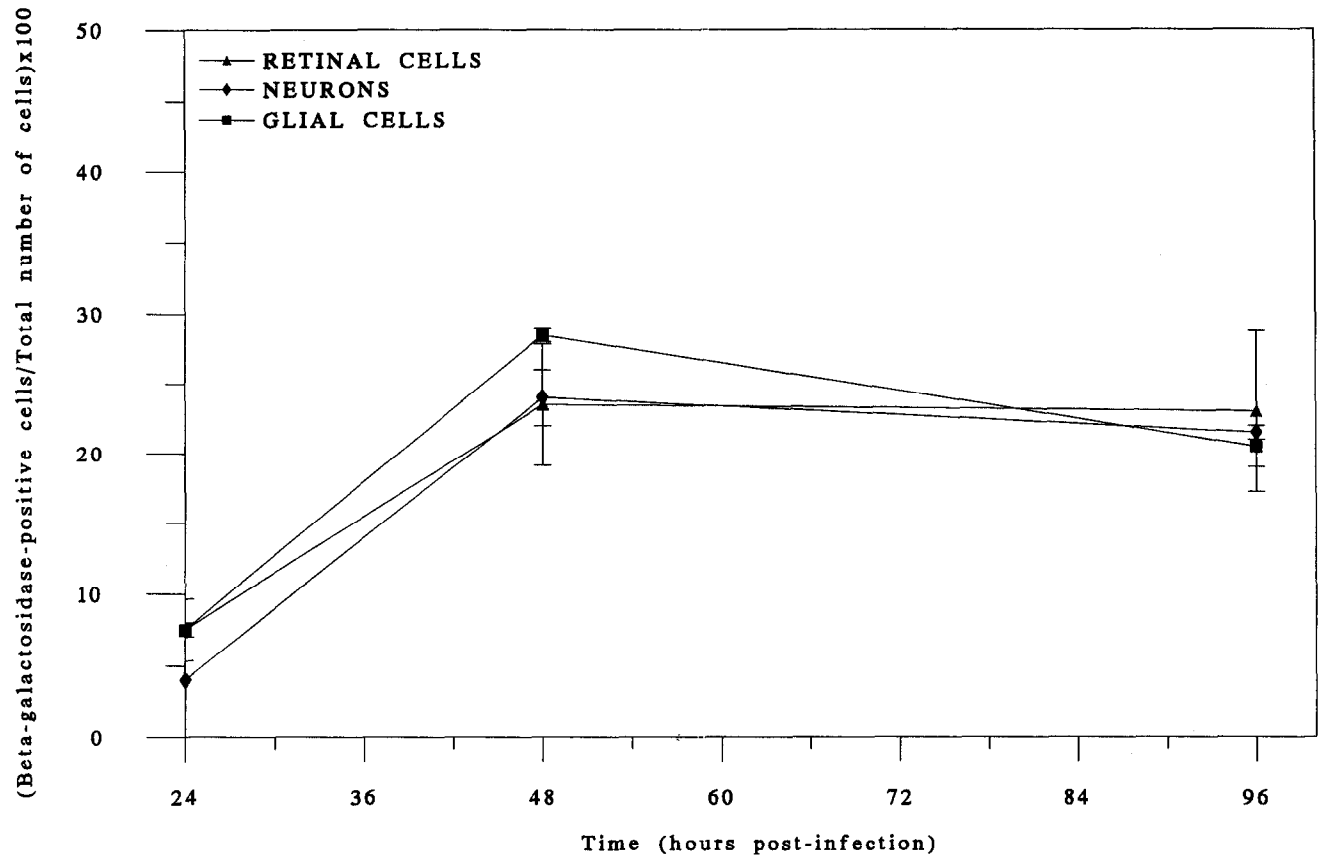

Fig. 2. Time course of transferred β-galactosidase expression in mouse retinal cell culture. Retinal cells were infected with 1.25 moi Ad2/CMV*lacZ*-1, and β-gal activity was evaluated at different times after infection by scoring the percentage of blue-stained cells. Neurons and glial cells are defined as those giving positive immunoreactivity with antibodies to NF and GFAP respectively. Values are the mean ± S.D. from three independent experiments.

