An Ocular Model of Adenovirus Type 5 Infection in the NZ Rabbit

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Ocular adenoviral infections occur worldwide and are associated with significant patient morbidity, including symptomatic distress with visual disturbances that can last for years. The economic and social impact of community epidemics is enormous. Not only is valuable time lost from work and school, but the spread of these infections within households and at the workplace is disruptive to normal family and commercial life. Furthermore, there is significant medical-legal liability to doctors and medical facilities that serve as inadvertent centers of transmission. Currently, there is no effective topical or systemic antiviral therapy to reduce the symptoms or duration of the disease, reduce viral shedding, or prevent vision-threatening subepithelial infiltrates (topical steroids only delay their appearance). In addition, no treatment will protect the second eye or prevent transmission within households, medical offices, and communities. Progress has been hampered by the absence of an animal model to evaluate new antivirals and to study ocular pathogenesis.

The goal of the current study was to develop a credible animal model of ocular adenoviral infection. After several years, we determined empirically that a clinical isolate of adenovirus serotype 5 (Ad5 McEwen) was able to infect the New Zealand (NZ) rabbit eye, replicate to high titers, and produce disease that mimics, in part, human disease.

This model is suitable for testing promising antivirals and initiating studies in pathogenesis.

Materials and Methods

Virus Strain

A clinical adenoviral isolate was cultured from a patient with typical adenoviral keratoconjunctivitis seen at the Eye & Ear Institute of Pittsburgh. The isolate was typed by immunofiltration and serum neutralization and was found to be type 5. The isolate, designated Ad5 McEwen, was grown in A549 monolayers at 37°C, 5% carbon dioxide-water vapor atmosphere, and titrated using a plaque reduction assay. The stock suspension was then frozen and stored in 1 ml aliquots at −70°C prior to use.

A549 cells were grown and maintained in Eagle’s minimum essential medium with Earle’s salts (Sigma
Cell Culture Reagents; St. Louis, MO), supplemented with 6% heat inactivated fetal bovine serum (Hazelton Biologies, Inc., Denver, PA), 2.5 µg/ml amphotericin B, 100 U penicillin G, and 0.1 mg streptomycin/ml (Sigma Cell Culture Reagents).

Animals

Six-week-old, 1 kg female New Zealand albino rabbits were obtained from Green Meadow Rabbitry, Murrysville, PA. All animal studies conformed to the ARVO Resolution on the Use of Animals in Research.

Inoculation Procedure

Four identical independent experiments were performed in the current study, involving 32 NZ rabbits. For each experiment, eight rabbits were anesthetized with intramuscular ketamine (33 mg/kg) and xylazine (9 mg/kg) and topical 0.1% proparacaine eye drops OU. Using a masked, paired-eye design with coded inocula, one eye was inoculated (total volume 100 µl) with 4 × 10⁵ plaque forming units (pfu) of Ad5 McEwen, while the fellow control eye was inoculated with control media. With the operating microscope, the central corneal was inoculated intrastromally with a 32 needle to form five focal blebs (dice pattern, total volume 50 µl). The cornea then was scarified superficially (eight scratches) with a 25 needle to form a square around the central intrastromal injection. Inoculation was completed by topically applying an additional 50 µl of the same coded inoculum the eye had already received as an intrastromal injection (either Ad5 McEwen or control media). The lids were closed, and the eye was massaged through the lids for 30 sec.

Experimental Protocol

Following 3 hr of viral adsorption, the residual viral inoculum was washed off with 30 ml balanced salt solution. Four hours later, the first swab (day 0) was taken for viral titration. Each eye was swabbed in the upper and lower fornices with a cotton applicator, and the swab was placed in 1 ml outgrowth media and frozen at −70°C pending titration. Daily ocular swabbing OU was carried out for 14 days. Ocular viral titers were determined by the plaque reduction assay.

Clinical disease was evaluated daily in a masked fashion and scored (scale 0 to 4) following flashlight and slit-lamp examination for acute conjunctivitis (conjunctival erythema, vascular injection, and increased mucus production) for 10 d. Delayed-onset presumed immunological disease was evaluated from day 10 through day 54. Blepharoconjunctivitis (lid crusting and conjunctivitis), iritis (engorged vessels with color change from blue to deep purple), corneal stromal edema (cloudiness), and subepithelial immune infiltrates (number of focal, discrete small white dots) also were graded in a masked fashion following flashlight and slit-lamp examination.

