

# Adenovirus Keratitis: A Role for Interleukin-8

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**PURPOSE.** Adenovirus type 19 (Ad19) infection of the human cornea results in a chronic, multifocal, subepithelial keratitis. Existing evidence suggests that early subepithelial corneal infiltrates are composed of polymorphonuclear neutrophils. In this study, the capacity of Ad19-infected human corneal stromal fibroblasts (HCFs) to produce neutrophil chemotactants (chemokines) was tested.

**METHODS.** HCFs grown from human donor corneas and passaged thrice were infected with a corneal isolate of Ad19 or mock-infected with virus-free media. Bioactivity of the cell supernatants was tested by a neutrophil chemotaxis assay. Supernatants were assayed by enzyme-linked immunosorbent assay for the neutrophil chemotactants interleukin-8 (IL-8) and GRO- $\alpha$ . Corneal facsimiles were generated with HCFs and collagen type I, infected with Ad19, and assayed by immunohistochemistry.

**RESULTS.** Ad19 infection of HCFs increased neutrophil chemotaxis from a baseline of  $0.4 \pm 0.7$  cells/high-powered field (hpf; mock-infected) to  $21.8 \pm 2.3$  cells/hpf (Ad19-infected). Chemotaxis was reduced by the addition of neutralizing antibodies against IL-8 and GRO- $\alpha$ . Infection of HCFs induced quantities of IL-8 protein 300- and 1000-fold over mock-infected controls at 4 and 24 hours, respectively (33 versus 11,813 pg/mL at 4 hours, and 57 versus 76,376 pg/mL at 24 hours,  $P \leq 0.001$  for both). In contrast, GRO- $\alpha$  protein levels were only sevenfold higher at 24 hours postinfection (118 pg/mL in mock-infected controls versus 880 pg/mL in Ad19-infected cell supernatants). Neither chemokine was induced by infection of an immortalized human corneal epithelial cell line. Immunohistochemistry of infected corneal facsimiles demonstrated IL-8 in the extracellular matrix within 3 days after infection.

**CONCLUSIONS.** Production of chemokines in infected tissues facilitates an early innate immune response to infection, and in the infected corneal stroma represents an elementary defense mechanism. Interleukin-8 may play a role in the development of subepithelial infiltrates in adenovirus keratitis. (*Invest Ophthalmol Vis Sci.* 2000;41:783-789)

**E**pidemic keratoconjunctivitis (EKC), caused most commonly by adenoviral serotypes 8, 19, and 37, is the only adenoviral syndrome associated with corneal inflammation. Keratitis in EKC characteristically presents with multiple corneal infiltrates in the subepithelial stroma beginning 1 to 2 weeks after onset of the conjunctivitis. The corneal infiltrates of EKC cause significant ocular morbidity; reduced vision, photophobia, and foreign body sensation may persist for months to years after infection.<sup>1</sup> Early cellular constituents of corneal infiltrates in the human with adenovirus keratitis remain unknown. However, the first inflammatory cell in human tears after the onset of conjunctivitis in EKC is the polymorphonuclear neutrophil,<sup>2</sup> and in experimental animal models of adenovirus infection, infil-

trates consisted of polymorphonuclear neutrophils in early stages<sup>3</sup> and lymphocytes later on.<sup>4</sup>

The cells in residence within the normal human corneal stroma, the keratocytes, maintain the corneal stroma extracellular matrix in a precisely organized and transparent state. In addition to their maintenance functions in the healthy eye, keratocyte responses to corneal wounding are critical to healing.<sup>5</sup> The significant inflammatory response to stromal infection by a diverse array of pathogens suggests an additional biological role for keratocytes: the capacity to amplify acute inflammation in the presence of infection. Indeed, keratocytes secrete proinflammatory chemokines, such as the neutrophil chemotactants interleukin-8 (IL-8)<sup>6</sup> and GRO- $\alpha$ ,<sup>7</sup> in response to a variety of chemical and infectious stimuli,<sup>8-10</sup> and may contribute to necrotizing corneal stromal inflammation due to herpes simplex virus<sup>11</sup> and Gram-negative bacteria.<sup>12,13</sup> Thus, keratocytes play a key role in the inflammatory retort to both corneal injury and invasion.

