Quantitation of Herpes Simplex Virus in Rabbit Corneal Epithelium

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The authors have developed an objective method for quantitation of herpes simplex virus in the corneal epithelium of rabbits. At appropriate times postinfection, full-thickness rabbit corneas were removed by trephination and subjected to one cycle of freezing and thawing. The corneal epithelium was then disrupted by sonication. The amount of infectious virus recovered from sonicated specimens was determined by an in vitro plaque assay, providing a measure of the quantity of virus present during the acute stage of herpetic keratitis. Using this technique, the authors found that the mean virus titer was reduced from $1.5 \times 10^5$ plaque forming units (pfu) per cornea in control rabbits to less than 200 pfu per cornea in rabbits treated topically for 2 days with 1% trifluridine. In contrast, instillation of 1% prednisolone acetate resulted in the persistence of higher levels of virus (275 pfu) than those observed in control rabbits (3 pfu) 4 days after the cessation of therapy. Invest Ophthalmol Vis Sci 26:873–876, 1985

Herpes simplex virus (HSV) is an important etiologic agent of ocular infection and a major cause of blindness in the United States. Following an initial episode of herpetic keratitis, approximately 40% of all patients experience one or more recurrences within 2 years.1,2 The repeated recrudescence of latent HSV results in active keratitis and corneal damage sufficient to cause visual impairment in many patients. Although topical therapy with antiviral antimetabolites is successfully employed for superficial herpetic keratitis, the search continues for drugs that can prevent stromal keratitis and eliminate latent virus. In the past, testing of antiherpetic drugs has generally relied upon biomicroscopic examination and qualitative ocular cultures of conjunctival/corneal swabs. The present study describes an objective, quantitative method for measuring infectious HSV in the rabbit corneal epithelium and for determining the effect of therapeutic agents on the replication of HSV during an acute keratitis.

Materials and Methods

Virus and Cells

The McKrae strain of HSV type 1 was kindly provided by Dr. D. Pavan-Langston. Virus pools were grown in human embryonic lung cells infected at a multiplicity of infection equal to 0.5. Infectious virus was routinely titered on TC-7 cells, a continuous monkey kidney cell line. All cells were grown at 37°C in Eagle’s minimal essential medium (Gibco) supplemented with 10% fetal calf serum (FCS, Whittaker MA Bioproducts; Walkersville, MD), 110 U/ml penicillin and 110 μg/ml streptomycin sulfate.

In Vivo Infections

The corneas of New Zealand white rabbits were bilaterally infected with approximately $10^6$ plaque forming units (pfu) of HSV in 0.035 ml phosphate buffer solution (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 8.3 mM glucose, and 1% FCS). Inoculated eyes were closed and gently massaged for 15 sec. Mock infected eyes were inoculated with 0.035 ml PBS.

Fluorescein stained corneas were examined daily using a slit-lamp biomicroscope and the extent of corneal epithelial involvement was scored on a scale of 0 to 3 as follows: 0, clear or non-distinct punctate staining; 1, dendritic processes involving less than 25% of the cornea; 2, multiple, well-developed dendrites covering up to 50% of the cornea; 3, geographic ulcers and dendrites covering more than 50% of the

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cornea. On the appropriate day, rabbits were killed with 3 ml intravenous pentobarbital (65 mg/ml). To avoid experimental bias, prenumbered rabbits were killed in numerical sequence. Rabbits which failed to exhibit biomicroscopically apparent lesions by the sixth day postinfection were excluded from further study.

Corneas were removed using a 10-mm trephine and stored frozen at −70°C in 3 ml PBS supplemented with antibiotics. Upon thawing the corneas were sonicated at 0.8 amps for 5 min in a Raytheon DF101 Sonic Oscillator (Raytheon; Waltham, MA). Rose bengal staining of sonicated corneas indicated that this treatment resulted in the sloughing of 80–90% of the corneal epithelium and produced cellular degeneration in the remaining 10–20%. The sonicated virus suspension was centrifuged for 5 min at approximately 600 × g to remove cellular debris and stored at −70°C until assayed for infectious virus. The pellet, resuspended in PBS, was also saved in order to determine the amount of virus which remained associated with cellular debris. A microscopic examination of resuspended pellets revealed no intact cells.

