Endothelial Barrier Function and Na⁺/K⁺-ATPase Pump Density in Herpetic Stromal Disease

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Purpose. Corneal edema is a significant component of the various forms of herpes simplex virus type 1 (HSV-1)-induced stromal disease. Maintenance of corneal thickness, a reflection of corneal hydration, depends on a physical barrier formed by endothelial cell-cell junctions and by the activity of Na⁺/K⁺-ATPase pumps that regulate ion flux and thus influence water movement through this cell layer. These functions were measured in corneas with increased corneal thickness caused by HSV-1-induced stromal disease to determine their contribution to the pathogenesis of the edema.

Methods. Stromal disease with corneal edema was induced in rabbits by intrastromal injection of the RE strain of HSV-1. At various times after infection, during the development of and recovery from stromal disease, endothelial barrier function and Na⁺/K⁺-ATPase pump sites were measured in excised rabbit corneas.

Results. The endothelial permeability coefficient, \( K_e \), for \(^{14}C\)-dextran, \(^{3}H\)-inulin, and \(^{14}C\)mannitol, were not altered significantly during periods of maximal corneal edema and stromal disease. Endothelial Na⁺/K⁺-ATPase pump density, as measured by ouabain binding, showed a statistically significant (\( P < 0.05 \)) decrease in HSV-1-infected corneas during peak edema compared to mock antigen-injected or un.injected control corneas. Pump density returned to baseline values by 24 days after infection, concurrent with the resolution of corneal edema.

Conclusions. These results indicate that corneal endothelial barrier function was not altered in this form of HSV-1-induced stromal edema; however, pump density was reduced significantly.
lie in dysfunction of the endothelial layer. Although endothelitis may occur in this form of disease, it is unclear whether it is caused by direct cytolytic attack of virus on a limited number of endothelial cells or as a result of effects of cytokines or other mediators of the immune and inflammatory responses on cell function. Data presented in the literature do not convincingly document that viral infection of the endothelium is the cause of the endothelial pathology observed.\(^5\),\(^6\) One recent study suggests that HSV DNA is not detectable in endothelial cells by immunoperoxidase methods at any time after infection, but plasma cells invaded to each eye. A 20-μl sample containing 10^3 plaque-forming units of HSV-1 was injected intrastromally into both eyes of a rabbit using a Hamilton microliter syringe and a 30-gauge needle as previously described by Metcalf and Kaufman.\(^31\) Control corneas received 20 μl of similarly diluted mock antigen. Gentamycin ophthalmic solution (Allergan Pharmaceuticals, Irvine, CA), one drop per eye, was administered immediately after injection. The use of animals in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with a protocol approved by the institutional animal care committee.

**Evaluation of Disease**

All rabbits were examined by slit lamp biomicroscopy three times a week. Corneal epithelial disease was assessed after fluorescein staining and quantitated on a 0 to 4+ scale based on the percentage of the cornea involved according to the method of Wander et al.\(^32\) Stromal disease was evaluated by measurement of cor-

In this study, we quantitate Na⁺/K⁺-ATPase binding sites by \(^3\)H-ouabain binding as an indicator of pump numbers\(^20\)–\(^28\) and evaluate endothelial permeability by diffusion of radiolabeled markers of differing molecular size as a measure of barrier activity.\(^27\)–\(^30\) Results document that the binding of \(^3\)H-ouabain is significantly decreased during periods of peak HSV-1-induced edema and that corneal barrier function is not compromised.

**MATERIALS AND METHODS**

**Virus**

The RE strain of herpes simplex virus type 1 (HSV-1) was subcultured in RK-13 cells, a rabbit kidney cell line. Virus was titrated by plaque formation on Vero monkey kidney cells. Mock antigen was prepared from uninfected RK-13 cell lysate. Virus was diluted in phosphate-buffered saline for intrastromal corneal inoculation.