We hypothesize that the inflammatory cell infiltrates in the adenovirus-infected cornea represent focal areas of stromal infection and manifest due to upregulation of neutrophil chemotactants by keratocytes. We examined adenovirus-infected human corneal cells for their capacity to induce neutrophil chemotaxis by the secretion of chemokines, and used an in vitro model of infection that mimics infection of the human corneal stroma in vivo.

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## METHODS

### Cells and Viruses

Primary keratocytes were derived from donor corneas (North Florida Lions Eye Bank, Jacksonville, FL) as previously described.<sup>9</sup> Briefly, after mechanical debridement of the corneal epithelium and endothelium, corneas were cut into 2-mm-diameter sections, and each section placed in individual wells of six-well Falcon Tissue Culture Plates (Fisher Scientific, Pittsburgh, PA) with Dulbecco's modified Eagle's medium (DMEM), containing 10% heat inactivated fetal bovine serum (FBS), penicillin G sodium, and streptomycin sulfate. Corneal fragments were removed before monolayer confluence. Cells were grown and maintained at 37°C in 5% CO<sub>2</sub>. The cell monolayers were used at passage three. After serial passage in serum-containing media, keratocytes maintain a fibroblast phenotype<sup>14</sup> and are referred to in the remainder of this article as human corneal fibroblasts (HCFs). A fibroblast phenotype was confirmed by indirect immunofluorescent staining with polyclonal anti-vimentin (positive reactivity) and anti-cytokeratin (no reactivity) antibodies by methods described previously.<sup>15</sup> Immortalized human corneal epithelial cells (HCECs) kindly provided by Araki-Sasaki, Suita, Japan,<sup>16</sup> were used as a control. HCECs were grown and maintained in Defined Keratocyte-Serum Free Media (Life Technologies) at 37°C in 5% CO<sub>2</sub>.

Corneal facsimiles<sup>17</sup> were generated by seeding HCFs at a final concentration of 10<sup>5</sup> cells/ml in rat tail collagen, type I (Becton Dickinson, Bedford, MA), prepared according to the company's instructions. While still in the fluid phase, the HCF/collagen mixture was plated in individual 6.5-mm Transwell tissue culture inserts (Costar, Cambridge, MA) at 300 [mu]l/insert, and the inserts placed in 12-well tissue culture plates. The facsimiles were allowed to gel briefly at room temperature, then fed with DMEM 10% FBS with antibiotics, and incubated overnight at 37°C in 5% CO<sub>2</sub>.

Human polymorphonuclear neutrophils were isolated by a technique adapted from that of Harvath et al.<sup>18</sup> Twenty-five milliliters of whole blood, harvested from volunteer donors, was overlaid onto 15 ml of Lymphocyte Separation Medium (Life Technologies), followed by centrifugation at 100g for 20 minutes. The neutrophil/erythrocyte layer was gently mixed with 8 ml of 6% dextran solution, followed by low-speed centrifugation and resuspension in 200 µl calcium/magnesium-free phosphate-buffered saline. The erythrocytes were lysed thrice by the addition of 10 ml ice-cold distilled water and 5 ml of 3.6% NaCl followed by a gentle vortex and low-speed centrifugation. Neutrophil viability was assessed by trypan blue staining.

Adenovirus type 19 (Ad19) cultured directly from the cornea of a patient with EKC was grown in human lung carcinoma cells (A549 cells, CCL 185; American Type Culture Collection, Rockville, MD) in minimum essential medium with 2% FBS, penicillin G sodium, streptomycin sulfate, and amphotericin B. The State of Oklahoma Department of Health confirmed the viral serotype. Typical adenoviral cytopathic effect, positive immunofluorescent staining for adenovirus hexon proteins, and increasing titers of virus within 1 week after infection of human corneal cells with this virus have been previously described.<sup>15</sup> Adenovirus stock was purified by cesium chloride gradient. The Tissue Culture Infectious Dose (TCID) of the purified Ad19 preparation was determined, and the virus stored at -80°C.