All rabbit experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

Drug Treatments

Two schedules for the administration of therapeutic agents were utilized. Three days postinfection, rabbits were microscopically examined and separated into groups composed of rabbits with equivalent levels of corneal involvement. On days 3 and 4 postinfection, eyes were treated with 0.035 ml 1% trifluridine (Virgoptic, Burroughs Wellcome; Research Triangle Park, NC), 1% prednisolone acetate (Econopred Plus, Alcon Laboratories; Fort Worth, TX), or balanced salt solution (BSS, Alcon Laboratories). Drugs were administered six times daily at 2-hr intervals. Rabbits treated in this manner were examined and killed on day 5. Rabbits on the second schedule were treated 6 times daily at 2-hr intervals on postinfection days 4 through 8, inclusive, and were examined and killed on days 10 or 12. In all instances, both eyes of each animal were treated with the same drug. The Mann-Whitney U-test was performed as described by Siegel to determine differences between treated groups. Values less than 0.05 were considered significant.

In Vitro Plaque Assay

Virus titers were determined using a plaque assay similar to that described by Zamansky et al. Confluent TC-7 cultures in 60-mm petri dishes were washed once with PBS prior to infection with 0.2 ml of appropriate dilutions of sonicated specimens. Following a 90-min absorption period at 37°C, virus was removed and the cells incubated in medium containing 0.03% human gamma globulin (Wyeth; Philadelphia, PA). Three days postinfection, the cultures were fixed with 95% ethanol and stained with 8% Giemsa stain. Using a dissecting microscope, virus plaques were counted in two to four plates for the determination of the virus titer of each cornea.

Results

The concentration of infectious virus recovered from the untreated rabbit cornea during the acute phase of herpetic keratitis is shown in Figure 1. Although the rabbits were inoculated with 10⁶ pfu, the amount of HSV recovered during the first two days of infection never exceeded 10⁴ pfu per individual cornea. The amount of virus synthesized increased rapidly after day 2 and reached a maximum titer between days 5 and 7. By day 11, very little virus could be recovered from most eyes. The high mean value observed on day 12 was caused by one of eight corneas that yielded 2.7 × 10⁵ pfu; five of the remaining seven corneas yielded no virus. The extent of corneal epithelial involvement, also shown in Figure 1, followed a pattern similar to that of virus synthesis. However, changes in the occurrence of lesions generally appeared to lag one day behind changes in virus titer. Less than 3% of the total recovered virus remained associated with cellular debris. Throughout this study no virus was recovered from mock infected eyes.

The effect of treatment with 1% trifluridine or 1% prednisolone acetate on HSV synthesis is shown in Table 1. Due to the broad range of infectious virus recovered within individual groups of rabbits, we have presented the median values as well as the geometric means of the virus titers in treated and untreated corneas. Topical treatment for only 2 days with 1% trifluridine, a well-established antitherpetic agent, resulted in a dramatic decrease in virus titer compared to rabbits receiving BSS. Only three of 18 trifluridine-treated corneas yielded more than 5,000 pfu, whereas 12 of 16 BSS-treated corneas contained more than 100,000 pfu. Treatment for 2 days with 1% prednisolone acetate, an anti-inflammatory agent, did not immediately effect virus synthesis (12 of 16 corneas yielded more than 100,000 pfu). However, longer treatments with prednisolone acetate resulted in significantly (P < 0.05) higher levels of virus for extended periods of time, increased virus titers persisting for at least 4 days after treatment had ceased. This was most dramatically demonstrated by corneas removed at 12 days postinfection. At this time, 16 of 20 BSS-treated corneas yielded no virus, whereas
prednisolone acetate treatment resulted in no virus in only four corneas and between 3,799 and 213,000 pfu in eight eyes.

**Discussion**

The assay described in the present study is an objective method by which HSV can be quantitated in the corneal epithelium of rabbits. The rabbit model system was selected because herpetic corneal infections in rabbits closely resemble those of humans, and the efficacy of drug therapy in rabbits has correlated well with that in humans.²⁻⁵⁻⁶ In the past, animal testing of antiviral agents has generally relied upon impressions obtained by biomicroscopic examination of treated eyes and/or the induction of cytopathic effects in cultured cells inoculated with swabs of the cornea and conjunctival fornix. More recently, Green and Dunkel described an assay in which HSV in the preocular tear film could be quantitated.⁷ This assay, which is based upon a mathematical prediction of virus titers from the rated degree of cytopathic effects observed following infection of cultured cells, has been utilized to evaluate the effect of acyclovir on the shedding of HSV into the preocular tear film of rabbits.⁷⁻⁸

It is clear from Table 1 that the amount of virus varied over a broad range within each group of rabbits killed on a particular day. Although we cannot be certain of the source of this variation, it may be influenced by such factors as the proportion of the initial virus inoculum that actually infects corneal epithelial cells and the individual rabbit’s immune system.