**Corneal Inoculation**

Specific pathogen-free male New Zealand white rabbits (each weighing 2 to 3 kg) were anesthetized by intramuscular injections of 5 mg ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) and 10 mg xylazine HCl (Phoenix Pharmaceuticals, St. Joseph, MO). Before infection, all eyes were examined by slit lamp and corneal thickness determined using an ultrasonic pachymeter (DGH Technology, Frazer, PA). Corneas that were clear and without evidence of infection or trauma were used in the study.

Before intrastromal injection of virus, topical 1% proparacaine (Alcon, Humacao, PR) was administered to each eye. A 20-μl sample containing 10^3 plaque-forming units of HSV-1 was injected intrastromally into both eyes of a rabbit using a Hamilton microliter syringe and a 30-gauge needle as previously described by Metcalf and Kaufman.\(^31\) Control corneas received 20 μl of similarly diluted mock antigen. Gentamycin ophthalmic solution (Allergan Pharmaceuticals, Irvine, CA), one drop per eye, was administered immediately after injection. The use of animals in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with a protocol approved by the institutional animal care committee.
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were used as selection criteria for appropriate stages of development and recovery of stromal disease. Animals were killed and corneas were removed for evaluation when edema was developing (corneal thickness = 450 to 500 μm), at peak edema (500 to ≥800 μm), and during recovery from disease (400 to 500 μm).

Endothelial Barrier Function
At the three stages during the development of and recovery from HSV-1-induced stromal disease, rabbits were killed with an overdose of pentobarbital (Beuthanasia-D Special; Schering-Plough, Kenialworth, NJ). Eyes were enucleated with lids and conjunctiva, and the corneal epithelium was removed with a Gill knife. Deepithelialized corneas with a 2- to 3-mm scleral rim were secured on teflon rings and mounted between two water-jacketed lucite perfusion chambers for endothelial permeability studies. Glutathione bicarbonate-Ringer's (GBR) solution was used to perfuse the deepithelialized side of the cornea. GBR containing 1 μCi/ml [3H]-inulin (MWt 5000 to 5500) and either 1 μCi/ml of a high-molecular weight marker molecule, [14C]-carboxy-dextran (MWt 50 to 70 kDa), or 1 μCi/ml of a low-molecular weight marker molecule, [14C]-carboxy-mannitol (MWt 182.2), were added to the endothelial side of the cornea. All radiolabeled markers were from DuPont NEN (Wilmington, DE), and the radiochemical purity was checked by high-performance liquid chromatography analysis in our laboratory. Constant mixing and pH of 7.3 were maintained by an air-lift siphon using a 95% air–5% CO2 mixture. A circulating water bath maintained a constant temperature of 37°C.

Corneas were allowed to equilibrate for 1 hour. Solution from the deepithelialized side or "cold" reservoir (1.5 ml) was then collected and discarded. This chamber was refilled with 1.5 ml GBR and immediately collected to provide a background count. At 30-minute intervals during a 3-hour period, the fluid in the epithelial-side reservoir was collected, and the reservoir was flushed with 1.5 ml GBR. Each sample was collected and weighed, and radioactivity was determined. After the final epithelial-side sample was collected, the endothelial-side reservoir (1.5 ml) was collected. As positive controls, normal, uninfected corneas (endothelium removed with cotton-tipped applicator before permeability studies; n = 11) and normal eyes (intact epithelia; n = 9) were perfused. All samples were counted using a scintillation counter with a dual-label program. Endothelial permeability, Ktrans, values were calculated for each isotope according to the method of Maffly et al using the following formula:

\[ K_{\text{trans}} = \frac{\text{increase in dpm on epithelial side}}{\text{concentration of dpm on endothelial side} \times \text{area of cornea} \times \text{time}} \]

A mean Ktrans value (±SEM) for HSV-1-infected and control corneas at each stage of development and recovery from stromal edema was determined.