### Adenoviral Infection of Human Corneal Cells

Cells grown to 95% confluence in 48-well plates were washed gently with OptiMEM (Life Technologies, Gaithersburg, MD). The plates were infected in duplicate or triplicate with purified Ad19 at a multiplicity of infection (MOI) of 10 or with OptiMEM without virus as a control. Virus was adsorbed at 37°C for 1 hour before the addition of additional OptiMEM to virus- and mock-infected cultures. At 4 and 24 hours after viral adsorption, cell supernatants were aspirated, centrifuged to remove cellular debris, and stored at -20°C for subsequent experiments. In viral growth curve studies, cells and supernatants were removed together at select times post-adsorption with the assistance of a cell scraper, freeze-thawed, centrifuged, and the resultant supernatants titered in triplicate in A549 cells. Corneal facsimiles were infected with purified Ad19 at a MOI of 10 based on the number of cells seeded in each Transwell tissue culture insert on the day before infection.

### Neutrophil Chemotaxis Assay

A neutrophil chemotaxis assay was performed according to previously published methods.<sup>18</sup> Four-hour infected or mock-infected HCF supernatants were placed in the bottom well of blind-well chambers (Poretics, Livermore, CA) and each well covered with a polyvinylpyrrolidone-free 3-µm pore size polycarbonate filter (Poretics). Monoclonal antibody to IL-8 (10 µg/ml, MAB208; R&D Systems, Minneapolis, MN) and GRO-α (5 µg/ml, MAB275; R&D) were added to the viral-infected supernatants in separate experimental chambers. Concentrations of monoclonal antibodies were chosen to maximally inhibit neutrophil chemotaxis. Freshly isolated human neutrophils with greater than 90% viability by trypan blue exclusion were diluted at 10<sup>6</sup> viable cells/ml in OptiMEM and placed above the polycarbonate filter for incubation of 1 hour at 37°C. The membranes were then gently removed, and any neutrophils on the top of the membrane gently scraped off with a scalpel. The membrane was then placed bottom side up on a glass slide and fixed in methanol for 2 minutes, air-dried, and stained with Diff-Quick (Dade Diagnostics, Aguada, PR). Neutrophils on the bottom of the membrane were counted in masked fashion in 10 high-powered fields (hpfs) for each slide with an Axiovert 135 microscope (Zeiss, Thornwood, NY). The capacity of virus- versus mock-infected cell supernatants to induce neutrophil chemotaxis was compared by Student's *t*-test. A value of *P* < 0.05 was considered significant.

### Enzyme-Linked Immunosorbent Assay for Neutrophil Chemotactants

In separate experiments, viral-infected and mock-infected, triplicate, 4- and 24-hour postinfection HCF and HCEC supernatants were assayed by enzyme-linked immunosorbent assay (ELISA) for IL-8 (Cytoscreen immunoassay kit; Biosource, Camarillo, CA) and GRO-α (Quantikine Immunoassay, R&D) proteins, according to the manufacturers' instructions. Tumor necrosis factor-α (TNF-α; Genzyme, Cambridge, MA), diluted in OptiMEM to 500 U/ml, was used as a positive control to stimulate chemokine production in the absence of virus.<sup>11</sup> The specificity of the ELISA was evaluated by the addition of anti-IL8 or anti-GRO-α at concentrations of 1 or 10 µg/ml (IL-8) and 0.05 µg/ml (GRO-α) before virus adsorption. Plates were read on an Emax microplate reader (Molecular Devices, Sunnyvale, CA) and analyzed with SOFTmax analysis software (Molecular

Devices). The means of triplicate ELISA values for each of the virus- or mock-infected wells were compared by Student's *t*-test.

### Immunohistochemistry on Corneal Facsimiles

At various times after infection with Ad19, facsimiles were removed from the tissue culture inserts and fixed in 10% neutral-buffered formalin before paraffin embedding and cutting of 5- $\mu$ m sections. Immunohistochemistry was performed for IL-8 using polyclonal goat anti-human IL-8 antibody (R&D), and for adenovirus capsid antigen using monoclonal mouse anti-adenovirus antibody (Cell Marque, Austin, TX) followed by a biotinylated rabbit anti-goat antibody (DAKO, Carpinteria, CA) or biotinylated anti-mouse antibody (BioPath, Oklahoma City, OK), respectively. The reaction was developed using an Ultra-LINK horseradish peroxidase detection kit (BioPath). Slides were counterstained with hematoxylin and photographed on an Axiovert 135 microscope (Zeiss).