Determination of Viral Titers

The virus suspension to be titered was thawed to room temperature and diluted serially (1:10) for seven dilutions. Each dilution was inoculated onto A549 monolayers (0.1 ml per well) in 10 replicate wells of a 96 well plate. The virus was adsorbed for 3 hr at 37°C in a 5% carbon dioxide-water vapor atmosphere. Following adsorption, 0.1 ml of A549 media was added to each well, and the plate was incubated at 37°C in a 5% carbon dioxide-water vapor atmosphere. The wells were examined for progressive cytopathic effect and stained with 0.5% gentian violet on day 7. The number of plaques per well were counted under a dissecting microscope (25 times). The titer of the virus suspension was calculated and expressed as plaque-forming units per milliliter (pfu/ml).

Neutralization Assay

Serological confirmation of adenoviral infection also was demonstrated by standard neutralization antibody studies of acute and convalescent sera on serial blood samples (days 0, 14, 28 after inoculation). Briefly, a 0.1 ml sample of 10-fold serial dilutions of rabbit sera was added to a well of a 96 well plate. 0.1 ml of 100 TCID50 of Ad5 McEwen was added to each well and shaken at room temperature for 1 hr. Positive and negative control wells were included. 0.15 ml of the neutralized sample from each well was then applied to A549 cells, incubated for 8 d, and stained with 0.5% gentian violet. The extent of cytopathic effect was examined microscopically and scored for each well. The change in serum antibody titer per animal was determined by comparing the results on days 0, 14, and 28 after inoculation.

Statistical Analysis

When each experiment was completed, the code was broken and the data were evaluated by the paired t-test and chi square analysis.

Results

The current study reports the results of our efforts to develop an animal model of adenoviral ocular infection using Ad5 McEwen in the NZ rabbit. The general time course of a typical experiment is summarized in Figure 1. With a paired-eye design, eight rabbits were randomly inoculated in one eye with Ad5
**Fig. 1.** Natural history of ocular adenoviral infection in New Zealand rabbit. Ad5 McEwen-infected eyes, solid lines; media-inoculated control eyes, dashed lines.

McEwen, while the fellow eye received control media. Each point represents the mean score of eight eyes. During acute productive infection, viral replication began on day 1 after inoculation, increased 100 fold to peak by day 3, and then continued through day 9. Viral replication was associated with acute conjunctivitis. While a small sample (n = 8) failed to distinguish between conjunctivitis due to viral replication from that due to the inoculation trauma, a larger sample (n = 32) clearly demonstrated significant differences (P < .02) between virus-infected eyes and media-control eyes (Table 1). On day 9 after the cessation of detectable replicating virus, presumed delayed-onset immunogenic disease began, as demonstrated by the sudden onset of blepharoconjunctivitis, iritis, and corneal stromal edema. These signs peaked on day 13 and gradually resolved by day 19. On day 17 after inoculation, fine, focal subepithelial infiltrates first appeared and persisted up to 8 weeks before resolving.

In preliminary experiments, following topical inoculation (10^3 pfu/eye) with different Ad5 serotypes (ATCC, Rucker, McEwen), ocular replication peaked on day 2 (3.6 x 10^4 pfu/ml) and persisted for 15 days. However, delayed-onset presumed immune-mediated disease could not be demonstrated in the absence of intrastromal injection. We believe that an immune response to intrastromal viral antigen is the most likely cause of blepharoconjunctivitis, iritis, stromal edema, and subepithelial infiltrates in the current model. However, we cannot rule out the possibility that some residual viral replication is occurring in the stroma and cannot be detected by surface swabbing.

Table 1 provides specific data that demonstrates reproducible results in four identical, independent experiments using the optimized protocol with Ad5 McEwen in the NZ rabbit ocular model. During acute productive infection, viral replication achieved high titers (5.64 x 10^3 pfu/ml) in 100% of virus-infected eyes (32/32) compared to 0% replication in media-inoculated control eyes (0/32, P < .0001). In virus-infected eyes, replication continued for 8.3 ± 1.5 days (range 6–12 days). While acute conjunctivitis was significantly more prevalent in the virus-infected eyes (24/32, 75%) than in fellow control eyes (14/32, 44%; P < .02), the mean conjunctivitis score was not able to distinguish between conjunctivitis due to viral replication from that due to the trauma of the inoculation procedure (1.28 vs. 0.91, P = .10).