Quantitation of Na+/K+-ATPase Pump Number
 Ouabain binds specifically and with high affinity to Na+/K+-ATPase pump sites on a one-to-one basis. [3H]-Ouabain (15.4 Ci/mmol, MWt 584.6) was analyzed by high-performance liquid chromatography to determine the purity using a C18-μm Bondapak column (10-μm particle diameter) (Waters Associates, Milford, MA) eluted at a flow rate of 1.0 ml/minute with 84% H2O:16% acetonitrile. Eluate was monitored by the absorbance at 220 nm and 240 nm and for radioactivity. Ouabain used in these studies was determined to be 97% pure.

Corneal endothelial Na+/K+-ATPase pump sites were measured as described by Geroski and Edelhauser. Briefly, eyes were enucleated, the corneal epithelium was removed with a Gill knife, and corneas were excised with a 1- to 2-mm scleral rim. Each deepithelialized cornea was incubated for 3 hours at 37°C in 3.5 ml of K+-free bicarbonate-Ringer’s solution consisting of the following (g/l): NaCl, 6.801; CaCl2·2H2O, 0.153; MgCl2·6H2O, 0.158; NaH2PO4, 0.103; NaHCO3, 2.458; and glucose, 0.903. [3H]-Ouabain was then added to 0.5 μM, a concentration at which ouabain-sensitive pump sites are saturated and nonspecific uptake is negligible. To estimate the nonspecific uptake of ouabain in the extracellular space, 1.0 μCi of inulin ([14C]-carboxyl, 2.3 mCi/g) was added to each 3.5-ml incubation mixture.

At the end of the 3-hour incubation period, the corneas were removed from the incubation medium and briefly rinsed three times in ice-cold ouabain-free, potassium-free incubation medium. A 9-mm corneal button was trephined from each cornea, and the endothelium plus Descemet’s membrane was stripped carefully from the stroma. Each endothelial sheet was solubilized overnight in 1.0 ml of 0.3 N NaOH at 37°C. Uptake of [3H]-Ouabain and [14C]-Inulin was measured by liquid scintillation counting. Na+/K+-ATPase pump density for each endothelial sheet was expressed as fmol/mn2.

Endothelial Cell Morphometry
Corneal endothelial cell area, density, and hexagonality were evaluated in alizarin red-stained flatmounts.
of endothelium and analyzed by computer-assisted digitization of 100 contiguous cells. 35

**Statistical Analysis**

Data were analyzed using Sigma Stat (Jandel Scientific, San Rafael, CA). Normally distributed data were tested for significant differences using one-way analysis of variance and Bonferroni t-test; data not normally distributed were evaluated by nonparametric analysis of variance (Kruskal–Wallis one-way analysis of variance), and groups were compared by Dunn’s method.

**RESULTS**

**Assessment of Barrier Function**

Corneal thickness in HSV-1-infected eyes increased significantly relative to either normal, uninjected controls or mock-antigen-infected controls, reaching a maximum thickness approximately 13 days after infection and returning to normal by 24 days after infection. 36 During the period of maximal edema, barrier function, as measured by $K_{\text{trans}}$ for a small molecule such as mannitol, was not significantly altered in edematous, HSV-1-infected corneas (mean thickness, 601 ± 24 μm, $n = 6$) compared to mock-antigen-injected controls of normal thickness (387 ± 5 μm, $n = 4$). $K_{\text{trans}}$ for mannitol in the HSV-1-infected corneas was $10.0 \pm 1.1 \times 10^{-6}$ cm/second, whereas in the control corneas it was $10.5 \pm 0.7 \times 10^{-6}$. Barrier function was not altered during development of disease 6 to 7 days after infection, at peak disease 12 to 14 days after infection, or during recovery from disease as measured with two larger molecular probes, inulin and dextran (Figs. 1, 2). When eyes were grouped according to the corneal thickness at the time of sacrifice, no significant differences relative to control values were detected. Corneas in the 400- to 450-μm thick range ($n = 8$) had inulin $K_{\text{trans}}$ values of $3.1 \pm 0.1 \times 10^{-6}$ cm/second and dextran $K_{\text{trans}}$ values of $0.17 \pm 0.004 \times 10^{-6}$ cm/second. Corneas with thickness values in the 450- to 600-μm range ($n = 7$) had $K_{\text{trans}}$ inulin values of $3.3 \pm 0.5 \times 10^{-6}$ cm/second and $K_{\text{trans}}$ dextran values of $0.17 \pm 0.05 \times 10^{-6}$ cm/second. These data indicate clearly that corneal barrier function remained intact in this form of disciform-like HSV-1-induced disease. In a second study, corneas taken at various stages of disease at 6, 12, 19, and 39 days after infection also showed no significant differences in barrier function among the groups. For example, at the time of peak disease 12 days after infection, when corneal thickness was 591 ± 11 μm in the infected group, the $K_{\text{trans}}$ for dextran was $0.23 \pm 0.03 \times 10^{-6}$ cm/second ($n = 4$), whereas mock-antigen-injected controls with a corneal thickness of 374 ± 5 μm had a $K_{\text{trans}}$ of $0.28 \pm 0.06 \times 10^{-6}$ cm/second ($n = 4$). The values of $K_{\text{trans}}$ found in our studies were similar to those previously reported for normal rabbit corneas and mechanically debrided corneas (Figs. 1, 2). 18,21,25