## RESULTS

### Ad19-Infected HCFs Secrete Neutrophil Chemotactants

Adenoviral titers in HCECs rose from 38 TCID/cell after 1 hour of viral adsorption to 190 TCID/cell 24 hours postinfection, an approximately fivefold rise. Over the same 24 hours, adenoviral titers in HCFs rose from 7 TCID/cell to 628 TCID/cell, an approximately 90-fold rise (Fig. 1). At 4 hours postinfection, no viral growth was evident in either HCECs or HCFs. Supernatants of 4-hour Ad19-infected HCECs elicited no neutrophil migration (data not shown). Supernatants from 4-hour viral-infected HCFs induced neutrophil chemotaxis of  $21.8 \pm 2.3$  cells/hpf, whereas 4-hour mock-infected HCF supernatants did not induce measurable neutrophil migration ( $P < 0.0001$  by Student's *t*-test; Fig. 2). Addition of saturating concentrations of monoclonal antibody to IL-8 reduced Ad19 infection-induced

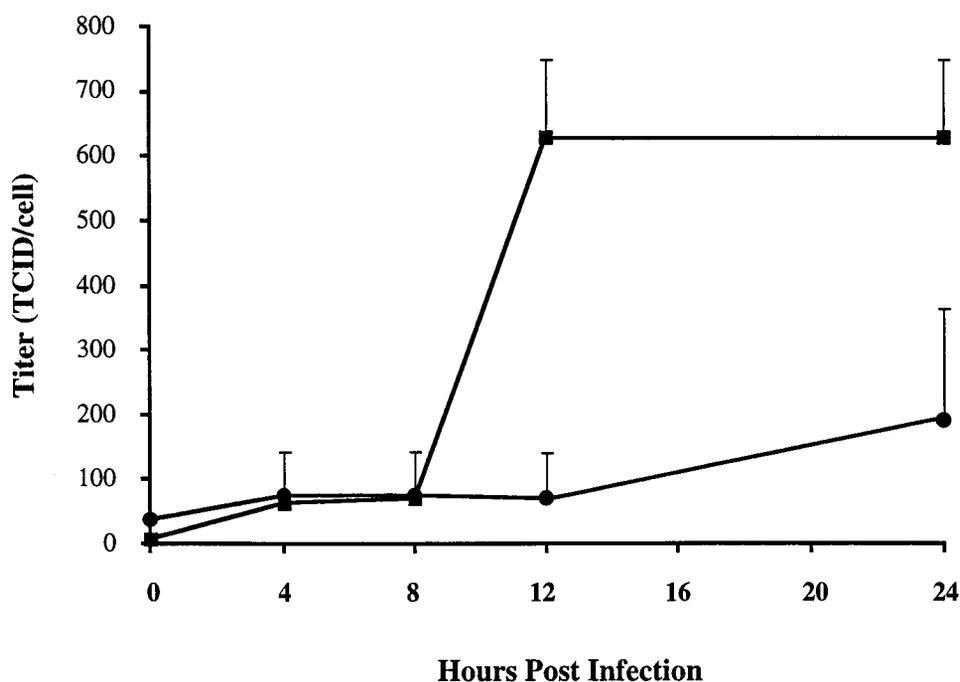
chemotaxis to close to uninfected levels. Anti-IL-8 and anti-GRO- $\alpha$  together completely inhibited neutrophil chemotaxis.

