Herpes simplex virus type 1 (HSV-1) can infect the cornea and achieve ganglionic latency. HSV-1 can later be activated by a variety of effectors although the exact mechanism of reactivation is unknown. Rabbits harboring latent HSV-1 strain McKrae can be induced to shed virus by ocular iontophoresis of epinephrine to the cornea. No studies have been done to investigate if corneal nerves are necessary for epinephrine induction of HSV-1 ocular shedding. We did penetrating keratoplasty (PKP) in one eye each of 23 rabbits; the other eye served as an unoperated control. The surgery effectively denervates the area of the transplant for up to 90 days. Eighteen rabbits carrying latent HSV-1 strain McKrae received corneas from uninfected rabbits. Five uninfected rabbits with no latent virus received corneas from rabbits harboring latent HSV-1. On days 10–14 after penetrating keratoplasty, 24 eyes in the HSV-1 latent group and all ten uninfected eyes received iontophoresis of 0.01% epinephrine (0.8 mAmps for 8 min or 0.6 mAmps for 6 min) once daily for 3 days by means of an eye cup whose diameter was less than the diameter of the transplant. Six rabbits in the HSV-1 latent group received intravenous injections of cyclophosphamide (75 mg/kg) and dexamethasone (4 mg/kg). Following iontophoretic or immunosuppressive induction, the eyes were swabbed daily for 9 days. Of the 12 rabbits with latent virus which were treated by iontophoresis, one of the transplanted eyes and eight of the nontransplanted eyes were induced to shed virus. The mean duration of shedding in the nontransplanted eyes was 3.25 days. Of the six rabbits with latent HSV-1 treated with cyclophosphamide and dexamethasone, three of the transplanted and six of the nontransplanted eyes were induced to shed virus with a mean duration of shedding of 4 and 4.5 days respectively. None of the ten eyes (PKP or non-PKP) in the five uninfected rabbits shed virus. The results of this study suggest that intact corneal nerves are necessary for epinephrine to induce shedding of latent HSV-1 in rabbits.

Herpes simplex virus type 1 (HSV-1) produces a primary ocular infection in humans and rabbits followed by colonization of the sensory and autonomic ganglia. Reactivation of latent virus by stimuli such as upper respiratory tract infection, ultraviolet radiation, radial keratotomy, and menses results in recovery of infectious virus from the ocular surface and, sometimes, recrudescent disease in the form of epithelial lesions, stromal disease and uveitis. It is presumed that the reactivation signal emanates from the peripheral site and reaches the ganglion. Then the reactivated virus travels from the ganglia within the neck, orbit and middle cranial fossa to the effector site via neuronal connections. Axons of the sensory and autonomic nerves probably act as the conduit for reactivation signals and HSV-1. The present study was undertaken to determine if corneal nerves are necessary to provide a means of stimulating the ganglionic neurons and to determine if corneal nerves are essential for recovery of virus at the ocular surface. Rabbits harboring latent HSV-1 were employed in the study and penetrating corneal transplantation was used as the method of denervating the rabbit cornea.

### Materials and Methods

#### Virus

McKrae strain HSV-1 was propagated on primary rabbit kidney (PRK) cell monolayers and titered by plaque assay on green monkey kidney cell monolayers. The virus was frozen in small aliquots at...
Rabbits and Viral Inoculation

The unscarified corneas of New Zealand albino rabbits (1.5–2.5 kg) were inoculated with 25 μl of a suspension of HSV-1, McKrae strain (2–4 × 10^6 PFU/ml). The closed eye with the viral suspension was massaged for 20–40 seconds with care taken to avoid leakage of the suspension. Primary corneal infection, mostly epithelial keratitis, was verified by slit lamp biomicroscopic examination (SLE) on postinoculation (PI) days 4–8. Spontaneous shedding during PI days 20–39 was determined by the eye swab procedure described by Berman and Hill.12 The care and maintenance of the rabbits used in all these experiments conformed to the ARVO Resolution on the Use of Animals in Research.

