Efficacy of Topical Immunoglobulins against Experimental Adenoviral Ocular Infection

Edward C. Nwanegbo,¹ Eric G. Romanowski,² Y. Jerold Gordon,² and Andrea Gambotto¹

PURPOSE. Presently, there is no U.S. Federal Drug Administration (FDA)-approved antiviral therapy for the treatment of adenoviral (Ad) ocular infections. The goal of the present study was to determine the antiviral efficacy of human immunoglobulin (Ig) against several wild-type and human ocular isolates of adenovirus types.

METHODS. The antiviral activity of human Ig against multiple adenoviral serotypes in vitro and in the Ad5 New Zealand White (NZW) rabbit ocular models. Further studies investigated the efficacy of topical immunoglobulin (Ig) against multiple adenoviral serotypes in vitro and in the Ad5/NZW rabbit ocular model. In vivo Ig antiviral results were compared with those obtained with topical 0.5% cidofovir and saline.

RESULTS. In three different epithelial cell lines, ≤6.25 mg/mL of the Ig neutralized several wild-type adenoviral serotypes that cause ocular infections. A dose of ≤10 mg/mL neutralized 88% of ocular isolates of the adenovirus serotypes. After treatment of infected animals, adenovirus-positive cultures per total cultures (days 1–14; P = 0.021), the duration of Ad5 shedding (P = 0.008), and the mean combined ocular viral titer during the early (days 1–5; P = 0.0001) and the late (days 7–14; P = 0.013) phases of infection were significantly lower in Ig-treated animals than in saline-treated animals and were similar to those in cidofovir-treated animals.

CONCLUSIONS. Ig demonstrated antiviral properties against multiple adenoviral serotypes in vitro and in the Ad5/NZW rabbit ocular model. Further studies needed to advance topical immunoglobulin for treatment and prophylaxis of ocular infections.

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and corneal epithelial cells in the eyes, decrease viral replication, and prevent transmission to other people. Ig is therefore a promising candidate for bacterial and viral causes of conjunctivitis, especially when the etiology of the eye infection is unknown. The anti-inflammatory property may also prevent the formation of subepithelial infiltrates responsible for decreased visual acuity in many patients with EKC. In theory, the combined antiviral and anti-immune properties of Ig make it attractive for topical treatment and prevention of EKC.

In this study, we investigated the neutralizing activity of Ig against multiple wild-type and clinical ocular isolates of adenoviral serotypes in different human cell lines. In addition, we evaluated topical ocular toxicity and antiviral efficacy in the Ad5/New Zealand White (NZW) rabbit ocular model in which Ad5 replication takes place in the corneal epithelium. Furthermore, we compared the antiviral result with the well-known antiadenoviral drug cidofovir. Apart from evaluating topical Ig’s effect on viral shedding and its duration in infected eyes, we also compared the efficacy of Ig, an extracellular neutralizing antibody, and cidofovir, a potent intracellular acting adenoviral DNA polymerase inhibitor.

**METHODS**

**Experimental Drugs**

Clinical grade Ig was purchased from Baxter (Westlake Village, CA). As required, lyophilized powder was aseptically reconstituted with water, filtered, and stored at 4°C until needed. Cidofovir (Vistide; Gilead Sciences, Foster City, CA) was purchased commercially. A 0.5% topical solution was prepared with 0.9% sodium chloride solution (Baxter Healthcare Corp., Deerfield, IL). The diluted drug was stored at 4°C until needed.

**Adenoviruses**

Wild-type adenovirus serotypes 3, 4, 5, 8, 11, 19, and 37 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The clinical adenovirus isolates were cultured from patients who had typical adenoviral ocular disease, at the Eye and Ear Institute of the University of Pittsburgh. The isolates were serotyped by serum neutralization, and found to be serotypes 1, 2, 3, 4, 5, 7, 8, and 19. Except for Ad8, all wild-type viruses were propagated with a human embryonic kidney cell line (HEK293). All human ocular isolates and wild-type Ad8 were propagated in a human bronchogenic carcinoma cell line (A549). Viruses were aliquoted and stored at -70°C until needed. Recombinant Ad5 encoding enhanced green florescent protein (Ad5EGFP) was generated in our laboratory, as described previously.