### Ad19-Infected HCFs Secrete IL-8

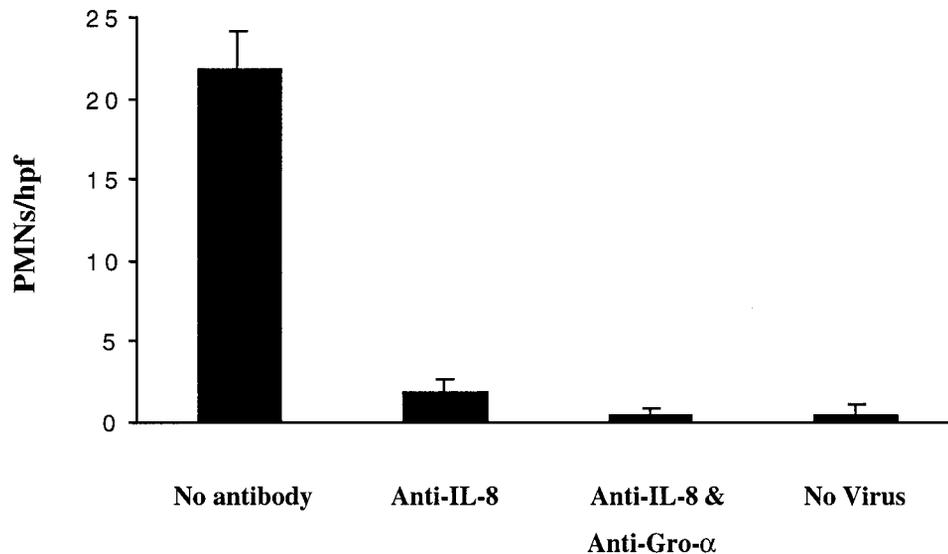
Viral- and mock-infected corneal cell supernatants were assayed by ELISA for neutrophil chemotactants. After 24 hours of infection, IL-8 secretion was greater in Ad19-infected HCECs than in mock-infected HCECs ( $P \leq 0.01$ ), but the level of IL-8 in infected HCEC supernatants reached only 80 pg/ml (Fig. 3A). GRO- $\alpha$  secretion was below the sensitivity of the assay for both infected and uninfected HCECs (data not shown). Infection of HCFs for 4 and 24 hours induced quantities of IL-8 protein 300- and 1000-fold, respectively, over mock-infected controls (33 versus 11,813 pg/ml at 4 hours,  $P \leq 0.001$ , and 57 versus 76,376 pg/ml at 24 hours,  $P \leq 0.001$ ; Fig. 3B). In contrast, GRO- $\alpha$  protein levels in infected HCF supernatants were only sevenfold higher than in uninfected controls at 24 hours and achieved much lower levels than seen for IL-8 (118 pg GRO- $\alpha$ /ml in mock-infected versus 880 pg/mL in Ad19-infected HCF supernatants at 24 hours,  $P \leq 0.002$ ; Fig. 4).

### Ad19 Infection of HCFs within an Extracellular Matrix Induces IL-8 Secretion into the Surrounding Tissue

By immunohistochemistry, IL-8 protein within extracellular matrix became apparent in corneal facsimiles 3 days after infection (Fig. 5B). At earlier times after infection, IL-8 was not apparent by immunohistochemical staining (data not shown). At 3 days postinfection, infected HCFs within the facsimiles appeared swollen and cytopathic and expressed adenovirus hexon proteins (Fig. 5E), signifying active viral replication. Uninfected facsimiles were negative for IL-8 (Fig. 5A) and adenovirus hexon protein (Fig. 5D). Infected facsimiles tested without primary antibody showed appropriately negative expression of IL-8 (Fig. 5C) and adenovirus (Fig. 5F).



**FIGURE 1.** Growth of Ad19 in human corneal cells. HCECs (●) and HCFs (■) were grown to 95% confluence in 48-well plates. Cells were infected in triplicate with Ad19 at a MOI of 10 or with media without virus as a control. Virus was adsorbed at 37°C for 1 hour. At 4, 8, 12, and 24 hours after adsorption, viral titers were determined. Error bars represent SD of the mean.



**FIGURE 2.** Neutrophil chemotaxis assay. HCFs were infected with purified Ad19 for 4 hours and the cell-free supernatants harvested. Freshly isolated human neutrophils were placed across from infected and mock-infected HCF supernatants in blind well chambers separated by 3- $\mu$ m pore polycarbonate membranes. After incubation for 1 hour at 37°C, membranes were stained with Diff-quick and mounted on glass slides. Neutrophils that migrated through the membranes were counted in masked fashion, and the number of cells in 10 hpfs averaged for each experiment. Error bars represent SD of the mean. Monoclonal antibodies to IL-8 (10  $\mu$ g/ml) and GRO- $\alpha$  (5  $\mu$ g/ml) were added as indicated ( $P < 0.0001$  for Ad19 HCF supernatants versus mock-infected control). The experiment was repeated twice with comparable results using cells from different human donors.