Delayed-onset presumed immunogenic disease was demonstrated in 100% of virus-infected eyes (32/32) and was significantly greater (P < .0001) than in media-inoculated fellow control eyes for all four parameters measured: blepharoconjunctivitis (mean score 1.48 vs. 0.14, P < .0001; 21/32 eyes vs 1/32 eyes, P < .0001), iritis (mean score 1.42 vs. 0.03, P < .0001; 29/32 eyes vs 2/32 eyes, P < .0001), corneal edema (mean score 2.66 vs. 0, P < .0001; 32/32 eyes vs 0/32 eyes, P < .0001), and subepithelial infiltrates (mean score 3.14 vs. 0.08, P < .0001; 30/32 eyes vs 2/32 eyes, P < .0001).

Subepithelial infiltrates demonstrated in the rabbit cornea (Fig. 2A) closely mimic the clinical appearance of immune-mediated precipitates characteristic of epidemic keratoconjunctivitis in humans. Histopathology in the rabbit cornea (Fig. 2B) confirms the presence of inflammatory cells localized beneath the epithelial layer.

Serological confirmation of adenoviral infection also was demonstrated by increasing neutralization antibody titers over time. Overall, 26 of 31 rabbits (84%) showed specific antiadenoviral antibodies in their sera on day 28 following inoculation. In a typical experiment (experiment no. 1), eight of eight rabbits (100%) had at least a four-fold increase in antibody titers on day 28 following inoculation. The median neutralization titer of eight rabbits on days 14 and 28

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**Table 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Virus-infected (n=32)</th>
<th>Media-control (n=32)</th>
<th>P-value</th>
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<tr>
<td>Conjunctivitis</td>
<td>24/32 (75%)</td>
<td>14/32 (44%)</td>
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<td>Mean Conjunctivitis Score</td>
<td>1.28</td>
<td>0.91</td>
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<td>Blepharoconjunctivitis</td>
<td>21/32 (65%)</td>
<td>1/32 (3%)</td>
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<tr>
<td>Mean Blepharoconjunctivitis Score</td>
<td>1.48</td>
<td>0.14</td>
<td>&lt; .0001</td>
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<tr>
<td>Iritis</td>
<td>29/32 (90%)</td>
<td>2/32 (6%)</td>
<td>&lt; .0001</td>
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<tr>
<td>Mean Iritis Score</td>
<td>1.42</td>
<td>0.03</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Corneal Edema</td>
<td>32/32 (100%)</td>
<td>0/32 (0%)</td>
<td>&lt; .0001</td>
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<tr>
<td>Mean Corneal Edema Score</td>
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<td>0</td>
<td>&lt; .0001</td>
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<tr>
<td>Subepithelial Infiltrates</td>
<td>30/32 (94%)</td>
<td>2/32 (6%)</td>
<td>&lt; .0001</td>
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<tr>
<td>Mean Subepithelial Infiltrates Score</td>
<td>3.14</td>
<td>0.08</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Expt.</td>
<td>Eyes (n)</td>
<td>Peak viral titer (pfu/ml) (day 3)</td>
<td>+ Eyes total (day 3)</td>
</tr>
<tr>
<td>-------</td>
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<td>Expt. 1</td>
<td>Ad eyes 8</td>
<td>8.48 ± 12.0 × 10^3</td>
<td>8/8</td>
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<td>Expt. 2</td>
<td>Ad eyes 8</td>
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<td>8/8</td>
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<td>P 0.0001</td>
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<td>0.0001</td>
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<td>Expt. 3</td>
<td>Ad eyes 8</td>
<td>5.70 ± 6.67 × 10^3</td>
<td>8/8</td>
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<td>P 0.0001</td>
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<tr>
<td>Expt. 4</td>
<td>Ad eyes 8</td>
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<td>Summary</td>
<td>Ad eyes 32</td>
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Discussion

In the current study, we provide initial data on the development of an ocular model of adenoviral infection in the NZ rabbit. Historically, the failure to develop a successful animal model of adenoviral infection may be due to the inherent characteristics of Adenoviridae (47 serotypes): species-specificity,\(^3\) organ-specificity,\(^4\) and rarity of host range mutants.\(^5\) Review of the literature demonstrated productive virus infections (permissive) with human adenoviruses in cells from other species: cotton rat,\(^6\) Sprague-Dawley rat,\(^7\) rabbit,\(^4\) African green monkey,\(^8\) Balb/c mouse,\(^8\) and C37B1/6J mouse.\(^7\) Our earlier studies demonstrated that different serotypes of human adenovirus (Ad5, Ad8, and Ad19) could be repeatedly passaged in nonhuman cell lines (rabbit cornea, rabbit kidney, rat lung, and rat kidney; Y. J. Gordon, University of Pittsburgh, 1989, unpublished data). Abortive infections with Ad5 also have been demon-

