Interferon Production and Sensitivity of Rabbit Corneal Epithelial and Stromal Cells

Jerry L. Taylor and William J. O'Brien

The induction of interferon and the ability of interferon to induce the antiviral state were studied using rabbit corneal epithelial and stromal cells which were cultured for fewer than five passages. Interferon titers in the range of 7000 units/ml were induced in epithelial cell cultures and 76,000 units/ml in stromal cell cultures treated with UV-inactivated bluetongue virus. The interferon induced was stable to pH 2.0 treatment and heating to 56°C for 16 hr. Infection of epithelial and stromal cell cultures with various strains of herpes simplex virus type 1 showed that all strains tested replicated to equivalent titers in the respective cell types, and that no detectable interferon was induced in stromal cells and only trace amounts in epithelial cells. Exogenously supplied rabbit interferon induced the antiviral state in cultures of both cell types restricting the replication of not only encephalomyocarditis virus but also herpes simplex virus. Sixty to ninety units of rabbit interferon reduced HSV-1 virus replication by 50%. Human interferons had less than 27% of the antiviral activity in rabbit cells than they had in a human cell line. The data indicate that exogenously supplied interferon may act to reduce the severity of herpetic keratitis by directly inducing the antiviral state in corneal epithelial and stromal cells. However, interferon endogenously produced by rabbit corneal cells in response to HSV-1 infection probably plays a minor role in the pathogenesis of ocular HSV-1 infections.

Interferons (IFNs) are natural antiviral proteins which can be produced by cells in response to a number of stimuli, including virus infection. In humans there are three classes of IFNs: (1) HuIFN-α which is generally produced by leukocytes in response to virus infection and is composed of the products of 13 or more genes, (2) HuIFN-β which is produced primarily by fibroblasts and other non-lymphoid cell types, and is coded for by only one or two genes, and (3) HuIFN-γ, a single gene product, which is produced by T-lymphocytes in response to mitogens or antigens. Although IFNs produced by cells from a particular animal species are generally most effective on the cells of that same species, naturally produced HuIFN-α does possess some cross-species activity. The antiviral activity of HuIFN-α on cells of other species may be due to the action of only certain of the multiple gene products of which it is composed, as determined by studies with recombinant IFNs produced in bacteria from cloned HuIFN-α genes.

The cross-species activity of natural HuIFN-α is probably the reason why it can be used effectively in the treatment of herpetic keratitis in rabbits if administered early in the course of infection. Studies have also shown that IFN therapy can be effective in conjunction with other antiviral agents or debridement in the treatment of established herpes simplex virus (HSV) ocular disease in humans. It has not been determined, however, that the beneficial effects of exogenous IFN are due to the induction of an antiviral state directly in corneal cells. IFNs have numerous other actions on cells, including immunomodulatory activities, and therefore it is possible that IFN may be acting through some mechanism to augment immune functions which are responsible for the observed therapeutic effects.

To determine the role of interferon in HSV-1 infections in the cornea, three specific questions were asked in the studies reported here. Can rabbit corneal epithelial and stromal cells produce interferon? Do these cells produce IFN in response to infection with HSV-1? Can these corneal cell types be protected by treatment with homologous species IFN against virus infection, especially with HSV-1?

Materials and Methods

Corneal Epithelial Cells

Corneal epithelial cells were harvested from freshly excised rabbit corneas as described by Gipson and Grill. The epithelium and anterior stroma were separated from the remainder of the cornea by stripping with forceps. The epithelium was obtained by incu-
Corneal Stromal Cells

Stromal fibroblasts were obtained by collagenase digestion of corneal stromal tissue. Corneas were scraped free of epithelial and endothelial cells, minced and digested with collagenase (154 U/ml Clostridium histolyticum, Gibco; Grand Island, NY) in Hanks' balanced salts solution (HBSS) for 2 hr at 37°C. The collagenase digests were harvested by centrifugation at 800 g for 10 min. The pelleted cells were washed once with DMEM and resuspended in DMEM containing 10% FBS and antibiotics. The particulate material from the digestion was then seeded into 25 cm² plastic flasks and incubated at 37°C until confluent cultures were obtained (generally about 5 days). The confluent cultures were passaged at a 1 to 3 split ratio until the third passage. Third passage cells were then subcultured for use in various experimental protocols. Stromal cells were tentatively identified by their characteristic fibroblastic morphology and further characterized by the lack of observable desmosomes and junction complexes in electron micrographs.