## DISCUSSION

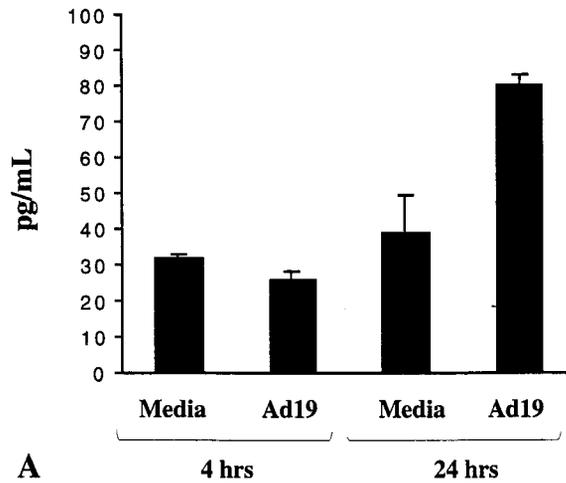
The corneal manifestations of EKC include epithelial keratitis, macro-epithelial erosions, and delayed-onset, multifocal, subepithelial infiltrates.<sup>1</sup> Jones<sup>19</sup> first suggested that subepithelial stromal infiltrates follow adenovirus infection of corneal epithelium, secondary viral antigen deposition in the superficial corneal stroma, and lymphocyte infiltration in a multifocal pattern at sites of antigen-antibody complexes. Jones' model does not take into account the potential contribution of keratocytes to the inflammatory cascade. We hypothesize that after corneal epithelial infection with adenoviruses,<sup>20</sup> multifocal subepithelial infiltrates in EKC develop due to stromal infection and upregulation of inflammatory cell chemotactants by corneal stromal cells.

Chemokines are produced by nearly all human cells and cause leukocyte chemotaxis with a high degree of specificity. Because of existing evidence that polymorphonuclear neutrophils infiltrate the corneal stroma in EKC,<sup>3</sup> we focused our attention on neutrophil-specific chemokines. Perhaps the best characterized chemokine, IL-8 is produced by a variety of human cells and strongly and selectively induces neutrophil chemotaxis and degranulation with a long duration of action.<sup>6</sup> Tissue infection, ischemia, and trauma each can lead to the induction of IL-1 and TNF- $\alpha$ , and these cytokines strongly induce IL-8 production by multiple cell types,<sup>21</sup> including HCFs.<sup>8</sup> Herpes simplex virus infection<sup>11</sup> and UV light<sup>22</sup> induce IL-8 synthesis by HCFs. These data suggest that IL-8 induction could be a final common pathway of corneal inflammation for a variety of corneal tissue insults. In our study, we demonstrated neutrophil chemotaxis toward supernatants of adeno-

virus-infected HCFs, with inhibition of chemotaxis in the presence of antibody against IL-8, and increased levels of IL-8 protein in infected HCF supernatants. Under our experimental conditions, infection induced only modest increases in secretion of GRO- $\alpha$ , another neutrophil chemotactant. Our data also showed no appreciable induction of neutrophil chemotaxis and minimal increases in IL-8 production by adenovirus-infected HCECs. Taken together, these data are consistent with the clinical observation of significant neutrophil migration into an infected cornea when the pathogen has breached the stroma.

