Multiple Adenoviral Serotypes Demonstrate Host Range Extension in the New Zealand Rabbit Ocular Model

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

PURPOSE. Although several human adenoviral serotypes demonstrated the genetic capability of replicating in New Zealand rabbit corneas in organ culture, only a single adenovirus (Ad) serotype, Ad5, has been reported to replicate in vivo in New Zealand rabbit eyes. The purpose of this study was to determine whether additional adenoviral serotypes could extend their host range to the New Zealand rabbit ocular model.

METHODS. Six rabbits per viral isolate were inoculated in each eye after corneal scarification with 1.5 x 10^6 plaque-forming units per eye with one of the following reference or clinical adenovirus isolates: Ad1 ATCC, Ad1 Kmetz, Ad2 ATCC, Ad2 Wolf, Ad5 ATCC, Ad5 McEwen, Ad6 ATCC, Ad 19 ATCC, and Ad8 Cray (five rabbits). Eyes were cultured on days 0, 1, 3, 4, 5, 7, 9, 11, 14, 16, 18, and 21 after inoculation, and their tear film viral titers were determined on A549 cells.

RESULTS. Ad19 ATCC and Ad8 Cray demonstrated no apparent viral replication. The mean duration of shedding was 1.5 and 0.3 days, respectively, and the total percentage of Ad-positive eyes was 13% and 3%, respectively. In contrast to Ad19 ATCC and Ad8 Cray, all other isolates demonstrated productive infection. The mean duration of shedding was 8 to 16 days (P < 0.0001), and the total percentage of Ad-positive eyes was 33% to 79% (P < 0.0002). The durations of shedding for Ad1 ATCC, Ad1 Kmetz, Ad2 ATCC, Ad2 Wolf, and Ad6 ATCC did not differ statistically from Ad5 McEwen, whereas Ad5 ATCC demonstrated a duration of shedding longer than all isolates (P < 0.0001).

CONCLUSIONS. This was the first demonstration of host range extension by additional clinical and reference isolates of adenovirus types 1, 2, 5, and 6 in the New Zealand rabbit ocular model. These results suggested that host specificity was less stringent than previously thought. (Invest Ophthalmol Vis Sci. 1998;39:532-536)

Ocular adenoviral infections occur worldwide and are associated with community and medical facility epidemics. Although not permanently blinding, ocular adenoviral infections are associated with significant patient morbidity, including symptomatic distress with visual disturbances that can last years. Of the 49 serotypes of known human adenovirus (Ad) serotypes, types 8, 19, and 37 are associated most commonly with ocular disease in the United States.

The study of the pathogenesis of ocular adenoviral infection has been limited by the narrow host range exhibited by human Ads. It has been determined that one serotype of human adenovirus (Ad) serotype 5 (Ad5), has the ability to extend its host range to permit replication in the eyes of New Zealand rabbits. The Ad5 McEwen—New Zealand rabbit ocular model has facilitated the development of topical cidofovir as a potential antiviral treatment for ocular adenoviral infections, and phase II clinical trials are now in progress. The Ad5 McEwen—New Zealand rabbit ocular model has also been used to establish possible clinical guidelines for the use of corticosteroids in the treatment of acute adenoviral ocular infections in the presence and absence of cidofovir.

The development of the Ad5 McEwen—New Zealand rabbit ocular model was made possible by first determining whether unadapted human Ads could extend their host ranges to permit replication in rabbit corneal cells. Previously, the replication of human Ads in vitro in rabbit corneal organ culture was demonstrated to be serotype dependent. Ad5 along with the other members of human Ad subgroup C (Ad1, Ad2, and Ad6) and Ad8 (subgroup D) were able to replicate in a rabbit corneal organ culture, whereas Ad3 and Ad7a (subgroup B), Ad4 (subgroup E), and Ad19 (subgroup D) could not. Of all of the clinical isolates tested, the Ad5 McEwen was chosen for the New Zealand rabbit ocular model because it demonstrated the best in vitro replication in rabbit corneal cells.