Tear Film Swabs

Precocular tear film was collected from the rabbit eyes on sterile Dacron-tipped swabs by gentle rotation of the swab in the upper cul-de-sac, gently across the cornea and then into the lower cul-de-sac, where the swab was allowed to absorb tear film in the fornix for 5 seconds. The swabs were immediately placed in tissue culture tubes containing confluent PRK cell monolayers and incubated for 18–24 hr at 37°C in a CO2 incubator. Subsequently, the swabs were squeezed against the side of the tubes to remove excess medium and removed. Eagle’s minimum essential medium (KC Biological, Lenexa, KS) with 2% fetal bovine serum (1 ml) was added for nutrition and pH adjustment. The tubes were monitored daily for 7–9 days for the appearance of cytopathic effects indicative of HSV-1.

Slit Lamp Biomicroscopic Examination

SLE after staining with fluorescein was done on all eyes to evaluate corneal epithelial lesions and the status of the transplant.

Penetrating Keratoplasty

Eighteen rabbits with latent HSV-1 and five uninfected rabbits had penetrating keratoplasty performed on one eye each; the opposite eye served as unoperated control. Rabbits were anesthetized by the intramuscular injection of ketamine (20 mg/kg body weight) and xylazine (10 mg/kg body weight). The rabbits received two drops of a mixture of 2% cycloproplolate, 10% phenylephrine, and 1% tropicamide preoperatively to both eyes. A sodium heparin solution (Elkins-Sinn, Inc., Cherry Hill, NJ; 200 units/ml normal saline) was injected (0.1 ml) into the anterior chamber immediately after entering the anterior chamber with the corneal trephine. In the latency group, a 7.5 mm corneal button was removed from the rabbit’s eye, and an 8.5 mm corneal button from an uninfected rabbit was sutured into place. A larger donor button was found necessary to ensure proper wound closure and restoration of the anterior chamber. Four cardinal sutures of 10-0 nylon were placed followed by a running 10-0 nylon suture of at least 16 bites. The cardinal sutures were removed. The anterior chamber was reformed by an injection of Healon (Pharmacia, Piscataway, NJ). Garamycin (Schering, Kenilworth, NJ) drops (3 mg/ml) were instilled once at the end of the procedure in both eyes. In the uninfected rabbits, a 7.0 mm corneal button was removed from the eye, and a 7.5 mm corneal button from one of the rabbits in the latency group was sutured into position using the technique described above. At least 2 days before iontophoresis, at 8–12 days after penetrating keratoplasty, the running suture was removed.

Iontophoresis

On days 10–14 after penetrating keratoplasty, 17 rabbits had epinephrine iontophoresis (ION) performed to both eyes, daily for 3 consecutive days. The rabbits were anesthetized as for the PKP above. A small eye cup (central diameter 6 mm) was centered on the eye so that the central cylinder containing the epinephrine solution was in contact with only the transplanted tissue in the PKP eyes to ensure the highest concentration of drug was delivered to the denervated transplanted corneas. Care was taken to ensure that the eye cup did not overlap onto the recipient cornea. In the non-PKP eyes, the eye cup was placed on the center of the cornea. Epinephrine, 0.01%, was freshly prepared daily from commercial epinephrine 1% drops (Epifrin, Allergan, Irvine, CA) and sterile triple distilled deionized water. The anode (+) was placed in contact with the epinephrine solution and the cathode (−) was attached to the ear over a saline soaked gauze pad. A Medtherm electromediator (Model AE1, Medtherm Corp., Huntsville, AL) was used for iontophoresis (0.8 mAmps for 8 min or 0.6 mAmps for 6 min). Stromal haze was noted following iontophoresis with the higher current and longer treatment. Therefore, a lower current and shorter treatment time were also used to minimize damage to the tissues.

Induction by Intravenous Cyclophosphamide and Dexamethasone

On day 12 after penetrating keratoplasty six other rabbits received an intravenous injection (IV) of cy-
Table 1. Viral shedding non-PKP eyes post epinephrine iontophoresis

<table>
<thead>
<tr>
<th>Condition of iontophoresis</th>
<th>Rabbit number</th>
<th>Eye</th>
<th>PI day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Total positive days 1–9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% Epinephrine, 0.8 mAmps, 8 minutes, small eye cup</td>
<td>M94</td>
<td>OS</td>
<td>58</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>C</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>0.01% Epinephrine, 0.6 mAmps, 6 minutes, small eye cup</td>
<td>P55</td>
<td>OD</td>
<td>59</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>5</td>
</tr>
</tbody>
</table>

* = Positive spontaneous eye swab, PI day 21–39.

p = Positive eye swab post-PKP to pre-ION.