RK-13 Cells

RK-13 cells, a continuous rabbit kidney cell line, were propagated in Eagle's minimum essential medium in Earle's salt solution (EMEM, Gibco; Grand Island, NY) containing non-essential amino acids and 10% FBS.

Vero Cells

Vero cells, a continuous monkey kidney cell line, were grown in DMEM with 10% newborn calf serum (KC Biologicals; Lenexa, KS).

Viruses

Several strains of herpes simplex virus type 1 (HSV-1) were used in these studies including McKrae (A. Nesburn; Los Angeles, CA); Shealey, RE, CGA-3 (Y. Centifanto-Fitzgerald; New Orleans, LA); and KOS (P. ShaefFer and D. Coen; Boston, MA). All virus stocks were prepared in RK-13 cells and titers were determined by plaquing in Vero cells. The vaccine strain of bluetongue virus (Colorado Serum Co.; Denver, CO) was propagated and inactivated by UV-irradiation as previously described.

Interferons

Rabbit interferons used were the National Institutes of Health reference reagent standard, G019–902–528, induced in rabbit kidney cells by bluetongue virus, and a rabbit interferon laboratory standard preparation made by the same procedure. Human interferon preparations used include HuIFN-α induced in human peripheral blood leukocytes by Sendai virus (K. Cantell; Helsinki, Finland), HuIFN-α (3 × 10⁶ U/ml) prepared by the same method but obtained from Meloy Laboratories (Springfield, VA), HuIFN-α (Ly) produced in the Namalva lymphoblastoid cell line induced with Sendai virus (Wellcome Research Laboratories; Beckenham, England). All of these HuIFN-α preparations were natural mixtures, probably of more than one HuIFN-α gene product. HuIFN-β was produced by poly I:C induction in human diploid fibroblasts (Dr. Rentschler Arzneimittel GmbH Co.; Laupheim, West Germany).

Interferon Assay

A microhemagglutination assay similar to that described by Jameson and Grossberg was adapted for...
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Fig. 1. Effect of human IFN-α on herpetic keratitis in rabbits. Scarified corneas of New Zealand white rabbits were infected with 10^6 pfu of HSV-1, McKrae strain. Therapy with HuIFN-α was initiated 6 (●), 24 (▲), 36 (●), or 72 (●) hr after infection (3.0 × 10^6 IU/ml). Controls (○) received phosphate buffered saline. Treatments continued 5 times per day until 7 days postinfection. Lesion scores were determined as described in Materials and Methods.

use in RK-13 cells, corneal epithelial cells and stromal fibroblasts. For this assay, cells were planted in 96 well plates to achieve confluent monolayers. The following day serial two-fold dilutions of the sample interferon preparations to be tested or standard interferon preparations were added to the cells. After 16 to 24 hr, IFN containing medium was removed, cells were washed with HBSS, and encephalomyocarditis virus (EMCV) was added to all wells of the plate. The challenge dose of EMCV for use was determined for each cell type as the minimal input multiplicity of infection which resulted in maximum virus yields after one day of incubation. Challenge doses of EMCV used for the assay are one plaque forming unit (pfu)/cell, 50 pfu/cell, and 5 × 10^-4 pfu/cell for RK-13, corneal epithelial, and stromal cells, respectively, based on plaque titration of EMCV in RK-13 cells. After 24 hr of incubation, yield of EMCV was assayed from each well by hemagglutination.