**Measurement of Na⁺/K⁺-ATPase Pump Sites**

The number of ouabain-binding sites represents the number of Na⁺/K⁺-ATPase molecules accessible to

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**FIGURE 1.** Corneal endothelial barrier function as measured by permeability to inulin ($K_{\text{trans}}$ in cm/second). Each bar represents the mean ± the standard error of the mean. Controls represent uninjected and uninfected corneas and untreated corneas from which endothelium was mechanically removed. Number of eyes per group: controls ($n = 4$), 6 days ($n = 8$), 12 to 14 days ($n = 6$), and 19 days ($n = 4$).

**FIGURE 2.** Corneal endothelial barrier function as measured by permeability to dextran ($K_{\text{trans}}$ in cm/second). Controls represent uninjected and uninfected corneas and untreated corneas from which endothelium was mechanically removed. Number of eyes per group: controls ($n = 4$), 6 days ($n = 8$), 12 to 14 days ($n = 6$), and 19 days ($n = 4$).

TABLE I. Ouabain Binding in HSV-1-Infected and Uninfected Rabbits

<table>
<thead>
<tr>
<th>Rabbit Status</th>
<th>Day Postinfection</th>
<th>Corneal Thickness (μm)*</th>
<th>Ouabain Bound (fmol/mm²)*</th>
<th>Number of Eyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>NA</td>
<td>370 ± 3</td>
<td>22.7 ± 2.6</td>
<td>7</td>
</tr>
<tr>
<td>&quot;normal&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock antigen</td>
<td>7</td>
<td>370 ± 7</td>
<td>26.2 ± 2.6</td>
<td>4</td>
</tr>
<tr>
<td>HSV-infected</td>
<td>7</td>
<td>485 ± 19†</td>
<td>20.0 ± 2.1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock antigen</td>
<td>13</td>
<td>402 ± 6</td>
<td>24.5 ± 1.8</td>
<td>11</td>
</tr>
<tr>
<td>HSV-infected</td>
<td>13</td>
<td>607 ± 42†</td>
<td>17.0 ± 0.9†</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock antigen</td>
<td>24</td>
<td>376 ± 6</td>
<td>20.0 ± 2.0</td>
<td>4</td>
</tr>
<tr>
<td>HSV-infected</td>
<td>24</td>
<td>431 ± 31</td>
<td>21.4 ± 2.6</td>
<td>4</td>
</tr>
</tbody>
</table>

NA = not applicable.
* Mean ± SEM.
† Significant difference determined by Kruskal–Wallis one-way analysis of variance comparing all groups by Dunn’s method to either "normal" corneal thickness or thickness of mock antigen-injected controls indicates corneas are significantly thicker (P < 0.05).
‡ Significant difference from uninjected "normal" and mock-antigen-injected controls taken 7 days after infection determined by one-way analysis of variance and comparison of all groups by Bonferroni test method (P < 0.05).