ture, the identification of specific cell types becomes essential. We characterized the infected cell types by using specific antibodies which distinguish between neurons and glial cells (respectively anti-NF and anti-GFAP), in conjunction with histochemical detection of β-gal expression. Light microscopic observations confirmed that the β-gal expression, resulting in a blue staining, was most intensely concentrated in the retinal cell nuclei. The expression of the *lacZ* gene is targeted to the nucleus due to the presence of the SV40 nuclear localization signal. Typically, expression is detectable in the perinuclear region, presumably at the nuclear pores. While some of the material may actually cross the nuclear membrane, the majority of the protein appears to remain outside. Loss of association with the nuclear membrane during fixation and staining leads to the appearance of 'leakage' into the cytoplasm (Fig. 3C). Based on an assessment of total cell numbers of infected and control cultures, no apparent cytotoxic effects due to virus infection or to expression of the transferred β-gal gene were evident. Infected retinal cells also showed no morphological changes compared with controls. The underlying monolayer of flat cells, on which the neuronal cells adhere, was labelled by anti-GFAP antibody (Fig. 3A,C,E). The filamentous nature of the antigen could be clearly discerned in the infected glial cells (Fig. 3C,E), but distinction between Müller cells and astrocytes, the major classes of retinal glial cells, remains to be established. GFAP-negative flattened cells (Fig. 3C), β-gal positive, were possibly endothelial or pigment epithelial cells or fibroblasts, as suggested in previous characterizations of retinal cultures [16,17]. Anti-NF antibody labelled cells with clearly differing morphologies. The class of cells seen most frequently consisted of small spherical cells associated in clusters and found aligned along the processes of other cells (Fig. 3B,D). A proportion of these cells could correspond to rod photoreceptors, lacking morphologically identifiable outer segments, as demonstrated by Akagawa and Barnstable in rat retinal cell culture [16]. A second class of NF-positive cells seen in these cultures had a typical neuronal morphology (Fig. 3B,F). These generally bipolar or tripolar cells produced long processes which ramified and formed a complex meshwork. However, their morphology alone did not allow subclassification into bipolar, amacrine and ganglion cells and further analysis using specific antibodies