One unit of interferon in this assay is defined as that amount of IFN which decreases virus yield from that obtained in untreated, virus-infected controls by 0.5 log_{10}. As is generally accepted for IFN assays in which serial twofold dilutions of IFN are used, a twofold variation in titer is not significant. The NIH rabbit IFN standard reference preparation, G019-902-528, was included in assays for comparative purposes.

The antiviral activity of IFN against HSV-1 was also determined. Epithelial or stromal cells in 12 well plastic dishes were treated with rabbit IFN at 25 to 2500 IU/ml for 24 hr. Medium containing IFN was then removed and cells infected with the McKrae strain of HSV at 1 pfu/cell for yield reduction assay. After 1 hr at 36°C for virus adsorption, unadsorbed virus was removed and medium added. Cultures were frozen after 24 hr and virus yield assayed by plaque formation in Vero cells. A plaque reduction assay was also performed in stromal cells. IFN treated cells were infected with McKrae strain HSV and plaque number determined after three days.

**Induction of IFN**

Rabbit corneal epithelial and stromal cells were grown to confluence in 25 cm² plastic tissue culture flasks or 24 well dishes. Cultures were infected with HSV-1 or with UV-inactivated bluetongue virus. Medium was removed from the cultures 24 hr after infection and frozen at −80°C until assayed for antiviral activity. At the time of assay, HSV-1 infected-cell supernatants were adjusted to pH 2 with HCl and held for 10 min or longer at 4°C to inactivate HSV-1. The samples were neutralized before IFN was assayed. IFN-induced was characterized as pH 2 stable by dialysis against 0.1 M KCl-HCl buffer pH 2 for 24 hr at 4°C. Samples were also held at 56°C for 16 hr to assess heat stability.

**IFN Treatment of HSV-1 Keratitis in Rabbits**

Both eyes of 2 to 3 kg New Zealand white rabbits were treated with dorsacaine and then scarified by making five overlapping circles 0.1 mm in depth on the corneas using a 5-mm trephine. The McKrae strain of HSV-1 (10^6 pfu) was dropped into the cul-de-sac of each eye, and the eye lids gently massaged over the corneal surface. Slit lamp examinations of fluorescein-stained eyes were made daily. Epithelial lesions were scored using 1+ = 25% of the corneal surface involved to 4+ = 100% involvement. Statistical evaluation of mean lesion scores of each group of five rabbits (10 eyes) was done using the Wilcoxon Rank Sum test.

IFN therapy with partially purified HuIFN-α (Me-loy-PIFA) was begun 6, 24, 36 or 72 hr after virus infection. Each eye received 20 µl of HuIFN-α (3 × 10^6 IU/ml) or phosphate buffered saline every 2 hr five times per day until 7 days post infection. All experiments which utilize animals described in this study conform to the principles outlined in the ARVO Resolution on the Use of Animals in Research.

**Results**

Treatment of herpetic keratitis in rabbits with HuIFN-α was effective in reducing the severity of the disease if the therapy was initiated early after infection. A significant reduction of lesion score at days 5 and 6 after infection was seen in rabbits treated beginning 6 hr after infection (Fig. 1). A delay in the time of ini-
tiation of therapy to 24 hr post infection decreased efficacy and, although lesion scores were reduced in this treatment group, the difference from control animal lesion scores was not significant. Initiation of treatment at times later than 24 hr after infection resulted in no apparent beneficial effect. These observations establish that our commercial source of HuIFN-α and the strain of rabbits utilized as a source of cells for culture have properties similar to those of other investigators.

### Sensitivity of Rabbit Corneal Cells to IFN

The significant therapeutic effects of HuIFN-α in the treatment of herpetic keratitis in rabbit eyes suggest that IFN induced an antiviral state in cells of ocular tissues. Therefore, the sensitivity of corneal cells to various IFNs, i.e., the ability of these IFNs to induce an antiviral state in epithelial and stromal cells, was studied. Antiviral activity titers on both corneal cell types were compared with those obtained on RK-13 cells which are routinely used to titer rabbit IFNs (Table 1). The rabbit corneal cell types were similar in their sensitivity to rabbit IFN when challenged with EMCV, although somewhat less sensitive than the RK-13 cells. Corneal epithelial cells were somewhat more sensitive in their response to several HuIFNs including HuIFN-α (a preparation similar to that used in the in vivo HSV keratitis studies), HuIFN-α (Ly), and HuIFN-β than stromal cells or RK-13 cells. The antiviral activity of HuIFNs on rabbit corneal cells was less than 27% of that found on human cells.

