Barbara, CA) and the ProTon Tonometer (Tomey Technology, Cambridge, MA) on mice. In our hands, these instruments could not reproducibly measure IOP in the very small eyes of mice. We are working toward the development of a noninvasive tonometer for mouse eyes. Our current procedure will allow calibration and validation of future noninvasive instruments.

In summary, we have developed and validated a system that accurately measures IOP in mouse eyes. We have used this system to identify IOP differences between genetically distinct strains of mice. These future studies will complement studies in humans and other species and will add considerably to a genetic understanding of glaucoma.

Key Words
genetics, glaucoma, intraocular pressure (IOP), mice

Acknowledgments

The authors thank David Beebe, John Krege, and Sharon Freedman for many valuable discussions; Norman Hawes, Charline Gunderson, Catherine Osborn, Marijo Vollmer, Joyce Worcester, and Susan Bean for their assistance; Les Kozak and Edward Leiter for critical reading of the manuscript; and Tomey Technology for the loan of a ProTon tonometer.

References


Topical Corticosteroids Reverse the Antiviral Effect of Topical Cidofovir in the Ad5-Inoculated New Zealand Rabbit Ocular Model

Eric G. Romanowski, Trinita Araullo-Cruz, and Y. Jerold Gordon

Purpose. To determine how the addition of topical corticosteroids would affect the anti-adenoviral inhibitory effect of topical cidofovir (S-HPMPC) in the Ad5 New Zealand (Ad5/NZ) rabbit ocular model.

Methods. In a series of experiments (two-eye design), Ad5-inoculated/NZ rabbits (10⁶ pfu/eye) were treated with 0% of 3 treatment regimens. Group 1 was adminis-
tered 1% cidofovir (CDV) twice a day for 3 days plus comfort tears four times a day for 14 days. Group 2 was administered 1% CDV twice a day for 3 days plus 1% Pred Forte four times a day for 14 days. Group 3 was administered vehicle twice a day for 3 days plus comfort tears four times a day for 14 days and served as the control. All eyes were evaluated for 21 days for serial eye titers, Ad5 positive eyes, and duration of Ad5 shedding.

Results. Compared to control eyes in the Ad5/NZ rabbit ocular model, CDV alone demonstrated a significant antiviral inhibitory effect: reduced mean Ad5 eye titer during the early phase of infection (days 3 to 7), fewer Ad5-positive eyes during the early and late (days 9 to 21) phases of infection, and shortened duration of shedding. However, concomitant treatment with both Pred Forte and CDV significantly reversed the antiviral
Adenoviral ocular infection remains the most common type of external ocular viral infection worldwide.\(^1\)\(^-\)\(^5\) Epidemic keratoconjunctivitis, pharyngeal conjunctival fever, and follicular conjunctivitis are three examples of highly contagious adenoviral ocular infection associated with community and medical facility epidemics, as well as with significant patient morbidity and loss of valuable time from school and work.\(^1\)\(^-\)\(^5\) There is no specific antiviral therapy for the treatment of these infections.

Cidofovir (CDV [S-HPMPC; (S)-9-(3-hydroxy-2-phosphonooxypropyl)cytosine]) is a highly promising broad-spectrum antiviral agent with significant inhibitory activity against a number of DNA viruses (human cytomegalovirus, herpes simplex virus (HSV)-1, HSV-2, varicella zoster virus, and adenovirus).\(^6\) We have demonstrated previously in prevention and treatment studies that the topical administration of CDV significantly reduced ocular viral titers and the duration of viral shedding in the Ad5/NZ rabbit ocular model\(^7\)\(^-\)\(^8\) and in the HSV-1/NZ rabbit ocular model.\(^9\)

The role of topical corticosteroids in the treatment of adenoviral ocular infections remains controversial. Routine use of topical corticosteroids generally is discouraged by most authorities because of the consequences of misdiagnosis (HSV-1, Acanthamoeba, fungi) and the complications of cataract, glaucoma, and superinfection associated with long-term use.\(^1\)\(^-\)\(^5\) Nevertheless, during the acute phase, antiinflammatory effects provide for patient comfort and reduce the signs of severe inflammation (lid edema, membranous and pseudomembranous conjunctivitis, chemosis, iridocyclitis, and decreased visual acuity). During the chronic phase, the immunosuppressive effects of topical steroids may reduce the symptoms associated with subepithelial immune infiltrates (decreased night vision, glare, and halos).