In accord with this prediction, Ad5 McEwen demonstrated the capacity to extend its host range by successfully replicating in the eyes of New Zealand rabbits. This result raised the question of whether other serotypes, those that replicated in a rabbit corneal organ culture (Ad1, Ad2, and Ad6 in subgroup C and Ad8 in subgroup D), could also extend their host ranges to permit replication in the eyes of New Zealand rabbits. The present study was designed to answer this question. Specifically, we determined whether reference (ATCC) strains of Ad1, Ad2, Ad5, and Ad6 and clinical isolates of Ad1, Ad2, and Ad8 could replicate in vivo in the eyes of New Zealand rabbits.
Zealand rabbits, and we compared the results to Ad5 McEwen, a positive replication control subject, and Ad19 ATCC, a presumed negative replication control subject (with no apparent viral replication in rabbit corneal organ culture).

**Materials and Methods**

**Virus Serotypes and Cells**

The reference Ad serotypes (ATCC Ad1, Ad2, Ad5, Ad6, and Ad19) were originally derived from human adenoidal tissue. These reference serotypes were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The clinical adenoviral isolates were recovered from patients at the Eye & Ear Institute of Pittsburgh who had typical adenoviral ocular disease. The isolates were serotyped by immunofiltration and serum neutralization and were found to be types 1, 2, 5, and 8. The clinical isolates, designated Ad1 Kmetz, Ad2 Wolf, Ad5 McEwen, and Ad8 Gray, along with the ATCC reference strains, were grown in A549 monolayers, and stocks were prepared as previously described, except for the Ad8 Gray. The Ad8 Gray virus stock was prepared by purifying and concentrating the virus using ultracentrifugation on a cesium chloride gradient. The clinical isolates chosen for this study demonstrated the best in vitro replication in rabbit corneal cells of all the clinical isolates tested for their respective serotypes.

A549 cells, epithelial-like cells derived from human lung carcinoma (CCl-185, ATCC), 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 (Sigma), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin (Sigma).

**Animals**

Six-week-old, 1-kg, female New Zealand albino rabbits were obtained from Green Meadows Rabbity (Murrysville, PA). All animal studies conformed to the tenets of 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 a series of three experiments using a total of 53 New Zealand rabbits. After appropriate systemic and topical anesthesia, 6 rabbits per viral isolate were inoculated into A549 monolayers (0.1 ml/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 A549 medium plus 0.5% methylcellulose or A549 medium alone (Ad8) was added to each well, and the plate was incubated at 37°C in a 5% CO2-water vapor atmosphere. After 7 to 11 days of incubation, the cells were stained with 0.5% gentian violet, and the plaques were counted under a dissecting microscope (×25). The viral titers then were calculated and were expressed as plaque-forming units per milliliter.

**Statistical Analysis**

After the completion of all experiments, the codes were broken and the data from each experiment were analyzed statistically. Because comparable results were obtained in each experiment, the data then were pooled to obtain a larger observation number and were analyzed using analysis of variance (Kruskal–Wallis or Duncan’s multiple comparisons, 2 X 2 tables (retrospective studies), and chi-square analysis. Significance was established at the P = 0.05 confidence level.

**Results**

### Adenoviral Eye Titers

The ocular samples to be titered were thawed and were diluted serially (1:10) twice. Each dilution and the undiluted original sample then were inoculated onto A549 monolayers (0.1 ml/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 A549 medium plus 0.5% methylcellulose or A549 medium alone (Ad8) was added to each well, and the plate was incubated at 37°C in a 5% CO2-water vapor atmosphere. After 7 to 11 days of incubation, the cells were stained with 0.5% gentian violet, and the plaques were counted under a dissecting microscope (×25). The viral titers then were calculated and were expressed as plaque-forming units per milliliter.