Replication of HSV-1 in both corneal cell types was inhibited by rabbit IFN (Fig. 2) with a 50% decrease in virus yield produced by treatment with 60–90 IU/ml. In stromal cells, antiviral activity was assayed both by yield reduction at a multiplicity of infection of 1 pfu/cell and by plaque reduction. The ED_{50} of IFN was similar by both assay methods.

### IFN Induction by Various Strains of HSV-1

The severity of herpetic keratitis in rabbits has been shown to depend to some extent on infecting virus strain. The ocular pathologies produced by infection with HSV-1 range from severe epithelial disease caused by the Shealey strain with greater than 50% of infected rabbits developing geographic lesions and one third showing stromal disease, to mild epithelial dendritic disease and no stromal involvement caused by CGA-3. We have confirmed these findings in our laboratory (data not shown) and have further shown that another HSV-1 strain, KOS, caused only epithelial disease characterized by punctate superficial lesions. Centifanto-Fitzgerald et al. have determined that some of the variation in pathology between strains is due to genetic information carried on the Bgl II 2F fragment of the HSV-1 genome. The gene function(s) encoded by this region of the genome are unknown. It is possible that the encoded functions result in viruses which differ in their ability to induce IFN in tissues of the eye, thus leading to disease of varying severity. To test this possibility, epithelial and stromal cells were infected with various strains of HSV-1 at multiplicities of infection

#### Table 1. The titers* of several interferon standards† in rabbit corneal cells and the RK-13 rabbit kidney cell line

<table>
<thead>
<tr>
<th></th>
<th>Rabbit IFN (units/ml)</th>
<th>Human IFN§ (units/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Lab std</td>
<td>NIH std‡</td>
</tr>
<tr>
<td>RK-13</td>
<td>160,000</td>
<td>4,180</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>40,000</td>
<td>2,100</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>24,000</td>
<td>1,000</td>
</tr>
</tbody>
</table>

* Titers of IFN measured in the three cell types with encephalomyocarditis virus as challenge virus. The titer reported are the reciprocal of the dilution of the stock of IFN which protects the specific cell type, i.e., reduces virus yield by 0.5 log_{10} in that cell type.
† IFN standards are prepared with different initial titers; therefore, comparison of titers can be made within columns, but not within rows.
‡ NIH GO19-902-528.
§ Titors in human A549 cells were HuIFNa 12,000 u/ml, HuIFNa 12,000 u/ml and HuIFNα (Ly) 25,000 u/ml.

Fig. 2. Effect of rabbit IFN on the replication of HSV-1 in rabbit corneal cells in culture. Corneal stromal (△) or epithelial (O) cells were grown in confluence, treated with rabbit IFN for 24 hr and then infected with HSV-1, McKrae strain, at an moi = 1 pfu/cell. Cultures were frozen after 24 hr and virus yield assayed on Vero cells. For plaque reduction assay (●), stromal cells were treated with IFN as above and then infected with HSV-1 to give 70 plaques/well in a 12-well plate. Plaques were counted after 3 days. All assays were performed in triplicate.
of 1 to 5 pfu/cell. At 24 hr after infection, medium was removed from cell monolayers, adjusted to pH 2 to destroy virus infectivity, and assayed for antiviral activity. The IFN yields were compared with those obtained using UV-inactivated bluetongue virus, a virus known to induce IFN in many types of cells.15