ouabain on a one-to-one basis. The literature indicates that in a "normal" adult rabbit, 20 fmol of ouabain/mm² specifically bind to Na⁺/K⁺-ATPase in endothelial cells.22 In normal, uninfected, and uninjected rabbit corneas, measurements of ouabain binding in our studies were found to be in good agreement with those previously published21-23 (Table 1). Endothelial cell number was not reduced at any time after HSV-1 infection in eyes with disciform disease, as we have previously reported.11 In a sample of eyes used in this study, there was no significant difference in cell density between three mock antigen-injected controls (3771 ± 21 cells/mm² and a corneal thickness of 383 ± 8 μm, n = 3) and HSV-infected corneas (3769 ± 60 cells/mm² and corneal thickness of 582 ± 84 μm, n = 3). We did observe, however, that significantly less ouabain was bound per mm² of corneas during the period of peak edema in infected corneas than in uninjected corneas. Pump site density returned to the normal range on return to normal corneal thickness. In further studies, an additional group of rabbits (n = 9) was infected and killed during the peak period of disease as measured by increased corneal thickness. These eyes had a mean corneal thickness of 517 ± 26 μm (n = 17 eyes) and bound 15.6 ± 1.4 fmol of ouabain/mm², a level significantly less than that detected in corneas within the normal thickness range (n = 16; corneal thickness = 566 ± 16 μm; ouabain bound = 19.9 ± 2.7 fmol/mm²).

DISCUSSION

Edema in corneas with intact epithelium can occur as a result of a breakdown in the physical barrier of the endothelium or as a result of the alteration of the metabolic activities in the endothelial cells. Our findings indicate that the latter mechanism occurs in HSV-1-induced stromal disease. Na⁺/K⁺-ATPase pump number in the corneal endothelium of HSV-1-infected rabbits with disciform-like disease was reduced by 22% to 33% during periods of maximal edema. By days 19 to 24 after infection, when corneas returned to normal thickness, pump number also returned to the range found in mock antigen-injected controls or uninjected "normal" corneas. The method of measurement of pump sites does show some degree of variation among populations of samples. Ouabain binding is highly accurate, but sample-to-sample variation exists. Thus, caution must be exercised in interpreting the meaning of small changes in pump site density.

Previous studies1,2,5,8-10,11 suggest that endothelial cell loss is not a part of disciform disease as it may be in more necrotic forms of disease. Some studies have implied that endothelial cell loss occurs in severe disciform forms of disease, but not in less severe disease in humans.6 Similarly, others have cited the isolation of virus from the anterior chamber as evidence of virus replication in the endothelium.5 Virus isolated from the anterior chamber, however, could arise from many other sites. Virus clearly can replicate in corneal endothelial cells in cell culture.36 We have demonstrated by in situ hybridization, immunocytochemical antigen detection, and isolation of virus that endothelial cells are infectable in excised corneas infected in situ, but we could not find virus in endothelium in our rabbit model using these techniques (O'Brien, unpublished data, 1990, and ref. 11). We also could not detect a
measurable loss of cells in the corneas of animals at the peak of edema.\textsuperscript{11,Corren data} The method used—digitization of alizarin red-stained endothelial flatmounts—should detect changes in cell number of approximately 10% to 15%.\textsuperscript{36,37} Therefore, it seems reasonable that virus did not destroy large numbers of endothelial cells and that the decrease in pump site density observed in our studies (maximum loss, 33%) was not caused by a loss of endothelial cells. One would anticipate that if approximately one third of the cells were lost, barrier function would change and/or morphometry would be altered, but neither was detected. In humans, if endothelial cell loss occurred, it would lead to decompensation of the cornea, a feature not usually seen in disciform disease. Therefore, if lytic viral infection of the endothelium occurs in disciform disease, it must be limited to prevent loss of large numbers of endothelial cells. Even if cell loss was clustered rather than generalized and, therefore, undetected by morphometric analysis, barrier function would still be detectably altered.\textsuperscript{38}