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### Results

<table>
<thead>
<tr>
<th>Ad Eye Titer Days 1–7 (PFU/ml)</th>
<th>Mean Duration of Shedding† (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ad1 ATCC</strong></td>
<td>2.2 ± 4.2 X 10⁰</td>
</tr>
<tr>
<td><strong>Ad1 Kmetz</strong></td>
<td>6.6 ± 9.1 X 10⁸</td>
</tr>
<tr>
<td><strong>Ad2 ATCC</strong></td>
<td>8.0 ± 13.8 X 10⁴</td>
</tr>
<tr>
<td><strong>Ad2 Wolf</strong></td>
<td>1.4 ± 3.3 X 10⁶</td>
</tr>
<tr>
<td><strong>Ad5 ATCC</strong></td>
<td>1.5 ± 2.0 X 10⁸</td>
</tr>
<tr>
<td><strong>Ad5 McEwen</strong></td>
<td>1.8 ± 3.0 X 10³</td>
</tr>
<tr>
<td><strong>Ad6 ATCC</strong></td>
<td>1.3 ± 1.8 X 10³</td>
</tr>
<tr>
<td><strong>Ad8 Cray</strong></td>
<td>0.3 ± 1.2 X 10⁶</td>
</tr>
<tr>
<td><strong>Ad19 ATCC</strong></td>
<td>2.3 ± 4.3 X 10⁶</td>
</tr>
</tbody>
</table>

Ad, adenovirus; PFU, plaque-forming unit.

*Adenoviral eye titers (days 1 to 7) were calculated by determining the mean ± SD and median of all eye culture titers from days 1 to 7 for each isolate of each serotype (n = 60 [12 eye cultures for 5 days] for each virus except Ad8 Cray, n = 50 [10 eye cultures for 5 days]).

†The duration of adenoviral shedding was ascertained for each eye by determining the final day a positive adenoviral culture was detected that was not preceded by 2 or more consecutive negative culture days and by calculating the mean ± SD for each isolate of each serotype (n = 12 for each virus except Ad8 Cray, n = 10).
PFU/ml, respectively; \( P = \) not significant). Ad1 ATCC, Ad2 ATCC, and Ad2 Wolf demonstrate higher mean Ad titers than Ad19 ATCC and Ad8 Cray, but these differences were not statistically significant. However, Ad1 Kmetz, Ad5 ATCC, Ad5 McEwen, and Ad6 ATCC had higher mean Ad eye titers days 1 to 7 \( (P < 0.03) \) than Ad19 ATCC and Ad8 Cray. Ad5 McEwen demonstrated a higher mean Ad titer \( (P = 0.05) \) than all clinical isolates and reference strains except for Ad5 ATCC (analysis of variance and Duncan's multiple comparisons).

All clinical isolates and reference strains of Ad1, Ad2, Ad5, and Ad6 had higher median Ad eye titers on days 1 to 7 \( (P < 0.000001) \) than Ad19 ATCC and Ad8 Cray. Adenovirus type 5 McEwen demonstrated a higher median Ad titer \( (P = 0.05) \) than all serotype clinical isolates and reference strains except the Ad5 ATCC isolate (Kruskal-Wallis analysis of variance and Duncan's multiple comparisons).

Figure 1 demonstrates the mean serial adenoviral ocular titers for each clinical isolate and reference strain of each serotype throughout the course of the study. These graphs demonstrate detectable mean adenoviral titers (mean titer \( > 1 \) PFU/ml) over a period of at least 9 days for all virus groups except the subgroup D Ads, Ad8 Cray and Ad 19 ATCC. Ad5 McEwen (days 1, 3, 4, 5, and 9), Ad5 ATCC (days 4, 5, 7, 9, 18, and 21), Ad6 ATCC (days 4 and 5), and Ad1 Kmetz (day 7) demonstrated higher mean adenoviral titers on individual days compared to Ad8 Cray and Ad19 ATCC during the course of the study \( (P = 0.05, \) Duncan's multiple comparisons).

