Studies of Adenovirus-Induced Eye Disease in the Rabbit Model


Purpose. To achieve a better understanding of the pathogenic processes associated with human adenovirus (Ad)-induced ocular disease.

Methods. Growth curves of Ad5 and Ad14 were performed in cell cultures derived from rabbit and human corneal epithelium (CE) and corneal keratocytes (CK). For in vivo studies, rabbit eyes were inoculated intrastromally and topically with $10^6$ plaque-forming units per eye of Ad5 and ultraviolet light-inactivated (UV-I) Ad5 or Ad14, and the clinical features of the eyes were evaluated by biomicroscopic slit lamp examinations. Duration and quantitation of virus in tear samples were monitored. Humoral response was evaluated by enzyme-linked immunosorbent assay and serum neutralization titrations. Histopathologic and immunocytochemical staining of frozen corneal tissues was performed to determine the expression of major histocompatibility complex (MHC) class I and II and the presence of CD4+ and CD8+ T lymphocytes and CD18+ cells after the immunopathologic response elicited by virus inoculation.

Results. Both Ad5 and Ad14 replicated in all human cell cultures studied. In cells of rabbit origin, Ad5 replicated in cultured CE and CK cells, whereas Ad14 replication appeared restricted. Virus titers in ocular samples from Ad5-inoculated eyes peaked on postinoculation days 3 through 4, with approximately a 100-fold increase in infectious virus in comparison to initial titers. The duration of Ad5 shedding was 8.9 ± 2.4 days. Ad5, Ad5 UV-I, and Ad14 induced seroconversion and subepithelial opacities. CD4+ and CD8+ T lymphocytes and CD18+ cells were present in these intrastral immune cell infiltrates. Expression of MHC class I and II was observed in keratocytes and immune cells; MHC class I also was expressed on CE cells in inflamed areas.

Conclusions. Ad5 is capable of replicating in both CE and CK cells of the rabbit eye. The presence of Ad antigens within the corneal stroma originating from infectious virus (Ad5), UV-inactivated virus (Ad5), or nonreplicating infectious virus (Ad14) can elicit indistinguishable immunopathologic responses in the stroma composed of CD4+, CD8+, and CD18+ cells. Invest Ophthalmol Vis Sci. 1995; 36:2740–2748.

Human adenoviruses are divided into six subgenera (A, B, C, D, E, and F) on the basis of DNA restriction enzyme analysis of genome typing. Approximately 47 different Ad serotypes are recognized; however, the current trend is not to serotype new isolates because of the inordinate number of new Ad hybrids being isolated from patients with AIDS. Adenoviruses are a common cause of eye disease in humans, and clinically they cause three basic syndromes: epidemic keratoconjunctivitis (EKC), pharyngconjunctivitis fever, and nonspecific follicular conjunctivitis. At least 19 of the known serotypes have been reported to be associated with sporadic or epidemic forms of EKC. Ad serotypes 8, 19, and 37, members of subgenus D, are the most common causes of EKC and pose the greatest medical challenge to the ophthalmic community, but members of subgenera A, B, and C (e.g., Ad12, Ad14, and Ad5, respectively) also are known to cause EKC. The epidemiology of EKC has been reviewed by Ford et al.
It has long been suspected that the presence of subepithelial cellular infiltrates (i.e., opacities) represents an immunologic reaction to viral antigens produced during the acute infectious stage of the disease. These infiltrates vary in size and number and are usually located in the anterior to mid stroma. They appear approximately 2 weeks after onset and may persist for weeks to months, or even, rarely, to years. Their presence may contribute to diminished vision.

An ocular model of Ad5 infection in the New Zealand rabbit has been described by Gordon et al. This model has been used successfully to evaluate experimental antiviral drugs and to study the role of early region 3 of human Ad5 genome in the pathogenesis of ocular disease. In this article, we present in vitro evidence that virus replication occurs in corneal epithelial and keratocyte cells and in vivo evidence that virus replication is not necessary for the establishment of subepithelial opacities. The presence of Ad antigenic stimulus within the stroma is sufficient to cause the formation of opacities containing CD4+ and CD8+ T lymphocytes and CD18+ cells. Most of the infiltrating cells are major histocompatibility complex (MHC) class I+. The infiltrate also contains an increased number of MHC class II+ cells, presumably mostly macrophages and B lymphocytes, and some of the keratocytes in the region also appear to be positive for MHC class II.