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Fig. 2. (A) Slit-lamp photograph of NZ rabbit cornea infected with Ad5 McEwen. Arrows denote subepithelial infiltrates similar to those seen in human EKC. (B) Histologic section of subepithelial infiltrate in NZ rabbit cornea infected with Ad5 McEwen (hematoxylin and eosin, original magnification ×250). Note focal deposit of inflammatory cells beneath the epithelium.

following inoculation was 1:24 with a range of 1:2 to 1:256.
strated in the mouse liver, and different serotypes of adenovirus have been recovered from asymptomatic animals after prolonged periods—Ad5 in rabbits and guinea pigs, and Ad 2, 3, 4, 7 in dogs. In addition, successful models of human adenoviral respiratory disease were developed in the cotton rat, hamster, and colostrum-deprived piglets.

Very early attempts to develop an ocular model of human adenoviral infection in different animals failed because of the lack of knowledge regarding the existence of different ocular adenoviral serotypes (Ad5, etc), the lack of microsurgical instrumentation for inoculation, and the failure or inability to document successful infection by slit-lamp evaluation and serial virus titers in human cell culture.

Successful demonstration of permissive viral infection of rabbit corneas in organ culture by adapted Ad serotypes (Ad8 and Ad19 serially passaged in rabbit iris cells) and a preliminary ocular model in immunosuppressed rabbits offered further encouragement that an ocular model was possible. We also have demonstrated that rabbit corneal organ culture will support productive replication of unadapted Ad5 and Ad8, but not Ad19.

Recently, preliminary studies in the cotton rat by Tsai et al demonstrated Ad8 replication associated with some eye disease. We demonstrated progressive ocular replication of Ad5 in the cotton rat, but failed to observe eye disease (Y. J. Gordon, University of Pittsburgh, 1989, unpublished data). Furthermore, the difficulties of breeding and handling these wild animals led us to favor the NZ rabbit as our laboratory animal.

Our current model of ocular adenoviral infection in the NZ rabbit using a nonadapted human serotype (Ad5 McEwen) has strengths and weaknesses. The strengths include the following. The NZ rabbit host animal is commercially available and has traditionally been the preferred lab animal in eye research because of the size of the eye and its similarity to the human eye. Also, progressive viral replication was achieved in 100% of eyes and the clinical time course was similar to human disease (14 days). Finally, delayed-onset subepithelial infiltrates that closely mimic human disease were observed clinically and characterized histologically. The subepithelial infiltrates observed in 2 of 32 control eyes were most likely the result of a low grade immune response to serum proteins in the control media. This result was significantly less (P < .0001) than the 100% response (32/32) observed in virus-inoculated eyes.

The weaknesses of the current model stem from the need for a combined inoculation (topical and intrastral) to achieve the desirable effects previously cited. The failure to isolate virus from the opposite uninfected eye is atypical of the clinical situation in which virus is usually transmitted to the fellow eye. The observed acute conjunctivitis may be due to viral disease or inoculation trauma, and the delayed-onset immunogenic iritis and corneal stromal edema is atypical of human disease. Finally, regarding the delayed-onset subepithelial infiltrates, because the control media used was prepared and inoculated directly, we cannot rule out the possible adventitious effects of A549 cell proteins as a confounding variable. However, increasing viral titers were demonstrated by positive culture in all virus-inoculated eyes, and this was the most likely source of antigen.

Despite these problems, the current model is suitable for determining the inhibitory effects of new antivirals on adenoviral replication in a living animal and for beginning pathogenesis studies. We are currently testing (S)-HPMPC—a broad spectrum antiviral effective against adenovirus in vitro—in our ocular model. This model also will be used to determine the mechanism of host range extension of Ad5 in the NZ rabbit. Using restriction enzyme analysis, we plan to determine whether Ad5 has a broader host range than previously suspected or whether selection of host range mutants occurred in the rabbit eye during replication.

In summary, an encouraging model of Ad5 ocular infection appropriate for antiviral and pathogenesis studies has been developed in the NZ rabbit. However, further refinements in the model are needed for better mimicking of human disease.

Key words: adenovirus, EKC, epidemic keratoconjunctivitis, keratoconjunctivitis, animal model.

References