**Duration of Shedding**

The duration of adenoviral shedding was ascertained for each eye by determining the final day on which a positive adenoviral culture was detected that was not preceded by 2 or more consecutive negative-culture days and by calculating the mean and standard deviation for each isolate of each serotype. These results are displayed in Table 1 and in Figure 2. All clinical isolates and reference strains of serotypes 1, 2, 5, and 6 demonstrated a longer mean duration of shedding \( (P < 0.0001) \) than Ad19 ATCC and Ad8 Cray. There was no difference in mean duration between Ad19 ATCC and Ad8 Cray. Adenovirus type 5 ATCC demonstrated a longer mean duration \( (P < 0.0001) \) than all clinical isolates and reference strains of all serotypes. There were no differences among Ad5 McEwen and Ad1 ATCC, Ad1 Kmetz, Ad2 ATCC, Ad2 Wolf, and Ad6 ATCC (analysis of variance and Duncan's multiple comparisons).

**Adenovirus-Positive Eyes**

The number of Ad-positive eyes per total was determined for each isolate of each serotype by ascertaining the number of eye swabs that demonstrated a positive adenoviral culture from day 1 to 21 after inoculation per the total number of cultures for each culture day and the total for the entire study (Table 2). All subgroup C clinical isolates and reference strains demonstrated a greater number of total Ad-positive eyes per total \( (P < 0.0002, \) chi-square analysis) than subgroup D serotypes (Ad19...
Ad5 McEwen had a longer mean duration of shedding ($P < 0.0001$) than subgroup D representatives, Ad19 ATCC and Ad8 Cray. ATCC and Ad8 Cray) throughout the course of the study. Within subgroup D, Ad19 ATCC demonstrated a greater number of total Ad-positive eyes ($P < 0.008$) than Ad8 Cray. Within subgroup C, Ad5 McEwen demonstrated a greater number of total Ad-positive eyes per total ($P < 0.01$) than Ad1 ATCC and Ad2 ATCC, but there were no statistical differences when Ad5 McEwen was compared with Ad1 Kmetz, Ad2 Wolf, and Ad6 ATCC. The reference strain, Ad5 ATCC, demonstrated a greater number of total Ad-positive eyes per total ($P < 0.02$) than all other clinical isolates and reference strains of all serotypes.

The number of Ad-positive eyes from all viruses on each culture day of the study is also shown in Table 2. The majority of the Ad-positive eyes are detected between day 1 and day 11 of infection. All subgroup C Ads demonstrated more Ad-positive eyes than Ad8 Cray ($P = 0.04$) on each of the culture days during this period (days 1, 3, 5, 7, 9, and 11) except for Ad1 ATCC (days 5 and 11) and Ad2 ATCC and Ad2 Wolf (day 11). Similar results are seen with the subgroup C viruses and Ad19 ATCC. All subgroup C viruses demonstrate more Ad-positive eyes ($P = 0.04$) on each culture day during that period except Ad1 ATCC (days 1, 3, 5, 9, and 11), Ad1 Kmetz (days 1 and 11), Ad2 ATCC (days 1, 9, and 11), Ad2 Wolf (days 9 and 11), and Ad5 McEwen (day 11). From days 14 to 21, only Ad5 ATCC demonstrated more Ad-positive eyes than Ad8 Cray and Ad19 ATCC ($P = 0.005$ on days 14 and 21 only). Within subgroup C there were some significant differences in the number of Ad-positive eyes detected on individual days during the course of the study. Ad1 ATCC demonstrated fewer Ad-positive eyes than all the other subgroup C viruses on day 3 ($P < 0.02$) and fewer than Ad1 Kmetz, Ad5 ATCC, and Ad5 McEwen on day 5 ($P = 0.01$). Ad1 Kmetz and Ad5 ATCC demonstrated more Ad-positive eyes than Ad2 ATCC ($P = 0.03$) on day 9, and Ad5 ATCC had more than all the other subgroup C viruses on day 14 ($P < 0.03$) and day 21 ($P < 0.03$), except for Ad5 McEwen ($P = $ not significant) on day 21. There was no significant difference in the number of Ad-positive eyes between the subgroup D viruses, Ad8 Cray and Ad19 ATCC, on any individual day of the study.