METHODS

Cell Cultures

Monolayers of A549 cells derived from a human lung carcinoma (CCL-185; American Type Culture Collection, Rockville, MD) were used for virus isolation and plaque assays. Cell cultures derived from rabbit and human corneal epithelium were grown by methods previously described. Rabbit and human keratocytes were provided by Dr. Won Ryong Wee (Doheny Eye Institute, Los Angeles, CA) as previously described. Tenets of the Declaration of Helsinki were followed; informed consent was obtained, and institutional human experimentation committee approval was granted.

Virus and Rabbits

Ad5 McEwen strain provided by Dr. Y. J. Gordon (Eye and Ear Institute of Pittsburgh, PA) was propagated in A549 cells as previously described. Ad14, originally isolated from the throat of a patient, (1091-VR; American Type Culture Collection) was used to prepare stocks of Ad14 in A549 cells. Cesium chloride-density, gradient-purified Ad5 was ultraviolet-inactivated with a UV model UVG-54 short-wave UV 254 nm lamp (UV Products, San Gabriel, CA). Purified Ad was placed in a 35-mm petri dish at a depth of less than 1 to 2 mm and irradiated at a distance of 5 cm for 6 minutes. The plate was rocked every 30 seconds during the exposure. Viral infectivity was verified to be at undetectable levels by plaque titration on A549 cells.

New Zealand white (NZW) rabbits, weighing 1.5 kg, were obtained from the Irish Farms Rabbitry (Los Angeles, CA). All animal investigations conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were examined for preinoculation defects. The rabbits were then anesthetized with a ketamine–xylazine mixture and topical 0.5% proparacaine hydrochloride eye drops (Alcaine, Alcon Laboratories, Fort Worth, TX). The eyes were inoculated intrastromally to form five focal blebs (10 μl per bleb) using a dice pattern, followed by scarification around the blebs and topical inoculation with 50 μl as described by Gordon et al. Total volume of the inoculum was 100 μl of 0.01 M Tris HC1 buffer, pH 8.0. The total number of eyes inoculated were as follows: 18 eyes (9 rabbits) received infectious Ad5; 14 eyes (7 rabbits) received UV-irradiated Ad5; 16 eyes (8 rabbits) received infectious AD14; and 18 eyes (9 rabbits) received sham inoculum as described. Three hours after inoculation, all inoculated eyes were irrigated with sterile saline to remove unadsorbed virus and were swabbed to determine residual virus present.

Examination and Culture

Slit lamp biomicroscopic ocular examinations were performed every other day by an uninformed observer immediately after virus cultures were collected. Conjunctivitis, corneal edema, and iritis scored as none (0), mild (1), moderate (2), or severe (3). The clinical parameter of subepithelial opacities were scored as follows: no opacities (0), one to five opacities (1), six to ten opacities (2), and more than ten opacities (3).

Eyes were cultured daily for virus isolation by the swab method before biomicroscopic examination, performed by an uninformed observer using a slit lamp. Swabs were then placed in 1 ml of Eagle’s minimum essential medium (Mediatech cellgro; Fisher Scientific, Tustin, CA), containing 6% heat-inactivated fetal bovine serum, freeze-thawed once, vortexed vigorously for 20 seconds, and inoculated onto duplicate wells in a 24-well plate containing A549 cells in 10-fold serial dilutions.

Virus Growth Curves

Rabbit and human CE and CK cell cultures were inoculated in triplicate in 24-well plates with the appropriate virus at a multiplicity of infection of 20 (i.e., MOI = 20) and incubated at 37°C for 1 hour. The inoculum was removed, and the monolayers were rinsed three times with Hank’s balanced salt solution, after which growth medium was added and the cells...
were incubated at 37°C. Virus yields were determined by plaque assay at 0, 12, 24, 48, and 72 hours after inoculation.  