Previously, we reported in the Ad5/NZ rabbit ocular model that a topical corticosteroid (1% prednisolone acetate) (Pred Forte [PF]; Allergan Pharmaceuticals, Irvine, CA) was an effective antiinflammatory and antimmune agent, but that it significantly enhanced Ad5 replication and prolonged Ad5 shedding.\(^10\) Presumably, the latter drug effect resulted from the inhibition of normal viral clearance mediated by the host immune system.

It is a common practice among some practitioners to use an antibiotic and, if necessary, a topical steroid to treat, for a short time, highly symptomatic bacterial blepharitis. In the current study, we determined whether a similar rationale could be applied to the treatment of adenoviral ocular infection. Specifically, we assessed how the addition of topical corticosteroid therapy would affect the antiadenoviral inhibitory effect of topical CDV in the Ad5/NZ rabbit ocular model. This outcome would indicate whether a treatment regimen that included both a topical corticosteroid and a topical antiviral drug would represent a rational approach to treating patients with symptomatic adenoviral ocular infection.

**METHODS. Virus Serotype and Cells.** A clinical adenoviral isolate was cultured from a patient with typical adenoviral keratoconjunctivitis. The isolate was serotyped 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% CO\(_2\)-water vapor atmosphere, harvested, aliquoted, and frozen as a stock virus solution at -70°C. Before use, the stock Ad5 McEwen virus was titrated using a standard plaque assay.

A549 cells, epithelial-like cells derived from human lung carcinoma (CCL-185; American Type Culture Collection, Rockville, MD), 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 (Harlan Bioproducts for Science, Indianapolis, IN), 2.5 μg/ml amphotericin B, 100 U penicillin G, and 0.1 mg streptomycin per milliliter (Sigma Cell Culture Reagents).

**Experimental Drugs.** Cidofovir (S-HPMPC) was synthesized by Antonin Holy (Prague, Czech Republic), evaluated as an antiviral agent by Erik De Clercq (Leuven, Belgium),\(^6\) and made available to us by Gil-ead Science (Foster City, CA). A topical ocular formulation was prepared as a 1% solution in a vehicle consisting of 1% carbomethylcellulose in phosphate-buffered saline (pH 6.2). Control eye drops for CDV consisted of the vehicle alone.

Pred Forte is a sterile corticosteroid suspension widely used as a topical ocular antiinflammatory agent. Concentrations of the preservatives in PF are BAC

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TABLE 1. Effect of Cidofovir, Pred Forte, and Combination Therapy on Ad5 Ocular Replication in the New Zealand Rabbit

<table>
<thead>
<tr>
<th></th>
<th>Group I Cidofovir + CT</th>
<th>Group II Cidofovir + PF</th>
<th>Group III Vehicle + CT</th>
<th>P &lt; I vs II*</th>
<th>P &lt; I vs III†</th>
<th>P &lt; II vs III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early phase (days 3–7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Ad5 eye titer (pfu/ml)†</td>
<td>3.7 ± 8.5 x 10²</td>
<td>1.1 ± 3.1 x 10³</td>
<td>1.2 ± 2.3 x 10³</td>
<td>0.034</td>
<td>0.024</td>
<td>0.89 (NS)</td>
</tr>
<tr>
<td>Ad5-positive eyes/total§</td>
<td>52/80 (65%)</td>
<td>72/80 (90%)</td>
<td>71/80 (89%)</td>
<td>0.00032</td>
<td>0.00074</td>
<td>0.99 (NS)</td>
</tr>
<tr>
<td>Late phase (days 9–21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Ad5 eye titer (pfu/ml)†</td>
<td>0.4 ± 3.3 x 10⁰</td>
<td>7.8 ± 29.1 x 10⁰</td>
<td>1.8 ± 7.8 x 10⁰</td>
<td>0.0006</td>
<td>0.95 (NS)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Ad5-positive eyes/total§</td>
<td>2/114 (2%)</td>
<td>47/120 (39%)</td>
<td>11/120 (9%)</td>
<td>0.000001</td>
<td>0.003</td>
<td>0.000001</td>
</tr>
<tr>
<td>Duration of Ad5 shedding (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD‡</td>
<td>5.5 ± 2.7</td>
<td>14.6 ± 6.0</td>
<td>8.4 ± 3.2</td>
<td>0.000004</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>Median∫</td>
<td>4.5</td>
<td>17.0</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Steroid reversal of the antiviral effect of cidofovir.
† Antiviral effect of cidofovir.
‡ Analysis of variance test, two means.
§ Chi-square analysis.
∫ Kruskal–Wallis median test.