**DISCUSSION**

Replication of human Ads has long been thought to be host and organ specific with no known animal reservoirs. However, there have been a number of recent reports of human adenoviral infections of laboratory animals. To date, all successful rabbit ocular models have been based on a single serotype, Ad5. In contrast, various serotypes have been shown to replicate in the lungs and eyes of cotton rats and to cause tumors in...
a variety of rodent species.\(^1\) Human Ads also have demonstrated productive virus infections from a variety of nonhuman cells in vitro.\(^2\,^9\)

In the present study, we provide additional data suggesting that the host specificity of human Ads is less stringent than previously thought. This study reports for the first time that the adenoid-derived ATCC reference strains of human Ad subgroup C (Ad1, Ad2, Ad5, and Ad6) and clinical ocular isolates of Ad1 and Ad2 demonstrate the capacity to replicate in New Zealand rabbit eyes. These results further validate the New Zealand rabbit ocular model by demonstrating that replication is not restricted to a single isolate of one serotype, Ad5 McEwen. This successful demonstration of host range extension now allows for the use of multiple adenoviral serotypes, including clinical isolates, to be used in future studies of adenoviral ocular pathogenesis and antiviral studies.

Although all subgroup C Ads seem to replicate well in rabbit eyes, representative subgroup D viruses, Ad8 and Ad19, did not demonstrate this genetic capability. The reasons for the failure of host range extension to the rabbit with these common clinical serotypes is unclear. It remains especially puzzling because Ad8 Cray was previously shown to replicate well in rabbit corneal organ culture.\(^7\) This result indicates that rabbit corneal epithelial cells possess the necessary receptors for Ad8 attachment and penetration. Furthermore, rabbit corneal epithelial cells in an organ culture clearly supported virus uncoating, DNA replication, and virion assembly. Other host factors such as local ocular defenses (mechanical lid function, lacrimal washout, and antimicrobial and immune factors in the tear film) must play a critical role in vivo in blocking Ad8 Cray replication and in restricting ATCC Ad19 to what seems to be an abortive infection. Finally, the time required for serotype replication may play some role in vivo. Ocular isolates of Ad8 required a longer time to achieve a complete cytopathic effect in A549 cells compared with other serotypes (8 days for Ad8 versus 4 days for Ad5) (unpublished data).

Group D Ads, Ad8, Ad19, and Ad37, have been shown to be the leading causes of adenoviral ocular disease worldwide.\(^1\) Although it would be ideal to have an animal ocular model of Ad replication and disease based on these serotypes, the present study does not support this possibility. In vitro antiviral assays will remain the primary tool for screening potential antiviral agents for inhibitory activity against the most common etiologic serotypes.\(^1\) Nevertheless, animal models based on Ad5 McEwen already have proven useful for antiviruse\(^5,^6,^12\) and pathogenesis studies.\(^7,^20\) Because Ad8 isolates have been shown to be as much as 10 times more sensitive to inhibition by antivirals than Ad5 in vitro,\(^12\) the Ad5 McEwen–New Zealand rabbit ocular model may serve as an effective surrogate for predicting clinical antiviral efficacy against more common (and more sensitive) Ad8 serotypes.

The application of this premise has successfully promoted the development of topical cidofovir in the Ad5 McEwen–New Zealand rabbit ocular model as a potential antiviral treatment for acute ocular adenoviral infections.\(^5,^6\) Current phase II clinical studies ultimately will determine the validity of this premise. In the future, other subgroup C Ads (Ad1, Ad2, and Ad6) may strengthen further antiviral research by providing additional adenoviral serotypes that can be tested in vivo to confirm the inhibitory activity previously demonstrated in vitro.

References