**Cell Culture**

A549 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM), as described previously. A human cervical cancer cell line (HeLa; ATCC) was grown and maintained like the A549 cell line. A conjunctival cell line (CCL-20.2 [Wong-Kilbourne derivative (D) of Chang conjunctiva]; ATCC) was grown and maintained with M199 medium (Mediatech, Herndon, VA) enriched with 0.68 mM of l-glutamine 10% fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B.

**In Vivo Antiviral Studies**

Animals. All animals used for the study (NZW rabbits) were obtained from Myrtle’s Rabbitry, Thompson Station, TN. Approval for the study was obtained from the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC; protocol number 0502953A-

**In Vitro Antiviral Studies**

**Flow Cytometric Neutralization.** This assay, which is described elsewhere, was designed to compare the neutralizing titer of two different lots of human IV-IgG solution. The test was conducted with serial final Ig concentrations of 50, 25, 6.25, 1.56, 0.39, 0.1, 0.02, and 0.01 mg/mL. Fifty microliters of the Ig dilutions were incubated in duplicate with 50 μL of 1 × 10⁶ virions/mL of Ad5EGFP in 96-well flat-bottomed plates for 1 hour at 37°C. Freshly harvested A549 cells (1 × 10⁵) were seeded onto the plate and incubated overnight. The cells were harvested and analyzed by flow cytometry (FACScan and CellQuest software; BD Biosciences, Mountain View, CA). The neutralizing titer is the reciprocal of Ig dilution at which >50% cell transduction was inhibited.

**Microneutralization.** This assay, described previously, was optimized to evaluate Ig-neutralizing activity against wild-type adenoviral serotypes 3, 4, 8, 11, 19, and 37. Fourfold duplicate serial dilutions corresponding to final concentrations of 25, 6.25, 1.56, 0.4, and 0.1 mg/mL of Ig (50 μL) were incubated with 50 μL of 1 × 10¹⁰ virions/mL of test virus in 96-well plates for 1 hour at 37°C. Freshly harvested cells (1 × 10⁵ cells/100 μL Ad5EGFP, HeLa, or conjunctival) were seeded into the wells and incubated for 3 days at 37°C and 5% CO₂. After incubation, the plates were stained with 0.5% crystal violet-formaldehyde solution and washed with distilled water. Complete neutralization of the test virus was associated with the persistence of cell adherence and staining of the cells. Infected cells in control wells with viral sample but without Ig did not stain, because of loss of cell adherence. The Ig concentration at which the cells were stained was taken as the titer. The mean Ig concentration titer in the three cell lines was calculated and plotted as shown in Table 1 and Figure 1.

**Log Reduction Neutralization.** This study was conducted with multiple human ocular isolates of adenoviral serotypes 1, 2, 3, 4, 5, 7, 8, 19, and ATCC type Ad37. The final viral concentration of 1 × 10⁶ pfu/mL was incubated with an Ig final concentration of 1000, 500, 100, 10, and 1.0 μg/mL. Further experiments were conducted with Ig final concentrations of 50, 10, 5, 1, and 0.1 mg/mL and 80, 50, 10, 5, and 1 mg/mL. After the viral-Ig mixture was prepared, it was incubated for 1 hour at 37°C in a water bath, followed by 10-fold serial dilutions of each mixture. The samples were subjected to a standard plaque assay using A549 cells as described in the next section. An Ig concentration that demonstrated at least a 1 log₁₀ decrease in Ad titer was considered to have significant antiviral activity.

