The Antiviral Resistance and Replication of Cidofovir-Resistant Adenovirus Variants in the New Zealand White Rabbit Ocular Model

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PURPOSE. To determine the antiviral resistance of three cidofovir (CDV)-resistant variants of adenovirus type 5 (Ad5) and their ability to replicate in the New Zealand White rabbit ocular model.

METHODS. Rabbits were inoculated topically in both eyes with the CDV-resistant variants R1, R2, and R3, and the Ad5 parental strain. On day 1, rabbits from each virus inoculation were divided into two topical treatment groups: 0.5% CDV and PBS control. Treatment was administered twice daily in both eyes for 7 days. All eyes were cultured for virus on days 0, 1, 3, 4, 5, 7, 9, 11, and 14. Using viral outcome parameters, CDV resistance was determined for each virus by comparing each CDV-treated virus group to its respective PBS control, and altered pathogenesis was assessed by comparing viral replication in the PBS control groups of the Ad5 parent and the three resistant variants.

RESULTS. Topical 0.5% CDV treatment demonstrated significant antiviral inhibitory activity in the Ad5 parental group (e.g., reduced total Ad5-positive cultures, reduced daily Ad5-positive cultures on days 5, 9, 11, and 14, and duration of ocular shedding), but had no effect on the three CDV-resistant variants. There were no significant differences in pathogenicity between the Ad5 parent and the CDV-resistant variants.

CONCLUSIONS. The Ad5 variants R1, R2, and R3 were resistant to topical treatment with 0.5% cidofovir in the rabbit ocular model. However, the acquisition of CDV resistance did not alter the replication of the three Ad5 CDV variants on the rabbit eye.

Adenoviruses (Ad) cause a number of clinical diseases ranging from eye infections of varying severity to infections of the respiratory, intestinal, and urinary tracts. Worldwide, adenovirus ocular infections, in the forms of epidemic keratoconjunctivitis (EKC), follicular conjunctivitis, and pharyngeal conjunctival fever, produce significant patient morbidity, resulting in lost time from school and work. Cidofovir, an analog of cytosine monophosphate, represented the first promising topical treatment for clinical adenoviral ocular infections. However, recently emerging toxicity issues after clinical trials raise questions about its safety and further development. Cidofovir has a potent and long-lasting inhibitory effect against a number of DNA viruses. It appears to mediate its effect directly on the DNA polymerases of human cytomegalovirus (HCMV), varicella-zoster virus (VZV), herpes simplex virus types 1 and 2 (HSV-1 and -2), and adenoviruses. Previous studies in animal models have demonstrated that cidofovir is promising as a broad-spectrum topical antiviral effective against both adenovirus and HSV-1–3, and HSV-1–3 ocular infections.

For most antivirals, the emergence of antiviral-resistant mutants creates a formidable clinical challenge after widespread clinical usage. Previously, we reported the successful isolation of three cidofovir-resistant variants of adenovirus type 5, designated as R1, R2, and R3, that were developed in the laboratory through stepwise passage in increasing concentrations of cidofovir. This was similar to the strategy used for the isolation of antiviral-resistant mutants of HSV, VZV, HCMV, and RNA viruses. In vitro studies from our laboratory demonstrated that the ability of the variants to infect cells was similar to that of the Ad5 parent wild-type isolate (unpublished data). DNA polymerase gene sequence analysis demonstrated two-base pair changes in the cidofovir-resistant variants from the Ad5 parent and the cidofovir-resistant variants.

CONCLUSIONS. The Ad5 variants R1, R2, and R3 were resistant to topical treatment with 0.5% cidofovir in the rabbit ocular model. However, the acquisition of CDV resistance did not alter the replication of the three Ad5 CDV variants on the rabbit eye.

METHODS

Viruses and Cells

The cidofovir-resistant variants, R1 (cidofovir IC_{50} 36.5 μg/ml), R2 (IC_{50} 36.7 μg/ml), R3 (IC_{50} 32.6 μg/ml) and their cidofovir-susceptible wildtype Ad5 parent (IC_{50} 6.2 μg/ml), were derived from the ATCC reference strain of Ad5 as previously described. The viruses were grown in A549 monolayers, harvested, aliquoted, and frozen as stock virus at −70°C. Before use, the stock viruses were titered using a standard plaque assay.

A549 human lung carcinoma cells, (CCL-185; American Type Culture Collection, Rockville, MD), were grown in Eagle’s minimum essential medium with Earle’s salts, supplemented with 6% fetal bovine serum, 2.5 μg/ml amphotericin B, 100 units/ml penicillin G, 0.1 mg/ml streptomycin, and 0.5 mg/ml gentamicin (Sigma Cell Culture Reagents, St. Louis, MO).