IFNs induced in cell cultures of corneal epithelial and stromal cells were titered on RK-13 cells (Table 2). None of the strains of HSV-1 induced any detectable IFN in stromal cells. Only very low levels of IFN (<25 units/ml) were produced in epithelial cells in response to the various strains of HSV-1, and no significant difference was detected. All strains of HSV-1 grew well in both cell types, but in general virus yields were somewhat higher from epithelial cells than from stromal cells. Corneal stromal cells, infected at multiplicities of infection of 10 to 0.01 pfu/cell with either the McKrae or KOS strains, produced no detectable IFN in culture supernatants harvested at the time of total cytopathic effect. McKrae strain HSV-1 which was inactivated by UV-irradiation at 4000 ergs/cm²/sec for 10 min showed only slightly greater induction of IFN (50 units/ml) in epithelial cells. UV-inactivated bluetongue virus, however, consistently induced IFN titers in the range of 4,000–7,000 units/ml in epithelial cells and 74,000–76,000 units/ml in stromal cells, demonstrating that both cell types do possess the ability to produce high titers of IFN. The IFNs produced by epithelial and stromal cells were characterized as typical virus-induced IFN, being stable to treatment at pH 2 or 56°C for 16 hours (Table 3).

### Discussion

It is not currently known what role, if any, IFN might play in the natural course of HSV keratitis in humans. In mice, the ability of certain strains to produce interferon within hours of intraperitoneal (ip) injection of HSV-1 correlates with resistance of those mouse strains to HSV infection.22-25 Further, administration of anti-mouse IFN serum to mice injected with HSV ip or subcutaneously results in an increase in mortality and a decrease in time to disease and death.26 Therefore, in mice IFN production is definitely a factor in limiting the pathogenesis of systemic HSV infections; however, this model may not reflect the role that IFN has in HSV keratitis where replication appears to be localized in ocular tissues.

Treatment with exogenous IFN can reduce the severity of HSV keratitis in rabbits and man. We have confirmed the findings of others1,3 that early initiation of therapy of HSV keratitis in rabbits with human IFN-α can reduce the severity of the disease. Treatment begun 6 hr after infection resulted in a significant reduction in lesion scores during the peak of disease. Delaying treatment to 24 hr postinfection reduced the effectiveness. Furthermore, delaying initiation of treatment until the time when lesions began to appear resulted in no apparent beneficial effects. The human IFN used to treat rabbits in our studies was capable of inducing less than 27% of the antiviral titer in rabbit cells as was detected in human cells.

The importance of utilizing the appropriate type of IFN in therapeutic studies was recently shown in the comparative studies of Sanitato et al.27 Their studies indicate that recombinant IFN-α A which is a gene product of a single human IFN-α gene was effective in treating herpetic keratitis in owl monkeys, but had no effect in rabbits, while natural HuIFN-α, a mixture of several gene products, was effective in both rabbits and monkeys. Smolin4 reported that two different recombinant HuIFN-α preparations differ in efficacy in treatment of keratitis in rabbits. Our data quantitatively demonstrates the relative insensitivity of both rabbit corneal cells and an established kidney cell line to human IFNs, although epithelial cells appear somewhat more sensitive than stromal fibroblasts or RK-13 cells.

The production of low titers of IFN by corneal epithelial cells in culture in response to HSV infection suggests that endogenous IFN may be produced in the eye during natural HSV infection. IFN activity has been detected in the tears of rabbits 24 hr after topical ap-