0.004%, PS80 0.0005%, and EDTA 0.013%. Comfort Tears (CT; Barnes–Hind, Sunnyvale, CA) artificial tears was used as the control drug for PF because it is formulated with the same preservatives as PF.

Animals. Six-week-old female New Zealand albino rabbits, each weighing 1 kg, were obtained from Green Meadows Rabbitry (Murrysville, PA). All animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Institutional approval was obtained, and institutional guidelines regarding animal experimentation were followed.

Experimental Design. This study was performed in duplicate, and 15 rabbits were used per experiment.

After the administration of appropriate general and topical anesthesia, NZ rabbits were inoculated with 50 µl (1.2 x 10⁶ pfu/eye) of Ad5 McEwen in both eyes after 12 cross-hatched strokes of a #25 sterile needle. Twenty-four hours later, after the establishment of Ad5 replication, five rabbits randomly were assigned to one of three treatment groups. Group 1 (CDV + CT) received 1% CDV twice a day for 3 days and CT four times a day for 14 days. Group 2 (CDV + PF) received 1% CDV twice a day for 3 days and 1% PF four times a day for 14 days. Group 3 (control) received vehicle twice a day for 3 days and CT four times a day for 14 days. Ocular swabbing was performed on days 0, 1, 3, 4, 5, 7, 9, 11, 14, 16, 18, and 21 after inoculation. Each eye was swabbed in the upper and lower fornices with a cotton-tipped applicator, and the swab was placed in 1 ml of media and frozen at −70°C pending titration. After completion of the study, ocular viral titers were determined by plaque assay.

Determination of Viral Titers. Ocular samples to be titered were thawed to room temperature and diluted serially (1:10) for two dilutions. Each dilution and the undiluted original sample were inoculated onto A549 monolayers (0.1 ml per well) in duplicate wells of a 24-well plate. The virus was adsorbed for 3 hours at 37°C in a 5% CO₂–water vapor atmosphere. After adsorption, 1 ml of A549 media plus 0.5% methylcellulose was added to each well, and the plate was incubated at 37°C in a 5% CO₂–water vapor atmosphere. Wells were examined for progressive cytopathic effect and stained with 0.5% gentian violet on day 7, and the number of plaques per well was counted under a dissecting microscope (x25). Viral titers of the ocular samples were calculated and expressed as plaque-forming units per milliliter.

FIGURE 1. Serial Ad5 ocular titers graphically demonstrate the antiviral effect of CDV + CT (□) when compared to the control (△). The addition of PF to CDV (○) induces the steroid reversal of the antiviral effect in the CDV + CT group. CDV = cidofovir; CT = comfort tears; PF = Pred Forte.
Statistical Analysis. After all experiments were completed, codes were broken and data from each experiment were analyzed statistically. Because comparable results were obtained in each experiment, data were then pooled to obtain a larger subject number and were analyzed using analysis of variance, the Kruskal–Wallis median test, and chi-square analysis. Significance was established at the $P < 0.05$ confidence level.

RESULTS. Antiviral Effect of Cidofovir. The effect of cidofovir again was demonstrated in this study in which CDV + CT-treated rabbits had a significantly lower mean Ad5 eye titer (the mean of all ocular cultures) during the early phase of infection (days 3 to 7) than the respective control (vehicle + CT) (Table 1, Fig. 1). Our data demonstrate that CDV + CT-treated rabbits had significantly fewer Ad5-positive eyes per total eyes than the vehicle + CT-treated rabbits throughout the entire study (days 0 to 21) (95 of 234 [41%] versus 122 of 240 [51%]; $P < 0.032$) and during the early and late phases (Table 1). Mean and median durations of Ad5 shedding also was significantly shorter for CDV + CT-treated rabbits than for the vehicle + CT-treated rabbits, as demonstrated in Table 1.

Steroid Reversal of the Antiviral Effect of Cidofovir. Complete reversal of the antiviral effect of cidofovir by the addition of treatment with topical Pred Forte also was demonstrated by the data summarized in Table 1 and Figure 1. During the early phase, the addition of PF therapy to the cidofovir-treated rabbits (group 2 — CDV + PF) resulted in a significant increase in the mean Ad5 eye titer (to control levels) compared to group 1 (CDV + CT) rabbits. Similarly, during the late phase, the addition of PF therapy to CDV therapy resulted in a significant increase in the mean Ad5 eye titer, the percentage of Ad5-positive eyes, and the mean and median duration of Ad5 shedding as compared to the CDV + CT rabbits.