**Humoral Antibody Response**

Before inoculation and at the time of euthanasia, serum samples were collected and frozen for antibody titration using a standard enzyme-linked immunosorbent assay (ELISA) with a modification of Voller’s procedure and a microtitration serum neutralization technique. Serology studies were performed on the following: 8 of 9 rabbits inoculated with infectious Ad5; 7 of 7 rabbits inoculated with UV-irradiated Ad5; 5 of 8 rabbits inoculated with infectious Ad14; and 9 of 9 sham inoculated rabbits.

**Immunohistochemistry**

Tissue fixation and immunohistochemical techniques have been described in detail elsewhere. Frozen 6-μm sections from Ad-infected corneal tissue from at least three animals of each study group were immunostained using monoclonal antibodies against rabbit MHC molecules I and II, CD4+ and CD8+, and CD18+ bone marrow-cell marker (Spring Valley Laboratories, Woodbine, MD).

**Statistical Analysis**

Kruskal–Wallis two group tests were used to test for differences in median conjunctivitis, edema, iritis, and corneal opacity scores between Ad5 and Ad5 UV-I groups at day 1 and every 2 days thereafter until day 21. Kruskal–Wallis tests also were performed on median titer and ELISA scores between groups. Two group Fisher exact tests were used to compare proportions of positive cultures, comparing the Ad5 group with the Ad5 UV-I, Ad14, and control groups for 7 days, as well as to compare proportions of opacities between groups. The overall significance level for all analyses was 0.05. When multiple comparisons were made, appropriate Bonferroni adjustments to the significance level were made. All data analysis was performed using Statistical Analysis System (SAS Institute, Cary, NC).

**RESULTS**

**In Vitro Studies**

A549 cell cultures were the most sensitive to Ad5 and Ad14 and, therefore, served as our reference cell culture for all infectivity titrations and growth curve studies. Infectious titers of Ad5 and Ad14 increased by 10,000-fold within 24 to 72 hours of inoculation in A549 cells (data not shown), as did Ad5 in CE cell cultures of rabbit (RCE) and human (HCE) origin (Figs 1A and 1B, respectively). Ad14 did not replicate in RCE and increased only 10-fold in HCE, which was an insignificant increase of infectious virus yield (P > 0.05). The kinetics of Ad5 and Ad14 replication in CK cultures established from rabbit (RK) and human (HK) tissues is presented in Figures 1C and 1D, respectively. In RK, Ad5 increased approximately 100-fold, whereas Ad14 did not show any increase. Human keratocytes gave a 10,000-fold increase with Ad5 and approximately a 10-fold increase of Ad14 replication.

**In Vivo Studies**

Mean duration of Ad5 and Ad14 shed into the tears was 8.9 ± 2.4 and 0.8 ± 1.0 days, respectively. Quantification of infectious Ad5, Ad5 UV-I, and Ad14 in rabbit ocular samples from eight infected eyes is presented in Figure 2. Ad5 infectivity titers peaked on day 5 after inoculation with approximately a 100-fold increase over input virus. No virus isolation was ever made from eyes inoculated with Ad5 UV-I viruses or sham inoculum. Significant pairwise differences were found between Ad5 and Ad14 inoculated eyes (P = 0.0002) and Ad5 and Ad5 UV-I and sham inoculated eyes (P = 0.0007). The occasional Ad14 isolation on days 1 (5 of 16 eyes positive) and 2 (1 of 16 eyes positive) was thought to be residual inoculum virus and not progeny virus because only a minimal amount of virus was detectable in ocular samples and no increase in virus titer could be demonstrated. Ad14 was not isolated after day 2.

Clinical disease data associated with rabbit eyes inoculated with infectious Ad5, Ad5 UV-I, and Ad14 are presented in Figure 3. The loss of Ad5 infectivity after UV-irradiation was confirmed by plaque titration (data not shown). Conjunctivitis was a prominent feature in eyes inoculated with infectious Ad5 for the first 15 days. Eyes inoculated with Ad5 UV-I and Ad14 displayed significantly less conjunctivitis throughout the study (P < 0.001). Minimal iritis was detected in eyes challenged with infectious Ad5, but not in eyes receiving Ad5 UV-I and Ad14. Absence of Ad5 infectivity after UV-irradiation coincided with a reduction in severity of conjunctivitis and iritis. Subepithelial opacities were observed in eyes inoculated with either infectious or UV-inactivated Ad5 or with Ad14 after approximately 11 to 15 days. The edema observed commonly was associated with the intrastromal administration of any inoculum.