Experimental Drugs

0.5% cidofovir (SHPMPC [(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cystosine]) was prepared in PBS from the 7.5% injectable form of...
cidofovir (Vistide; Gilead Sciences, Inc., Foster City, CA). PBS served as control drops.

Animals
Two- to three-pound female New Zealand White (NZW) rabbits were obtained from Myrtle’s Rabbitry (Thompson Station, TN). All animal studies conformed to the ARVO Statement on the Use of Animals in Ophthalamic and Vision Research. University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) approval was obtained, and institutional guidelines regarding animal experimentation were followed.

Experimental Design
This study was performed using a total of 20 rabbits for each of the cidofovir-resistant variants, and 40 rabbits for the Ad5 Parent. After appropriate systemic and topical anesthesia, NZW rabbits were inoculated with 50 μl (1.5 × 10^6 pfu/eye) of the appropriate virus in both eyes after 12 cross-hatched strokes of a No. 25 sterile needle. Inoculation of both eyes of the rabbits allowed us to reduce the number of animals needed without jeopardizing statistical validity in accordance with the Animal Welfare Act Policy No. 12 (Consideration of Alternatives to Painful/Distressful Procedures, June 21, 2000). Twenty-four hours later a total of 10 rabbits each for the variants and 20 for the Ad5 parent were randomly assigned to one of two topical treatment groups: I, 0.5% cidofovir; and II, PBS control. Rabbits were treated in both eyes twice daily for 7 days. On days 0, 1, 3, 4, 5, 7, 9, 11, and 14 after inoculation, after topical anesthesia with proparacaine, a single cotton-tipped swab was placed into the lower fornix, rolled over the cornea into the upper fornix to recover adenovirus from the tear film and corneal and conjunctival surfaces. The swabs from each eye were placed individually into tubes containing 1 ml of outgrowth media and were frozen at −70°C pending viral assay.

Viral Assay
The ocular samples to be assayed were thawed and diluted 1:10. One-tenth milliliter of both the undiluted sample and the dilution and were inoculated onto duplicate wells of a 24-well multilayer containing A549 monolayers. The virus was adsorbed for 3 hours at 37°C in a 5% CO_2–water vapor atmosphere without constant rocking. The plates were rocked intermittently to keep the cells from drying. After adsorption, 1 ml of media plus 0.5% methylcellulose was added to each well, and the plates were incubated at 37°C in a 5% CO_2–water vapor atmosphere. After 7 days of incubation, the cells were stained with 0.5% gentian violet, and the viral cytopathic effect was assessed under a dissecting microscope (25×).

Statistical Analysis
After the completion of all experiments, the data from each experiment was analyzed statistically. As comparable results were obtained in each experiment for each virus, the data were pooled to obtain a larger subject number, reduce costs, and minimize the number of animals needed to complete the studies. The data were analyzed statistically using True Epistat (Epistat Services, Mesquite, TX) statistical software using t-test, analysis of variance (ANOVA) with Duncan’s multiple comparisons, Fisher Exact Test (FET), and χ² analyses. Significance was established at the P ≤ 0.05 confidence level.

RESULTS
Cidofovir Antiviral Studies
Figure 1 shows the percentage of serial Ad5-positive cultures of the Ad5 wild-type parent and CDV-resistant variants R1, R2, and R3 after inoculation and subsequent treatment with 0.5% CDV, twice daily for 7 days, compared with the PBS control. For the Ad5 wild-type parent group (n = 40/group), 0.5% cidofovir treatment resulted in significantly fewer positive cultures on days 5, 9, 11, and 14. In contrast, no significant differences between PBS control and the CDV-treated groups (n = 20/group) were detected for R1, R2, and R3 viruses at any time point (FET).

Comparison of the total number of positive cultures from days 1 to 14 (Table 1) indicates that the only significant difference between the CDV-treated and control animals was for the Ad5 wild-type parent. All CDV-resistant viruses showed no significant differences between treated and untreated control groups (χ²), indicating that resistance to CDV in vitro is faithfully reflected in vivo in the rabbit ocular model.

The duration of shedding was estimated by determining the last day on which adenovirus-positive cultures were obtained. Table 1 also shows the mean duration of shedding from infected eyes for the Ad5 wild-type parent and CDV-resistant variants under treatment and control conditions. For the Ad5 wild-type parent, treatment with CDV resulted in a shorter duration of shedding compared with the PBS control group (Ftest). However, variants R1, R2, and R3 demonstrated no significant differences in duration of shedding between control and treated groups. These results indicate that CDV treatment has little effect on the length of ocular shedding of the resistant variants.

Replication Studies
The capacity to replicate on the ocular surface is reported for each virus tested. The natural history of viral replication was assessed under the nontreated conditions (PBS control groups in Table 1) for the Ad5 wild-type parent and the resistant variants. There were no significant differences among any of the groups for total Ad5-positive eye cultures (χ²) and duration of ocular shedding (ANOVA). We conclude that, within the limitations of the Ad5/NZW rabbit ocular model, the development of resistance of Ad5 to CDV is not accompanied with any loss of pathogenicity as measured by ocular viral replication.