+ = Positive eye swab.

- = Negative eye swab.

C = Contaminated.

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cyclophosphamide (75 mg/kg) followed 24 hr later by an IV injection of dexamethasone (4 mg/kg) on day 13 after PKP. This procedure has been shown to induce HSV-1 shedding in rabbits. These rabbits received no direct ocular manipulation. Eye swabs were performed every day for 9 days and cultured for the presence of HSV-1.

Corneal Co-cultivation

At the termination of the experiment, selected transplanted corneas originally from rabbits carrying latent virus were removed using aseptic technique and were sectioned into four equal pieces. Corneas from rabbits harboring latent virus that were not transplanted were also co-cultivated as described. Each piece was placed in a tube or well containing Eagle’s minimum essential medium with PRK indicator cells. The specimens were incubated for 21 days to monitor for cytopathic effects.

Experimental Design

Twenty-three rabbits had PKP performed on one eye; eighteen of these harbored latent HSV-1, strain McKrae. In the latency group, PKP was performed on the side that had previously shed HSV-1 spontaneously at least once during PI days 20–39. This indicates the development of latency and spontaneous reactivation on that side. This theoretically increases the likelihood of iontophoretically induced HSV-1 ocular shedding on that side. The exception was rabbit No. M94 which had not shed in either eye spontaneously during PI days 21–39. Five rabbits were used as uninfected controls. Seventeen rabbits received iontophoresis of 0.01% epinephrine to both eyes as described above. The tear film was sampled to assay for HSV-1 by daily collection on a Dacron swab for 10 days starting on the first day of iontophoresis, prior to treatment. Epinephrine iontophoresis was done beginning from PI days 44–83 (mean = 60 days). Tables 1 and 2 show PI days for these individual rabbits.

Results

During PI days 20–39 spontaneous shedding was confirmed (at least one eye with at least one positive episode) in all but one HSV-1 infected rabbit. For the PKP eyes, the ratio of positive spontaneous swabs (pre-PKP) per total swabs was 27/221 (12.2%); for the non-PKP eyes, it was 23/246 (9.3%). By chi square analysis there was no significant difference between PKP and non-PKP eyes (P > 0.05). For the PKP eyes 17/18 shed virus pre-PKP while 8/18 eyes in the non-PKP eyes shed spontaneously pre-PKP. By chi square analysis there was a significant difference between PKP and non-PKP eyes (P < 0.05). Whenever possible, the PKP eye was chosen as one which had shed, thus confirming latency on that side and accounting for the difference in spontaneous shedding between PKP and non-PKP eyes. Post-PKP and pre-treatment 4/18 non-PKP and 5/18 PKP eyes in the latent HSV-1 group shed HSV-1 with a mean duration of shedding of 1 and 1.4 days respectively. By chi square analysis, there was no significant difference...
### Table 2. Viral shedding PKP eyes post epinephrine iontophoresis

<table>
<thead>
<tr>
<th>Condition of iontophoresis</th>
<th>Rabbit number</th>
<th>Eye</th>
<th>PI day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Total positive days 1-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% Epinephrine, 8 minutes, small eye cup</td>
<td>M94</td>
<td>OD</td>
<td>58</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M95</td>
<td>OD*</td>
<td>58</td>
<td>C</td>
<td>C</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>M96</td>
<td>OS*</td>
<td>44</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>M99</td>
<td>OD*</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
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<tr>
<td></td>
<td>P4</td>
<td>OD*</td>
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<td>P10</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>0.01% Epinephrine, 0.6 mAmps, 6 minutes, small eye cup</td>
<td>P55</td>
<td>OS*</td>
<td>59</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>P59</td>
<td>PD*</td>
<td>59</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td></td>
<td>P61</td>
<td>OD*</td>
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<td>-</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>P63</td>
<td>OS*</td>
<td>59</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>P64</td>
<td>PD</td>
<td>59</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Eyes +/Total Eyes: 0/10 0/10 0/9 0/12 0/12 1/10 0/8 0/8 0/10 1/100

* = Positive spontaneous eye swab, Pl day 21-39.
p = Positive eye swabs post-PKP to pre-ION.
+ = Positive eye swab.
- = Negative eye swab.
C = Contaminated.
| = Iontophoresis.