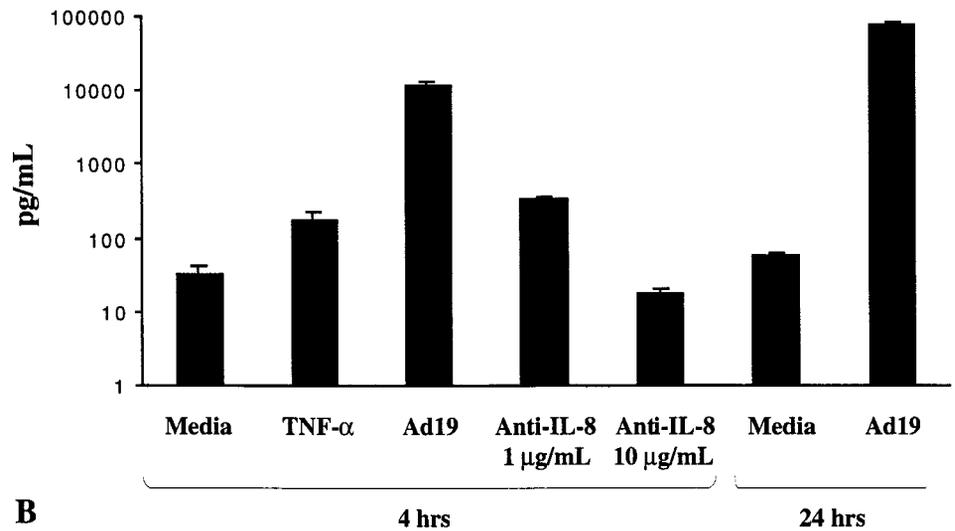
It remains unclear whether a complete viral replicative cycle is necessary for adenovirus to induce chemokine synthesis by HCFs. Trousdale and coworkers<sup>4</sup> demonstrated subepithelial infiltrates in rabbit corneas after intrastromal injection of a replication-deficient adenovirus strain, suggesting that production of infectious virus is not a prerequisite for stromal inflammation. Indeed, Bruder and Kovessi<sup>23</sup> showed IL-8 production by A549 cells in the presence of UV-inactivated but not heat-inactivated adenoviral gene vectors, suggesting that the interaction between the adenovirus and its cellular receptor is necessary and sufficient to induce IL-8 gene transcription. These observations are consistent with one aspect of Jones' theory<sup>19</sup> of subepithelial infiltrate formation: The presence of nonreplicating viral components in the corneal stroma may be sufficient to induce an inflammatory signal.

Finally, because of a lack of immunologic reagents for available animal models of adenovirus corneal infection,<sup>3,24</sup> we used the cornea facsimile model<sup>17</sup> to study adenovirus infection of human corneal cells in a simulated tissue microenvironment. Although an oversimplification of the human cornea,