### Table 2. Induction of IFN in corneal cells with HSV-1

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Cell</th>
<th>IFN yield (units/ml)</th>
<th>Virus yield (pfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 McKrae</td>
<td>stromal</td>
<td>~5</td>
<td>1.1 ± 0.8 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>epithelial</td>
<td>18</td>
<td>1.2 ± 0.1 x 10⁴</td>
</tr>
<tr>
<td>HSV-1 KOS</td>
<td>stromal</td>
<td>&lt;5</td>
<td>1.9 ± 0.8 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>epithelial</td>
<td>12</td>
<td>9.0 ± 2.1 x 10⁹</td>
</tr>
<tr>
<td>HSV-1 Shealey</td>
<td>stromal</td>
<td>&lt;5</td>
<td>5.7 ± 1.7 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>epithelial</td>
<td>25</td>
<td>1.1 ± 0.3 x 10⁷</td>
</tr>
<tr>
<td>HSV-1 CGA-3</td>
<td>stromal</td>
<td>&lt;5</td>
<td>5.9 ± 2.1 x 10⁹</td>
</tr>
<tr>
<td></td>
<td>epithelial</td>
<td>12</td>
<td>9.8 ± 3.8 x 10⁹</td>
</tr>
<tr>
<td>HSV-1 RE</td>
<td>stromal</td>
<td>&lt;5</td>
<td>6.5 ± 5.1 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>epithelial</td>
<td>17</td>
<td>8.4 ± 3.0 x 10⁸</td>
</tr>
</tbody>
</table>

* IFN assayed in RK-13 cells.
† Virus yield assayed in Vero cells by plaque assay.

* IFN assay in RK-13 cells.
† IFN induced in 25 cm² flasks with UV-inactivated bluetongue virus, harvested at 24 hr.

### Table 3. Induction of IFN in corneal cells*

<table>
<thead>
<tr>
<th>Producer cell type</th>
<th>IFN (units/ml)</th>
<th>pH 2 treated</th>
<th>56°C 16 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial cells</td>
<td>7,000</td>
<td>7,000</td>
<td>5,600</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>76,000</td>
<td>42,500</td>
<td>33,000</td>
</tr>
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</table>

* IFN induced in 25 cm² flasks with UV-inactivated bluetongue virus, harvested at 24 hr.
† IFN titered in RK-13 cells.
plication of heat inactivated HSV or 2 days after treatment of HSV-2 keratitis with corticosteroids. IFN has also been detected in the tears of patients with coxsackie viral infection, but it was not determined which cell type was responsible for synthesis of the IFN. The production of high titers of IFN in corneal cells following induction with the potent IFN inducer, UV-inactivated bluetongue virus, shows that corneal cells do have IFN-producing capability. Thacore et al were able to induce IFN production by cells of unknown type (presumably fibroblasts) derived from human corneas. After 50–60 passages in culture, their cells could be induced to synthesize IFN when induced with Newcastle’s disease virus, but the IFN had little antiviral activity against HSV. It remains to be determined, however, what effect endogenous IFN may have on HSV keratitis.

Several strains of HSV-1 which produce markedly different pathologies in the rabbit eye were found to induce very low levels of IFN in corneal epithelial cells and no detectable IFN in stromal cells. In addition, there were no differences among the virus strains tested in their ability to replicate in either epithelial or stromal cells. Overall et al examined several strains of HSV-1, although not those strains examined in this report, for their sensitivity to IFN and found no significant difference between strains. These results suggest that the difference in clinical disease caused by the different virus strains does not result from an inherent difference in the ability of the various strains to replicate in cells of the cornea, or from their ability to induce different amounts of IFN in these cells.

Our results suggest that the establishment of an antiviral state in corneal epithelial and stromal cells as a result of IFN-production by these cells in response to HSV infection is probably not a major factor in the pathogenesis of HSV ocular disease. However, these results do not eliminate the possibility that IFN may play a role in limiting the disease process. Higher concentrations of IFN produced by other cell types, for example lymphocytes responding to HSV infection, could induce an antiviral state in ocular cells. Endogenous IFN produced by epithelial cells in response to HSV infection or IFN induced in other cell types may act through a different mechanism than direct establishment of antiviral activity in corneal cells. These IFNs could act through one of the many immunomodulating activities of IFNs to reduce disease. Only α or β type IFNs have been examined in these studies; γ IFN could be important, particularly in recurrent disease as suggested by the work of Rasmussen et al and Cunningham and Merigan.

Key words: Herpes simplex virus type 1, interferon, herpetic keratitis, corneal epithelial cells, corneal stromal fibroblasts

References