Corneal hydration is influenced by the physical barrier formed by cell–cell gap and tight junctions in the endothelium. Studies indicate that it is the tight junctions in the apical membrane region that principally compose the barrier to diffusion of molecules larger than water.\textsuperscript{17} We detected no effect of HSV-1 infection on this barrier function. The $K_{\text{trans}}$ values for inulin, dextran, and mannitol determined in our laboratory for both uninfected and infected corneas were similar to those reported by others\textsuperscript{21,20} for normal rabbit corneas using the lucite block diffusion technique. Therefore, it is clear that diffusion (or leak) of molecules as small as mannitol to molecules as large as dextran was not altered during the periods of edema induced by HSV-1 infection. Based on wound healing studies of others using rabbits, one would not anticipate normal barrier function to become established while the cornea was at or near maximum thickness.\textsuperscript{38} It has been shown that treatment of the cornea with ouabain, resulting in inhibition of the Na$^+$/K$^+$-ATPase, does not alter the permeability barrier,\textsuperscript{17,39} clearly indicating that pump function can be inhibited independently of an effect on barrier function.

Although there is a correlation between decreased pump number and increased corneal thickness, these findings do not prove absolutely that the decrease in Na$^+$/K$^+$-ATPase pump number is entirely responsible for the increased corneal thickness. Corneal hydration clearly is influenced by several factors. Other endothelial ion exchangers, in addition to Na$^+$/K$^+$-ATPase, may have functional roles in the regulation of corneal hydration. Some evidence suggests that a CI$^-$/HCO$_3$- exchanger, as well as a Na$^+$/H$^+$ exchanger, contribute to corneal dehydration.\textsuperscript{18,40} Our studies would not detect changes in these exchangers because ouabain binds specifically to Na$^+$/K$^+$-ATPase pumps and would not be expected to bind to other exchange molecules.

The cause for the loss of Na$^+$/K$^+$-ATPase is unclear. One study indicated that HSV-1 infection can cause the loss of ouabain-inhibitable Na$^+$/K$^+$-ATPase activity in Vero cells in culture,\textsuperscript{41} but the mechanism was not determined. Because we cannot find evidence for virus replication in endothelial cells in our model, the direct inhibition of Na$^+$/K$^+$-ATPase activity by virus does not appear likely. Ocular inflammation, such as that induced by intracameral injection of bovine serum albumin, can result in corneal edema associated with Na$^+$/K$^+$-ATPase loss in the absence of morphometric changes in the endothelium.\textsuperscript{21} It has been hypothesized that mediators of inflammation can influence endothelial cell function. Arachidonic acid metabolites, such as prostaglandin E$_2$, can alter endothelial cell shape and presumably junctional complexes.\textsuperscript{16} However, morphometric parameters of the endothelial cells and junctional complexes appear not to be influenced in our studies. Another arachadonic acid metabolite, 12(R) hydroxyeicosatetraenoic acid, has been shown to influence specifically the activity of the corneal endothelial Na$^+$/K$^+$-ATPase and to induce corneal swelling.\textsuperscript{15} It is unknown whether significant amounts of this compound or other mediators of inflammation are produced after HSV-1 infection of the cornea. In addition to arachadonic acid metabolites, various cytokines and lymphokines are produced by cells on infection by HSV-1.\textsuperscript{12,14} Some of these molecules, such as interferon-$\gamma$ and tumor necrosis factor-$\beta$, could influence gene expression in endothelial cells and may, therefore, influence endothelial cell function.

Our data document that corneal edema associated with HSV-1 corneal infection is likely the result of inhibition of Na$^+$/K$^+$-ATPase function. Endothelial layer barrier function was not measurably compromised, endothelial cell loss was not measurable, and direct cytolysis of the virus on the endothelium was not demonstrable. We think the data support the hypothesis that corneal edema is the result of decreased Na$^+$/K$^+$-ATPase pump function caused by effects secondary to direct virus infection of endothelial cells.

**Key Words**

corneal edema, herpes simplex keratitis, Na$^+$/K$^+$-ATPase, virus infection

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