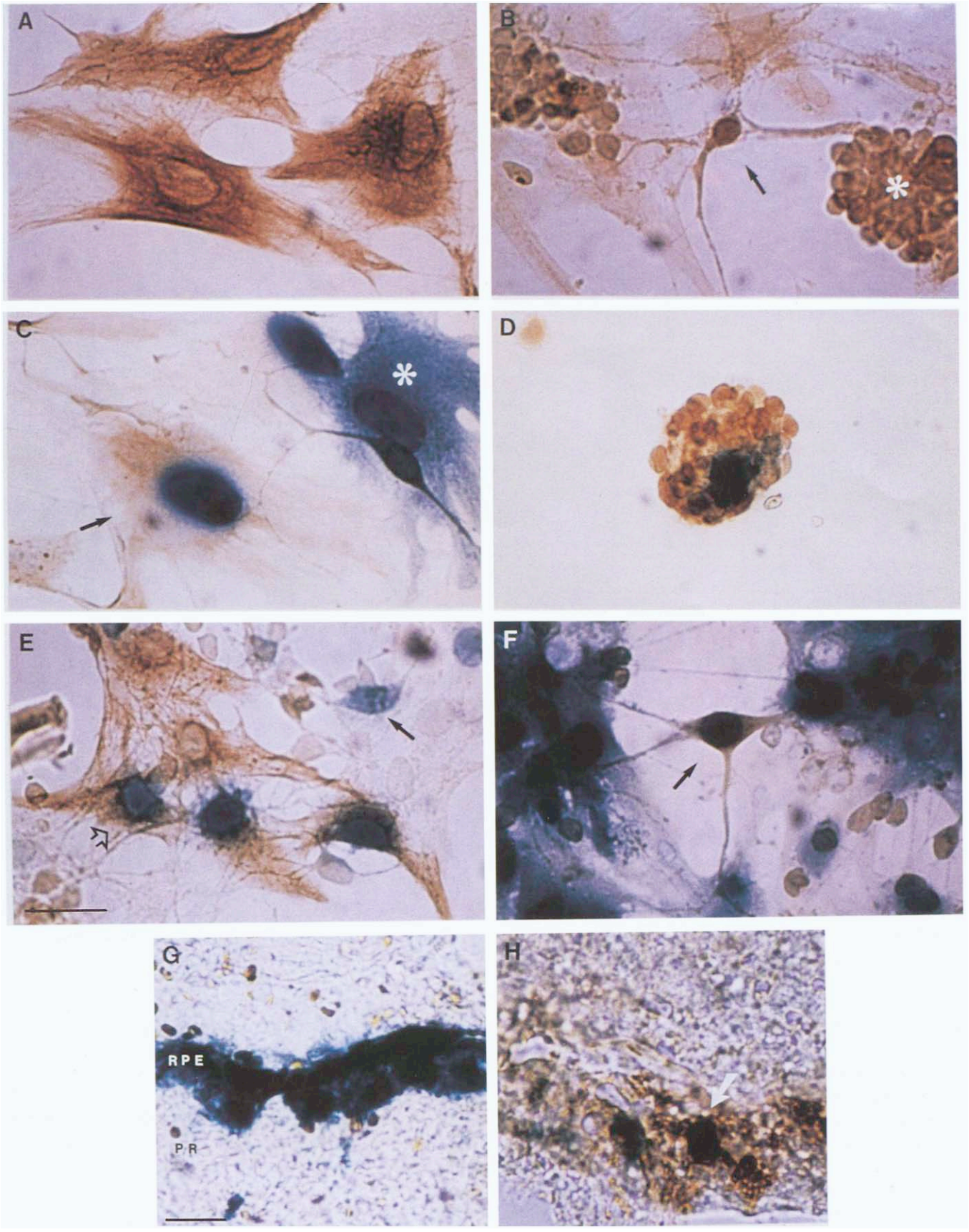
A
B
C
D
E
F
G
RPE
PR
H

Fig. 3. Transferred β-galactosidase expression in vitro and in vivo. Mouse retinal cell cultures were infected with Ad2/CMV*lacZ*-1 at 2.5 moi (C-F; uninfected controls: A, B). Cultures stained immunohistochemically after incubation with anti-GFAP (A,C,E) and anti-NF (B,D,F) antibodies. Glial cells labelled with anti-GFAP, showed the filamentous nature of the antigen (A,C,E). Immunoreactive glial cells displayed a nuclear X-gal staining (C: arrow; E: open arrow) and diffusion in the cytoplasm (C: asterisk). Clusters of small neuronal cells (B and D) attached occasionally (B asterisk) to cellular processes. Bipolar (E: filled arrow) and multipolar cells (B and F: arrows) with long processes and occasionally with a pyramidal morphology (F: arrow) were observed. Retinal frozen sections (10 μm) from mice (n = 3; 24 h post-intraocular injection with Ad2/CMV*lacZ*-1 at 3×10^6 pfu) were stained for β-gal and then labelled with anti-NF antibody (G,H). Retinal pigment epithelium (RPE) cells, adjacent to photoreceptors (PR), were heavily stained for β-gal (G). Ganglion cells were double labelled with X-gal blue staining and anti-NF antibody (H: arrow). A–H: bar = 50 μm.
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for each category [17–20] will be needed. Occasionally, multipolar cells with more pyramidal morphology were observed (Fig. 3F) and could represent axonless horizontal cells [16].

3.3. *Adenovirus gene transfer in vivo*

Pilot experiments were carried out to evaluate the ability of Ad2/CMV*lacZ*-1 vector to infect retinal cells in vivo in adult BALB/c mice. No β-gal activity was detectable in the retinas of control-injected animals (data not shown). By 24 h post-intraocular injection of adenovirus, a patchy distribution of β-gal-expressing cells was detected in frozen retinal sections. Retinal pigment epithelium was densely stained (Fig. 3G). Relatively large blue-stained cells in the ganglion cell layer also stained positively with anti-NF antibody, indicating that ganglion cells were infected with the adenovirus (Fig. 3H). In control sections lacking primary antibody, no non-specific immunoreactivity was observed (data not shown). While the efficiency of infection of retinal cells appears to be lower in vivo, the conditions are not directly comparable with those of the in vitro state. In particular, age-related changes in infectability of ocular cells must be considered, since the cultured cells were derived from early postnatal animals. Additional factors may contribute to the difference, including reduced accessibility of the virus to the different retinal cell layers, possibly partly due to the presence of interphotoreceptor matrix (IPM), and clearance of the injected virus by the ocular blood circulation. Increasing the viral titre in the injection may compensate at least partially for these factors; enzymatic perturbation of the IPM could also increase efficiency of in vivo adenovirus-mediated gene transfer [21]. Extended expression of transferred genes is likely to be a requirement for rescue of neuronal cells, including photoreceptors, affected by progressive degenerative disease. Characterization of long-term expression of the reporter gene in vivo is currently in progress, and the introduction of endogenous cell-specific promoter and enhancer elements may extend transgene expression. Repeated treatment at regular intervals may also serve to extend expression. In this regard, Ad2 vectors have been shown to be safe for repetitive administration to intrapulmonary airway epithelia in primates and cotton rats [22], and a comparable assessment in the eye will be warranted.

In summary, these results demonstrate that Ad2/CMV*lacZ*-1 is an effective vector to achieve in vitro transfer and expression of the *lacZ* gene in a wide range, and possibly all types, of retinal neurons and glial cells. In vivo results suggest that adenovirus type 2 vectors are likely to be powerful tools for transduction of gene expression and may ultimately be suitable for correcting inherited or acquired retinal diseases.

While this paper was in preparation, we became aware of reports from other groups indicating that adenoviruses could be used for gene transfer to ocular tissues in vivo [21,23,24].

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