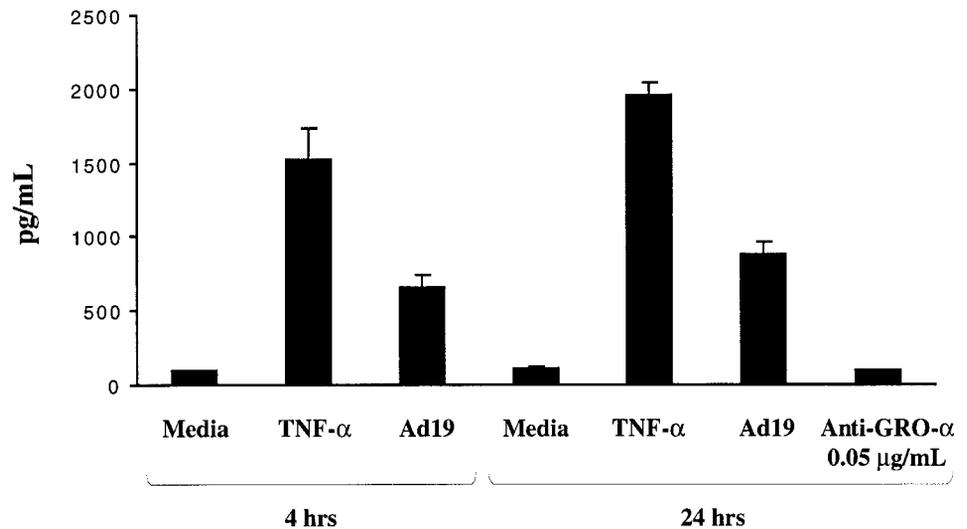


**A**

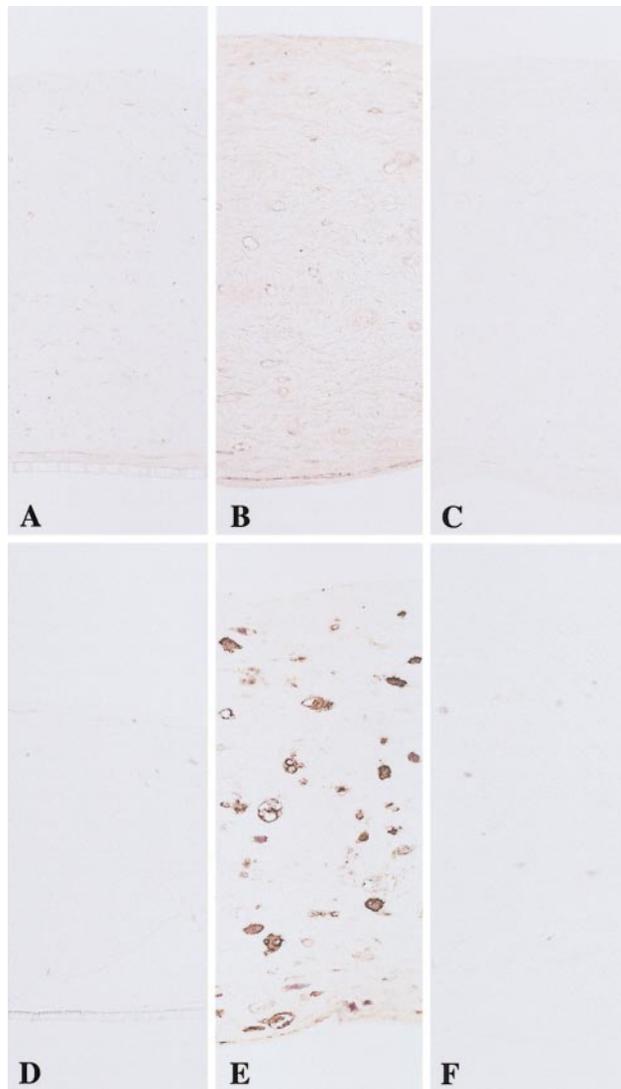
**FIGURE 3.** Interleukin-8 production by Ad19-infected HCECs (A) and HCFs (B). Cells were infected in triplicate for 4 or 24 hours with Ad19 at a MOI of 10, mock infected with virus-free media, or treated with TNF- $\alpha$  500 U/ml. Specificity of the ELISA was tested in HCFs at 4 hours by addition of anti-IL-8 at concentrations of 1 or 10  $\mu$ g/ml before virus adsorption (Ad19-infected versus mock-infected controls,  $P \leq 0.01$  for HCECs at 24 hours;  $P \leq 0.001$  at both 4 and 24 hours for HCFs). This experiment was performed three times with similar results.



**B**



**FIGURE 4.** GRO- $\alpha$  production by Ad19-infected HCFs. Cells were infected for 4 or 24 hours with Ad19 at a MOI of 10, mock infected with virus-free media, or treated with 500 U/ml TNF- $\alpha$ . Specificity of the ELISA was tested at 24 hours by addition of anti-GRO- $\alpha$  at a concentration of 0.05  $\mu$ g/ml before virus adsorption (Ad19-infected versus mock-infected controls,  $P \leq 0.002$  for HCFs at 24 hours). This experiment was performed twice with similar results.



**FIGURE 5.** Immunohistochemistry for IL-8 and adenovirus in Ad19- and mock-infected corneal facsimiles. Corneal facsimiles were infected with Ad19 at a MOI of 10 (B, C, E, F) or mock-infected with virus-free media (A, D) and incubated at 37°C in 5% CO<sub>2</sub> for 3 days. The facsimiles and underlying membranes were removed from the Transwell tissue culture inserts and fixed in 10% neutral-buffered formalin before paraffin embedding and cutting of 5- $\mu$ m-thick sections. Immunohistochemistry was performed for IL-8 (A through C) or adenovirus capsid hexon protein (D through F). Primary antibody was omitted as a negative control (C, F).

this allowed us to test directly the capacity of HCFs to secrete IL-8 into a surrounding extracellular matrix. The demonstration of IL-8 within the "stroma" of an infected corneal facsimile is significant, because IL-8 does not reach appreciable intracellular levels and does not specifically bind to collagen. We propose that the subepithelial location of adenovirus-induced inflammatory infiltrates in the cornea may be due to the presence of potential binding sites for IL-8 in the region of Bowman's and epithelial basement membranes, as has been suggested by investigations in other systems.<sup>25</sup>

In summary, the induction of chemokines by infected tissues amplifies the innate immune response to the infection. In the infected cornea, chemokine production and

subsequent leukocyte infiltration may confine the pathogen and potentially prevent its entrance into the interior of the eye. In adenovirus keratitis, the induction of neutrophil migration into the cornea might serve to limit the extent and duration of infection. Our data support the potential role of IL-8 in the pathogenesis of adenovirus-induced subepithelial corneal infiltrates.

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