Enzyme-linked immunosorbent assay and neutralizing serum antibody titers resulting from the intrastromal inoculation of infectious and UV-inactivated Ad5 and Ad14 are presented in Table 2. The mean ELISA and neutralizing antibody titers elicited by infectious Ad5 were 4800 ± 4079 and 115 ± 50, respectively, whereas UV-inactivated Ad5 had an ELISA titer of 2686 ± 2700 and a neutralizing titer of 160 ± 120. The ELISA and neutralizing antibody levels
Adenoviruses and Subepithelial Opacities

FIGURE 1. Growth kinetics of Ad5 WT300 and Ad14 in (A) rabbit and (B) human corneal epithelial and (C) rabbit and (D) human corneal keratocyte cultured cells grown at 37°C in a 5% CO₂ atmosphere.

FIGURE 2. Quantification of Ad5, Ad5 ultraviolet-inactivated, and Ad14 from eye specimens collected from animals by the plaque assay method after topical and intrastromal inoculation.

elicted by Ad14 were significantly less ($P = 0.0001$) at $1833 \pm 2284$ and $144 \pm 104.3$, respectively. Rabbits that were not inoculated with virus did not develop a detectable humoral response to Ad.

**Immunohistochemistry**

MHC class I and II antigen expression (Fig. 4) and the distribution of CD4⁺, CD8⁺ and CD18⁺ (Fig. 5) antigen-positive cells in Ad-inoculated corneas were evaluated 21 days after inoculation using immunohistochemical staining. Immunohistochemistry results appeared indistinguishable for Ad5, Ad5 UV-I, and Ad14 inoculated eyes. Subsequently, only Ad5 data are presented and compared to the sham group. MHC class I expression was variable in the corneal epithelium of sham-inoculated eyes. Figure 4A presents an area of epithelium in which no MHC class I antigen expression was detectable. The stroma of sham-inoculated eyes did not stain for MHC class I antigen expression was detectable. The stroma of sham-inoculated eyes did not stain for MHC class I. Positive staining for MHC class I antigen was present in CE cells and was localized on the surfaces of keratocytes and
immune cells in the inflamed areas of Ad5 inoculated corneas. The keratocyte population appeared more dense in areas of the anterior stroma. Keratocytes not in an area infiltrated with immune cells did not stain for MHC class I or II. MHC class II staining also was observed on the surfaces of keratocytes and immune cells when present, but not on normal rabbit CE cells. The staining of serial sections from corneas with Ad5- and Ad14-induced subepithelial opacities for CD4+ and CD8+ T lymphocytes and CD18+ cells revealed each to be present. CD8+ T cells appeared to outnumber the CD4+ T cells in the opacities. In normal areas of the cornea, CD4+ and CD8+ T cells rarely were found.

**DISCUSSION**

In our studies, we used two different Ads (Ad5 and Ad14), both of which are known to cause ocular disease in humans. Ad5, but not Ad14, replicated in our cell cultures derived from rabbit and human CE and CK cells; yet equal amounts of either Ad5 or Ad14 were capable of inducing subepithelial opacities in a similar time frame when inoculated intrastromally. The intrastromal cellular infiltrates induced by Ad5 and Ad14, which appeared after approximately 14 days, contained cells that expressed CD4+, CD8+, or CD18+ antigens. It appears that virus replication is not necessary to cause the formation of infiltrates because even UV-inactivated Ad5 (data confirming the loss of infectivity after UV-irradiation not shown) and the nonreplicating Ad14 were capable of inducing subepithelial opacities. The extent of pathologic response is related to the dose and route of virus inoculation. We have found that Ad5 inocula of less than 10^4 plaque-forming units per eye did not induce the formation of opacities (data not shown). Similar findings were reported by Ginsberg and Prince for Ad5 infections producing disease in cotton rat lungs, and, as reported by Gordon et al., eyes were less likely to develop opacities in the absence of intrastromal inoculation. The formation of opacities after a challenge with replicat-