Between PKP and non-PKP eyes (P > 0.05). After epinephrine iontophoresis in the infected rabbits, PKP eyes had a ratio of positive swabs to total swabs of 1/100, (1%), non-PKP eyes had a ratio of 26/102, (25%). By chi square analysis the difference was statistically significant (P < 0.01). The mean duration of shedding for the non-PKP eyes was 3.25 days.

After intravenous treatment with cyclophosphamide and dexamethasone in the group of six HSV-1 latently infected rabbits, PKP eyes had a ratio of positive swabs to total swabs of 12/53 (23%), non-PKP eyes had a ratio of 27/54 (50%). By chi square analysis the difference was statistically significant (P < 0.01). The mean duration of shedding was 4 and 4.5 days for the PKP and non-PKP eyes respectively.

When compared to the rabbits which received iontophoresis of epinephrine, the PKP eyes (1/100) differed significantly (P < 0.001) from those PKP eyes treated with intravenous cyclophosphamide and dexamethasone (12/53). By chi square analysis non...

### Table 3. Viral Shedding PKP and non-PKP eyes after intravenous injection of cyclophosphamide and dexamethasone

<table>
<thead>
<tr>
<th>Group</th>
<th>Rabbit number</th>
<th>Eye</th>
<th>PI day</th>
<th>CX</th>
<th>DX</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Total positive days 0-8</th>
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<tbody>
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<td>PKP</td>
<td>T74</td>
<td>OD*</td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T77</td>
<td>OD*</td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T78</td>
<td>OD*</td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>2</td>
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<tr>
<td></td>
<td>T88</td>
<td>OS*</td>
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<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Eyes +/Total Eyes</td>
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<td>0/6</td>
<td>1/6</td>
<td>2/5</td>
<td>3/6</td>
<td>2/6</td>
<td>2/6</td>
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<td>1/6</td>
<td>12/53</td>
<td></td>
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<tr>
<td>Non-PKP</td>
<td>P74</td>
<td>OS*</td>
<td>64</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>4</td>
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<td>OS*</td>
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<td>P85</td>
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<tr>
<td></td>
<td>P88</td>
<td>OD*</td>
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<td>Eyes +/Total Eyes</td>
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<td>4/6</td>
<td>3/6</td>
<td>3/6</td>
<td>27/54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = Positive spontaneous eye swab, Pl day 21-39.
p = Positive eye swabs post-PKP to pre-cyclophosphamide.
+ = Positive eye swab.
- = Negative eye swab.
C = Contaminated.
| = Cyclophosphamide (CX) 75 mg/kg IV.
| = Dexamethasone (DX) 4 mg/kg IV.

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PKP eyes which received iontophoresis (26/102) did not differ from the PKP eyes which were treated with intravenous cyclophosphamide and dexamethasone (12/53) \((P > 0.1)\). In the uninfected rabbits, the ratio of positive swabs to total swabs was 0/37 and 0/37 for the PKP and non-PKP eyes respectively \((P > 0.05)\).

Corneal co-cultivation was performed using both corneas from four latently infected rabbits, the transplanted corneas from five latently infected rabbits and the transplanted corneas from four uninfected rabbits that had received corneas from latently infected animals. After 21 days of monitoring for cytopathic effects, there were 13 negative cultures and four cultures contaminated with bacteria. None of the corneas was positive for HSV-1.

**Discussion**

Rozsa et al\(^{14}\) have shown that following denervation of the cornea by 180° perilimbal incisions, no new axons are noted to penetrate scar tissue until 60 days after injury. The repair process is slow so that at least 90 days are necessary for reinervation of the cornea with remodeling of the subepithelial neuronal plexus. In the present study, penetrating keratoplasty was performed on one eye of all rabbits, completely severing axons subserving sensory and autonomic modalities to most of the cornea. The neuronal communication between most of the cornea and ganglia was lost. The data above demonstrate that this type of denervation is effective in interfering with recovery of HSV-1 from tear film swabs following iontophoresis of epinephrine. Care was taken to ensure that only transplanted, denervated cornea received epinephrine via the iontophoretic treatment. Although cornea peripheral to the eye cup received some iontophoretically delivered epinephrine, this strengthens the validity of the finding of decreased shedding in the PKP eyes since treatment to the undisturbed recipient cornea would be expected to cause HSV-1 shedding, which did not occur.