Finally, comparisons between group 2 (PF + CDV group) and group 3 (untreated vehicle + CT control group) yielded data also summarized in Table 1. During the early phase, there were no statistically significant differences between groups 2 and 3 with respect to mean Ad5 titers and percentage of Ad5-positive eyes. In contrast, during the late phase, the addition of PF to CDV (group 2) did result in significant increases compared to the control (group 3) in the mean Ad5 eye titer, the percentage of Ad5-positive eyes, and the mean and median duration of Ad5 shedding.

DISCUSSION. We have demonstrated previously that the topical administration of cidofovir significantly reduced adenoviral ocular titers and the duration of adenoviral shedding in treatment studies in the Ad5/NZ rabbit ocular model. In contrast, we have reported in the same model that the topical administration of PF significantly enhanced Ad5 replication and prolonged Ad5 shedding. It is common practice among some practitioners to treat highly symptomatic bacterial blepharitis for a short time with topical antibiotics and topical steroids to kill the etiologic bacterial agent and to provide symptomatic relief for the patient. The current study evaluated whether the addition of a typical corticosteroid treatment regimen of four times a day for 14 days to the established antiviral treatment regimen of 1% CDV twice a day for 3 days would continue to provide the enhanced viral clearance seen when CDV was administered alone. We instituted a longer treatment regimen for PF (14 days) to capture the immunosuppressive effect that would be present during the early and late phases of Ad5 infection in the model. In a patient, this treatment strategy presumably would provide inhibition of viral replication, symptomatic relief, and possible reduction of the extent and frequency of subepithelial corneal infiltrates. In the current study, the limitations of the replication model did not allow us specifically to assess for possible symptomatic relief (antiinflammatory effect) or reduction in subepithelial immune infiltrates.

The results of this study demonstrate that topical therapy with cidofovir significantly inhibited the magnitude and duration of adenoviral replication, but the addition of a typical treatment regimen using a topical corticosteroid (1% Pred Forte) to the cidofovir regimen completely reversed the antiviral effect of cidofovir and appeared to delay clearance of the adenovirus by the host immune system. In clinical terms, the increased viral replication and longer duration of adenoviral shedding in the animals may presage a potential public health problem in humans. An increased risk of transmission could promote further the number and extent of community and medical facility epidemics.

The reversal of the antiviral effect of CDV by PF in our experimental model again suggests the critical role played by the normal immune system in viral clearance from the eye. The presence of viridical doses of CDV, a potent viral DNA polymerase inhibitor, were not sufficient to kill adenovirus when presumably topical steroid concurrently blocked local immune mechanisms involved in virus clearance. Under normal circumstances, this result suggests that a probable synergy exists between the immune system and CDV to produce the antiviral inhibitory effects seen in this and previous studies.

Although our experimental data provide additional support for the continued development of cidofovir as a topical broad-spectrum antiviral agent, it does not support a treatment regimen that includes...
a topical corticosteroid in addition to topical cidofovir as a desirable strategy for the treatment of symptomatic adenoviral ocular infection.

Key Words
adenovirus, antiviral drugs, cidofovir, conjunctivitis, corticosteroids

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Effect of Transforming Growth Factor Beta-1 in Endotoxin-Induced Uveitis
Bo Peng, Qian Li, Francois G. Roberge, and Chi–Chao Chan

Purpose. Transforming growth factor beta-1 (TGFβ-1) can modulate inflammation. Endotoxin-induced uveitis (EIU) is characterized by acute ocular inflammation related to the release of cytokines, including interleukin (IL)-6. The authors investigated the effect of TGFβ-1 on EIU in mice.

Methods. Three independent experiments were performed. Endotoxin-induced uveitis was induced in C3H/HeN mice by an injection of 200 μg of lipopolysaccharide (LPS). Two micrograms of TGFβ-1 in 0.1 ml phosphate-buffered saline (PBS) or 0.1 ml PBS alone was administered intraperitoneally at 8 hours after LPS injection. Twenty-four hours after LPS injection, the aqueous humor of the right eyes was collected for leukocyte count, protein concentration, and IL-6 assay. Left eyes were processed for routine histology.

Results. TGFβ-1-treated mice showed less ocular inflammation histologically than to the animals that were given PBS. This was confirmed by decreases in leukocyte count, protein concentration, and IL-6 level in the aqueous humor.