**Data Table**

<table>
<thead>
<tr>
<th>Serotypes</th>
<th>HeLa Cell Line</th>
<th>A549 Cell Line</th>
<th>CJ Cell Line</th>
<th>Mean IG ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad3</td>
<td>1.56</td>
<td>0.1</td>
<td>0.4</td>
<td>0.69 ± 0.62</td>
</tr>
<tr>
<td>Ad4</td>
<td>1.56</td>
<td>0.1</td>
<td>0.4</td>
<td>0.69 ± 0.62</td>
</tr>
<tr>
<td>Ad8</td>
<td>0.1</td>
<td>6.25</td>
<td>0.1</td>
<td>2.15 ± 2.9</td>
</tr>
<tr>
<td>Ad11</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25 ± 0</td>
</tr>
<tr>
<td>Ad19</td>
<td>6.25</td>
<td>6.25</td>
<td>0.03†</td>
<td>4.18 ± 2.93</td>
</tr>
<tr>
<td>Ad37</td>
<td>6.25</td>
<td>6.25</td>
<td>1.56</td>
<td>4.68 ± 2.21</td>
</tr>
</tbody>
</table>

Data are milligrams per milliliter.

TABLE 1. Neutralization of Adenoviral Serotypes in Three Epithelial Cell Lines by IG

* Conjunctival cell line.
† Infectivity of Ad19 in conjunctival cells was poor.
Results

In Vitro Antiviral Efficacy

Ig Lots and Antiviral Activity. In this study we compared the antiviral properties of two different Ig lots (03I17AX21 and 02F30AX12). Each lot was serially diluted and incubated with Ad5 encoding green fluorescent protein. Inhibition of an A549 cell line infection was analyzed by flow cytometry, as described previously. The two solutions demonstrated similar neutralization of Ad5EGFP. In the two lots, the neutralizing titer concentration of Ig was 0.02 mg/mL.

Common EKC Serotypes. We investigated the viral-neutralizing activity against wild-type common EKC adenoviral serotypes in three cell lines. In the A549 and HeLa cell lines, 5 \times 10^3 virions/cell of all test viruses produced maximum cellular infection. A similar rate of infection was seen with all test viruses in the conjunctival cell line, except Ad19. Despite using 5 \times 10^4 virions/cell in the conjunctival cell line assay, Ad19 was neutralized at an even lower (0.03 \pm 0 mg/mL) concentration of Ig. As shown in Table 1, the mean neutralizing Ig concentration was 0.1 \pm 0 mg/mL against the Ad3 and Ad4 serotypes and 6.25 \pm 0 mg/mL against Ad8, Ad11, Ad19, and Ad37, in an assay using the A549 cell line. Results from similar experiments conducted with HeLa and conjunctival cell lines are shown in Table 1. Ad11, Ad19, and Ad37 demonstrated similar results in both HeLa and A549 cell lines, whereas the same neutralizing titer was seen against Ad8 in HeLa and conjunctival cell lines. Ad11 neutralizing titers remained unchanged in the three cell lines. Less than 10 mg/mL of Ig neutralized all the wild-type, common EKC serotypes in the three cell lines (Fig. 1).

Human Ocular Isolates. We evaluated the in vitro direct antiviral inhibitory activity of different concentrations of Ig against human ocular isolates of adenoviruses. In this study, 0.1 mg/mL of Ig reproducibly produced \(>1\log_{10}\) decrease in titers of multiples isolates of Ad1, Ad2, Ad3, Ad4, and Ad5. Also, 0.1 mg/mL of Ig produced \(>1\log_{10}\) decrease in titers of multiple isolates of Ad7, Ad19, and ATCC Ad37. For three isolates of Ad8, 10 mg/mL of Ig demonstrated a similar result. However, for another two isolates of Ad8, 50 mg/mL of Ig was necessary to produce \(>1\log_{10}\) decreases in viral titers (Fig. 2). In all, 10 mg/mL or less of Ig demonstrated significant antiviral effect on more than 88% of all isolated ocular serotypes.

Statistical Analysis

For the in vivo efficacy study, the masking codes were broken after each experiment was completed, and the data were calculated. Data from the two studies (R1 and R3) were combined, analyzed by analysis of variance (ANOVA; MiniTab Statistical Software; State College, PA) and \(\chi^2\) analyses. Significance was established at \(P < 0.05\).
In Vivo Safety and Efficacy

Ocular Toxicity. Ocular toxicity studies were conducted in four groups of animals, as described earlier. All the animals tolerated the drugs at all concentrations. The maximum mean total scores (MMTSs) were 0 in all groups, and all Ig concentrations and the placebo (albumin) were considered nonirritating. Ophthalmic examinations during the study demonstrated no corneal involvement, conjunctival redness, chemosis, discharge, or iritis.