In the cyclophosphamide/dexamethasone treated rabbits, there was significantly less shedding in the transplanted eyes. However, the shedding (12/53) was not significantly different than the non-PKP eyes in the iontophoresis treated group (26/102) \((P > 0.1)\). This indicates that while the transplant surgery and attendant loss of corneal innervation may interfere with viral shedding in this model of HSV-1 latent infection and reactivation, the rabbit has not lost the ability to shed HSV-1. Only the reactivation circuit has been interrupted or slowed down. Therefore a corneal stimulus does not reach the ganglion while a central stimulus such as intravenous immunosuppressive agents is still capable of interrupting latency. Our data suggest that the corneal nerves probably are the conduit for the reactivated HSV-1. In the systemically reactivated rabbits, HSV-1 may be released from the undamaged conjunctival and lacrimal nerve terminals but since the corneal nerve terminals are absent, a decrease in viral recovery is obtained. We have shown that performing a corneal transplant and thus interrupting the axonal connections results in a decreased release in shed HSV-1. It is also possible that other factors such as local corneal wound healing contribute to this decrease.

Iontophoresis of epinephrine has been shown to be effective in inducing HSV-1 shedding in both the rabbit and mouse.\(^{15-25}\) The exact mechanism of this type of HSV-1 reactivation is not known. Various chemical agents such as epinephrine, 6-hydroxydopamine or timolol have been proven effective in inducing HSV-1 reactivation\(^{26,27}\) and HSV-1-specific recurrent corneal epithelial lesions.\(^{28}\) Recently, Elena et al\(^{29}\) have shown that beta and alpha adrenergic receptors are present in the epithelium of both pigmented and nonpigmented rabbits. It is possible that the large concentrations of drugs that are delivered by iontophoresis are capable of interfering with the receptor balance and thus signal the dormant HSV-1 in the ganglion to reactivate. Alternatively, perhaps we are observing an effect of the medication with direct damage to the nerve terminals which in some fashion indirectly stimulates the neurons latent with the virus. This question remains to be answered.

Iontophoresis of epinephrine in unoperated rabbit eyes has been shown to be effective in causing HSV-1 shedding in more than 75% of treated eyes.\(^{15-17,19}\) Every effort was made to choose the eye for PKP which had shed HSV-1 spontaneously in order to establish that HSV-1 latency had occurred on that side of the rabbit. This selection could have biased the data in favor of increased shedding in the PKP eyes since it is known that eyes which have previously shed spontaneously are more easily adrenergically induced.\(^{18}\) This did not occur. Only one of the transplanted eyes in the iontophoresis group shed virus for 1 day following iontophoresis. Data from the cyclophosphamide and dexamethasone treated rabbits demonstrate that HSV-1 can be induced to shed and that after depletion of the majority of corneal nerves virus can still be recovered from the tear film.

All of the co-cultivated corneas removed at the time of transplantation or necropsy were negative for HSV-1. This indicates that the loss of neuronal connection and not the removal of latently infected tissue accounted for the decrease in viral shedding in the transplanted eyes. We conclude that corneal nerves are necessary for reactivation of HSV-1 from the rabbit eye. The data suggest that a complete neuronal
circuit must be present either for transmission of the trigger for reactivation to the ganglion or for relay of the infectious HSV-1 particles from the central to peripheral site. Perhaps the neuronal connections are necessary for both aspects of reactivation.

In addition, the uninfected rabbits did not shed virus following epinephrine iontophoresis, even though they received corneal tissue from HSV-1 latent rabbits. No co-cultivated corneas were positive though they received corneal tissue from HSV-1 latency rabbits, as mentioned above. This supports the concept that the cornea in itself is not a site of latent virus in eyes with disease restricted to the epithelium. Clinically, these results could explain why not all herpes patients who undergo corneal transplantation develop recurrent disease since in these patients corneal innervation is absent for a significant period following surgery. 

**Key words:** HSV-1 reactivation, corneal nerves, adrenergic, rabbit, penetrating keratoplasty

**References**