**Table 1.** Effect of drug therapy on virus titer in the corneal epithelium

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Day killed</th>
<th>No. of eyes</th>
<th>Median value (pfu)</th>
<th>Geometric mean</th>
<th>P values‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced salt solution</td>
<td>5</td>
<td>16</td>
<td>281,250 (195-873,750)</td>
<td>147,911</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Trifluridine 1% Days 3 and 4</td>
<td>5</td>
<td>18</td>
<td>71 (0-240,000)</td>
<td>182</td>
<td>ns§</td>
</tr>
<tr>
<td>Prednisolone acetate 1% Days 3 and 4</td>
<td>5</td>
<td>18</td>
<td>210,000 (8,250-1,061,250)</td>
<td>87,096</td>
<td>ns§</td>
</tr>
<tr>
<td>Balanced salt solution Days 4 through 8, inclusive</td>
<td>10</td>
<td>20</td>
<td>214 (0-42,600)</td>
<td>269</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Prednisolone acetate 1% Days 4 through 8, inclusive</td>
<td>10</td>
<td>18</td>
<td>21,750 (0-331,500)</td>
<td>3,311</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Balanced salt solution Days 4 through 8, inclusive</td>
<td>12</td>
<td>20</td>
<td>0 (0-39,000)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Prednisolone acetate 1% Days 4 through 8, inclusive</td>
<td>12</td>
<td>20</td>
<td>229 (0-213,000)</td>
<td>275</td>
<td></td>
</tr>
</tbody>
</table>

* Six topical doses (0.035 ml) were administered at 2-hr intervals on the days indicated.

† Median values, extremes given in parentheses; for determination of the geometric mean, 0 values were set equal to 1.

‡ P values refer to comparisons between rabbits treated with balanced salt solution or the indicated drugs for the same period of time and killed on the same day.

§ ns: not significant.
response to infection. The low level of virus recovered during the first 2 days postinfection (Fig. 1) probably reflects the small amount of virus that achieves the requisite contact with corneal epithelial cells to permit viral attachment and penetration. As Figure 1 demonstrates, changes in the severity of epithelial lesions appeared to lag 1 day behind changes in virus titers; the maximal clinical effect was observed 1 day after maximal virus titers were attained. This 24-hr interval appears necessary for virally induced cellular damage to become clinically apparent. During the course of our experiments, we occasionally encountered large discrepancies between clinical appearance and virus titers. For example, we observed eyes that failed to exhibit lesions on the day of killing but yielded as much virus as eyes in which multiple dendrites were present. These results were not caused by the presence of metaherpetic infections since differentiation between active herpetic disease and metaherpeic ulcers was made during biomicroscopic examination using the criteria described by O'Day. Although the reason for such discrepancies remains unclear, it is possible that lower than anticipated virus titers in severely affected eyes may occur if the keratitis is characterized by a significant loss of epithelium.

We investigated the effect of 1% trifluridine and 1% prednisolone acetate on corneal virus titers to determine if our results would agree with the clinical experience in humans. Topically applied trifluridine, a clinically effective treatment for herpetic dendritic keratitis, significantly (P < 0.002) reduced the amount of virus in the cornea of rabbits. Corticosteroids are sometimes used to reduce inflammation during stromal keratitis. However, the physician must exert great caution in using these drugs since they appear to exacerbate epithelial infections. We have found a persistence of significantly (P < 0.05) more infectious virus for extended periods of time in rabbits exposed to prednisolone acetate for 5 days. This result confirms earlier studies on the effect of corticosteroid in rabbit herpetic keratitis. It was not possible to determine from our limited testing if the persistence of virus resulted from an enhancement of HSV replication or an inhibition of the immune response. These experiments suggest, however, that our quantitative assay will prove to be very useful in testing the efficacy of new antiviral agents as well as the effect that anti-inflammatory agents may have on viral epithelial infections in the cornea.

**Key words:** herpes simplex virus, keratitis, herpetic keratitis, quantitative assay

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**References**