Efficacy Studies. The results of the combined studies are summarized in Table 2 and Figures 3 and 4. The number of Ad5-positive cultures per total was determined for each treatment group by ascertaining the number of eye swabs that demonstrated a positive Ad5 culture per total number of cultures taken per group. These data were divided into the early phase (days 1–5) of infection, during which most of the adenovirus replication takes place, and the late phase (days 7–14) of infection, during which the normal immune and antiviral aided clearance of adenovirus occurs. A comparison of the total number of Ad5-positive cultures per total number of cultures taken per group over the entire course of the study (14 days) demonstrated that both Ig and 0.5% cidofovir significantly decreased the number of Ad5-positive cultures per total cultures (Table 2) compared with the control. Breaking these data down into the early and late phases of infection (Table 2) showed that Ig and cidofovir exerted significant antiviral activity compared with the control, but during only the late phase of infection. The daily reduction in percentage of Ad5-positive cultures in all treatment groups is presented graphically in Figure 4. Ig and cidofovir treatment resulted in significant decreases in the number of Ad5-positive cultures on days 7 and 9, compared with the saline-treated eyes.

The mean combined Ad5 ocular titers represent a global measure of adenovirus replication during the early and late phases of infection. These were determined by calculating the mean and SD of all ocular cultures from each treatment group during the early and late phases (n = 80 for all groups). The results are presented in Table 2. During the early phase of infection, the mean Ad5 ocular titers were significantly decreased when the eyes were treated with Ig and cidofovir (P = 0.0001, power 0.7599, ANOVA). Similar results were demonstrated during the late phase compared with the control (P = 0.013, power 0.9998, ANOVA).

FIGURE 2. One log_{10} reduction in titer by neutralization of multiple clinical ocular isolates of adenoviral serotypes. Shown is the Ig concentration that decreased titers of multiple clinical ocular isolates of adenoviral serotypes and ATCC type Ad37 by 1 log_{10} pfu/mL. The numbers in the x-axis labels represent the adenovirus serotype, whereas the letters represent multiple isolates of the same serotypes.

FIGURE 3. Percentage of Ad5-positive cultures in the total cultures for each culture day for eyes treated with 100 mg/mL Ig (n = 20), 0.5% cidofovir (n = 20), and saline (n = 20). Swabs were taken on days 0, 1, 3, 4, 5, 7, 9, 11, and 14 PI. Both 100 mg/mL or Ig and 0.5% cidofovir demonstrated significantly fewer Ad5-positive cultures compared with the saline control on days 7 and 9 (χ²). There were no significant differences among the groups on any other day.

TABLE 2. Viral Outcome Measures of 100 mg/mL IG in the Ad5/NZW Rabbit Ocular Model

<table>
<thead>
<tr>
<th>Time Period</th>
<th>IG (100 mg/mL)</th>
<th>Cidofovir (0.5%)</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoviral positive culture/total (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (days 1–14)</td>
<td>89/160 (55.6)*</td>
<td>78/160 (48.7)*</td>
<td>109/160 (68.1)</td>
</tr>
<tr>
<td>Early phase (days 1–5)</td>
<td>75/80 (93.75)</td>
<td>72/80 (90.0)</td>
<td>79/80 (98.75)</td>
</tr>
<tr>
<td>Late phase (days 7–14)</td>
<td>14/80 (17.5)*</td>
<td>6/80 (7.5)*</td>
<td>30/80 (37.5)</td>
</tr>
<tr>
<td>Mean combined Ad5 titer (pfu/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early phase (days 1–5)†</td>
<td>9.9 ± 14.6 × 10^{-4}</td>
<td>1.4 ± 2.1 × 10^{-5}</td>
<td>2.3 ± 2.4 × 10^{-5}</td>
</tr>
<tr>
<td>Late Phase (days 7–14)‡</td>
<td>2.5 ± 10.1 × 10^{-4}</td>
<td>0.8 ± 0.4 × 10^{-6}</td>
<td>9.6 ± 35.0 × 10^{-4}</td>
</tr>
<tr>
<td>Duration of Ad5 shedding (days)‡</td>
<td>6.4 ± 1.7*</td>
<td>5.3 ± 1.3*</td>
<td>8.1 ± 1.4</td>
</tr>
</tbody>
</table>

χ² was used for the analysis of Ad5-positive cultures/total. ANOVA was used for the analysis of mean combined Ad5 titer and duration of Ad5 shedding.