DISCUSSION
The development of antiviral resistance may be accompanied with a reduction in pathogenicity in emergent mutant viruses. Consequently, drug-resistant viruses may represent much less of a clinical problem in immunocompetent patients, but still retain pathogenicity in immunocompromised hosts. For HCMV, a number of CDV or other drug-resistant mutants have been isolated, some of which show growth attenuation, whereas others do not.15–17 In the present study, the rabbit model of adenovirus ocular replication was used to assess replication of the CDV-resistant viruses, both in the context of untreated (natural history) and CDV-treated conditions (antiviral resistance).

Specifically, we established here, the in vivo efficacy of topical cidofovir treatment and the pathogenicity of three Ad5 variants that were previously isolated and shown to be resistant to CDV.10 The importance of this work is that it provides an important correlation between in vitro and in vivo models of adenovirus replication and the study of resistance. Our assessment of these variants in the Ad5/NZW rabbit ocular model indicates that Ad5 CDV-resistant variants retain their capacity to replicate efficiently in the eyes of immunocompetent animals during acute infection. Because clinical signs of ocular infection have not proven to be a reliable measure in the rabbit model, ocular viral replication was used in the current studies as the measure of pathogenicity.

Review of the literature reveals that many HSV and HCMV viruses resistant to CDV have been detailed14,16,18–20 and clinically resistant CMV is a major problem in the treatment of CMV retinitis in AIDS patients.17,21–23 However, most clinical
HCMV mutants resistant to CDV have arisen after long-term treatments in immunocompromised (AIDS) patients. The limited duration of acute adenovirus conjunctivitis, and EKC (<14 days) in a generally healthy population suggests that antiviral resistance to topical cidofovir treatment is unlikely to emerge. However, the possibility of long-term parenteral therapy with cidofovir in severely immunocompromised patients (e.g., after bone marrow transplantation) for life-threatening systemic adenoviral infections suggests that favorable conditions for the emergence of antiviral resistance may occur.

The current studies were all performed with adenovirus type 5, an etiologic agent of adenovirus conjunctivitis. Other subgroup C adenoviruses, Ad1 and 6, also replicate in the rabbit ocular model, and are also effectively inhibited by topical CDV treatment. The principal etiologic agents of ocular disease, Ad8, 19, 37, 3, 4, and 7, do not replicate effectively in the NZW rabbit ocular model but are all effectively inhibited by CDV in vitro. It remains to be determined whether these other ocular serotypes can also develop resistance to CDV.

### Table 1. Viral Outcome Measures

<table>
<thead>
<tr>
<th></th>
<th>Ad5 Parent</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ad5-positive cultures/total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(days 1-14)</td>
<td></td>
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<tr>
<td>0.5% CDV</td>
<td>170/320 (53%)</td>
<td>102/160 (63%)</td>
<td>100/160 (63%)</td>
<td>105/160 (65%)</td>
</tr>
<tr>
<td>PBS control</td>
<td>235/320 (73%)*</td>
<td>116/160 (72%)*</td>
<td>103/160 (64%)*</td>
<td>106/160 (66%)*</td>
</tr>
<tr>
<td><strong>P (CDV vs. PBS)</strong></td>
<td>&lt;0.00001</td>
<td>NS†</td>
<td>NS†</td>
<td>NS†</td>
</tr>
<tr>
<td><strong>Duration of Ad5 shedding (days)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% CDV</td>
<td>6.38 ± 3.04*</td>
<td>7.6 ± 2.7</td>
<td>7.9 ± 3.0</td>
<td>7.8 ± 2.5</td>
</tr>
<tr>
<td>PBS control</td>
<td>9.35 ± 3.08*</td>
<td>8.9 ± 2.4*</td>
<td>8.5 ± 2.4*</td>
<td>7.8 ± 1.6*</td>
</tr>
<tr>
<td><strong>P (CDV vs. PBS)</strong></td>
<td>0.000042</td>
<td>NS†</td>
<td>NS†</td>
<td>NS†</td>
</tr>
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</table>

* Not significant (ANOVA) comparing differences among all virus groups treated with PBS (replication).
† Not significant comparing CDV treatment with its respective PBS Control for each virus group.
Finally, recent events in the clinical development of topical cidofovir have raised serious questions about its future in ophthalmology. Toxicity and marketing issues have led Bausch and Lomb, Inc. to abandon its development and return the drug to Gilead Sciences. Because of these developments, “off label” usage by clinicians should be discouraged in those contemplating it and discontinued by those currently using it until more human data become available to clarify emergent safety concerns (YJG).

References