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**FIGURE 3.** Severity of (A) conjunctivitis, (B) corneal edema, and (C) iritis was scored as follows: 0 = none; 1 = mild; 2 = moderate; and 3 = severe. Subepithelial opacities were scored as 0 = none observed; 1 = 1 to 5 opacities; 2 = 6 to 10 opacities; and 3 = >10 opacities.
FIGURE 4. Cryosections of corneal tissue from sham (A,C) and Ad5-infected (B,D) rabbit eyes 21 days after intrastromal inoculation with Tris HCl buffer or density gradient purified Ad5 were immunostained for major histocompatibility complex (MHC) class I and II antigen expression. The cornea from sham-inoculated tissue did not stain for MHC class I (A). In areas of opacities induced by Ad5, the epithelium, keratocytes, and immune cells stained positively for MHC class I (B). MHC class II staining was observed in sham-inoculated (C) and Ad5-inoculated (D) epithelia, as well as in keratocytes and immune cells in the corneal opacities of infected eyes.

ing and nonreplicating virus or inactivated virus may occur by different mechanisms because different immunologic mechanisms are effective against varying forms of antigens, depending on the way viral antigens are encountered. The possibility exists that Ad5 UV-I, Ad14, or both, might undergo an abortive infection with the expression of some early gene products. However, reverse transcriptase polymerase chain reaction studies of early gene 1A (E1A) gene expression by UV-inactivated virus has failed to support this possibility (Simonelli PF, unpublished data, 1995). Another possibility would be that in vivo complementation might occur with the high-titered UV-irradiated Ad5 inoculum. This complementation process might be responsible for inducing disease.

Even though Ad-induced ocular disease in an animal model differs from the natural disease in human eyes in several ways, such experimental investigations provide an opportunity to elucidate the pathogenesis of EKC. A notable difference between the model and the actual disease is that in the model, high-titered virus is introduced directly into the stroma, whereas in nature, Ad-induced ocular disease theoretically is initiated by an infectious virus coming into contact
with a susceptible cell and undergoing replication. We speculate that the degree of success of virus replication with consequent antigen production would influence the severity of the chronic phase of the disease. The extent of virus antigen amplification and the host cell population involved may determine whether a sufficient immune response is elicited to initiate the influx of immune cells into the stroma and the formation of subepithelial opacities. As reported earlier by Gordon et al,5 we found that the appearance of infiltrates was more frequent in corneas subjected to intrastromal inoculation than in corneas subjected only to topical inoculation with superficial scarification of the corneal epithelium (data not shown). We reason that viral antigen clearance would be slower after intrastromal inoculation, and the extended presence of the antigenic challenge in the cornea may maximize the local Ad-specific immune response. Our observation that Ad antigen introduction into the stroma is associated with a higher incidence of subepithelial opacities is compatible with Jones’ hypothesis18 that Ad antigens enter the corneal stroma below overlying infected epithelium and react in situ if and when hypersensitivity develops. From our in vitro studies, we now know that Ad5 can replicate in corneal epithelial cells and in keratocytes. If keratocytes are infected in the natural disease in humans, it raises the possibility that viral antigen production in the stroma by keratocytes might be necessary or at least might enhance the chances for the formation of subepithelial opacities.

Ginsberg et al16 reported with their Ad-induced pneumonia model that the induction of tumor necrosis factor alpha and other cytokines plays an important role in the early phase of the pathogenic process and that infiltration of cytotoxic T cells is a major cellular component in the second phase of the inflammatory response to infection. They speculated that cytokines play an important role in the pathogenesis of Ad-induced disease.19 Some differences in the sequence of events in the pathogenesis of Ad-induced pneumonia and Ad-induced ocular disease might be anticipated because lungs are highly vascularized and corneas normally are avascular. We speculate that optimal treatment of Ad-induced ocular disease eventually may involve the suppression of virus production and spread with an antiviral agent, as well as the selective interruption of the immunopathologic response. The latter step eventually may be accomplished by blocking one or more of the cytokines.

Key Words
adenovirus, cornea, keratoconjunctivitis, pathogenesis

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References