* P < 0.05 when compared with the control.
† n = 80.
‡ n = 20.
administration.16 Ad5/NZW rabbit ocular model, and (3) safety after topical virus serotypes that infect the eye, (2) antiviral efficacy in the infections: (1) antiviral activity against a wide range of adenoviral conjunctivitis. First, topical Ig may accelerate clearance of the virus from infected eyes, leading to a more rapid cure. Second, because of rapid decreases in ocular titers in the early phase of the infection, transmission to susceptible hosts will be limited thereby, reducing local epidemics. Third, the prophylactic use of topical Ig in susceptible persons may prevent additional clinical infections. Although Ig and cidofovir were equivalent in most outcome parameters, cidofovir demonstrated a significantly shorter duration of viral shedding (Table 2), presumably because of its intracellular-mediated adenoviral DNA polymerase-blocking activities38 and prolonged tissue half-life after rapid uptake into cells.

Because commercial Ig is produced from serum pooled from many donors, the problem of product consistency should be addressed during future development of an ophthalmic topical antiviral preparation. Data from the current in vitro studies indicate that different lots of Ig demonstrated similar antiviral features, indicating that antadenoviral activity was consistent from lot to lot (data not shown). Future studies to test the antiviral activity of Ig from different manufacturers may also be informative.

In summary, the current experimental study represents the first successful evaluation of topical antiviral properties of Ig against etiologic agents of adenoviral ocular diseases, both in vitro and in vivo. Because of its many beneficial properties, a topical solution containing Ig may provide anti-inflammatory, anti-immune as well as antiviral activity against EKC. Furthermore, because of its broad-spectrum antimicrobial properties, topical ocular application of Ig may be effective against other viral and bacterial causes of conjunctivitis. The potential risk of transmission of infectious diseases has been minimized by current methods of producing Ig. Also the risk of anaphylaxis is minimal because of presumed very low levels of ocular absorption. The topical ophthalmic use of Ig may be of immense benefit in the ophthalmology units, pediatric units, community clinics, and global public health. These potential benefits and our preclinical data support further studies of topical Ig.

DISCUSSION

In this study, Ig met the previously suggested minimal criteria for development of an antiviral treatment for ocular adenoviral infections: (1) antiviral activity against a wide range of adenovirus serotypes that infect the eye, (2) antiviral efficacy in the Ad5/NZW rabbit ocular model, and (3) safety after topical administration.16

In general, Ig demonstrated antiviral activity that was equivalent to cidofovir despite major differences in their mechanisms of inhibitory action. Although cidofovir is a nucleoside analogue that works intracellularly to block DNA replication, Ig works by neutralization of free infectious virus on the ocular surface. Ig was remarkably effective during the critical early phase of infection (days 1–5) as demonstrated in the significant reduction of mean daily ocular titers on days 1, 3, and 4 (Fig. 4). Ig also reduced the combined ocular titers during the early phase of infection compared with both the cidofovir and saline treatments (Table 2). These findings support that Ig acts rapidly through extracellular viral neutralization on the ocular surface. The clinical implications may be summarized as follows: First, topical Ig may accelerate clearance of the virus from infected eyes, leading to a more rapid cure. Second, because of rapid decreases in ocular titers in the early phase of the infection, transmission to susceptible hosts will be limited thereby, reducing local epidemics. Third, the prophylactic use of topical Ig in susceptible persons may prevent additional clinical infections. Although Ig and cidofovir were equivalent in most outcome parameters, cidofovir demonstrated a significantly shorter duration of viral shedding (Table 2), presumably because of its intracellular-mediated adenoviral DNA polymerase-blocking activities38 and prolonged tissue half-life after rapid uptake into cells.

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References


