absolutely required to initiate pock formation.[6] Studies currently in progress will examine the role of donor and recipient effector cells.

Our findings demonstrate that sensitization to lymphocyte antigens may lead to allograft rejection against donor corneal endothelial cells, and this has important implications for human graft recipients who have received previous blood transfusions. This new, simplified model of corneal allograft rejection will permit controlled sensitization to a variety of corneal and histocompatibility antigens, which may be responsible for initiation of graft rejection. The model is ideal for studying the sensitization of isolated lymphocyte populations, and their interactions with cells of the corneal endothelium in vivo and allows for experimental manipulations during the sensitization phase, which may lead eventually to a means of completely abrogating the corneal allograft reaction.

**Key words:** in vitro sensitization, corneal rejection, endothelial cell pock formation

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# *HSV-1 Quantitation from Rabbit Neural Tissues after Epinephrine-Induced Reactivation*

Yoshikazu Shimomura,* Jo Beth Dudley,* Louis P. Gangarosa, Sr.,† and James M. Hill*

**Epinephrine iontophoresis into the eye can induce ocular herpes simplex virus type-1 (HSV-1) shedding with a high frequency from latently infected rabbits. The present study was designed to qualify and quantify infectious HSV-1 from neural tissues of latently infected rabbits after ocular epinephrine iontophoresis. Epinephrine iontophoresis was performed daily for 3 consecutive days on selected days during 220–227 days postinoculation. The induced ocular shedding was detected in the tear film with a frequency of 83% (10/12) within 72 hr after the initial iontophoresis. The rabbits were killed 24 hr after the last iontophoretic treatment, and the corneas and neural tissues were homogenized *immediately*. The *cell-free* supernatants were inoculated on primary rabbit kidney cell monolayers for qualitative assays of infectious virus and later titrated on CV-1 monolayers. The frequencies of the recovery of infectious HSV-1 from the cell-free homogenates were 0% of the corneas (0/12), 83% (10/12) from the superior cervical ganglion (SCG), 100% (12/12) from the trigeminal ganglion (TG), 42% (5/12) from the ophthalmic branch of the trigeminal nerve (TN), 8% (1/12) from the root entry zone of the trigeminal nerve into the brain-stem (REZ), and 0% (0/12) from the cerebellum. The authors conclude that epinephrine iontophoresis can reactivate the latent HSV-1 in neural tissues and infectious virus can be quantified from the cell-free homogenates. To the best of our knowledge, this is the first report to quantify HSV-1 with a high frequency from neural tissues following induced reactivation. Invest Ophthalmol Vis Sci 26:121–125, 1985**

Investigations to elucidate the mechanisms of HSV latency, reactivation, and recurrences require a reproducible and reliable animal model. We have demonstrated that epinephrine iontophoresis to the cornea induced ocular HSV-1 shedding reliably from latently infected rabbits.[1-3] In ocular HSV infections, two methods are available to detect reactivation; one is to determine ocular shedding,[1-6] and another is to determine the presence of infectious virus in the neural tissues.[7-9] During latency, infectious virus cannot be detected in cell-free homogenates of ganglia but can be detected by explant co-cultivation. In our previous study, infectious HSV-1 was found only in cell-free homogenates of neural tissues after an in vitro 18–24 hr amplification.[1] We failed to detect infectious virus from direct (no amplification) homogenization of neural tissues in latently infected rabbits that were killed 4 days after the last epinephrine iontophoresis. These results suggest that an earlier killing might provide more information about an

alteration in the viral state in the latently infected neural tissue. Therefore, in the present study, rabbits were killed 1 day after the last epinephrine iontophoresis so that the state of HSV-1 in an earlier phase of reactivation could be assessed.

***Materials and Methods.*** *Animals and virus inoculation:* New Zealand white rabbits (1.5–2.0 kg) were inoculated by drops in both unscarified corneas with a 50 μl suspension of HSV-1 McKrae strain (1–5 $\times 10^6$ PFU/ml). These investigations using rabbits conformed to the ARVO Resolution in the Use of Animals in Research. Viruses were prepared in primary rabbit kidney (PRK) cell monolayers and titrated on CV-1 cell monolayers. Primary ocular infections (typical corneal epithelial dendritic ulcers) were confirmed in all eyes by slit-lamp examination using fluorescein stain on day 3 postinoculation.

*Detection of HSV ocular shedding:* HSV-1 ocular shedding was determined from eye swabs taken with sterile, dacron-tipped applicators as previously described.[1] All rabbits used in this experiment had at least one positive spontaneous HSV-1 shedding from both eyes. Ocular sheddings were determined for 4 consecutive days before the initial procedure for iontophoresis and for 3 consecutive days after the initial procedure.

*Procedure for ocular iontophoresis:* Epinephrine iontophoresis was used to induce reactivation of latent HSV in rabbits between 220–227 days postinoculation. As a control, NaCl iontophoresis was employed in rabbits between 170–220 days postinoculation (mean = 201). Rabbits were anesthetized by separate intramuscular injections of xylazine (4 mg/kg) and then ketamine (20 mg/kg). An eye cup containing 0.01% epinephrine or 0.01% NaCl was centered with its periphery within the limits of the corneal limbus. The anode (+) made contact with the solution of epinephrine or NaCl and the cathode (−) was attached to a shaved area of dorsum. A direct current (0.8 mA; 6–7 V) was applied for 8 min. Iontophoresis was performed once a day for 3 consecutive days. Prior to iontophoresis, only ocular swabs and slit-lamp examinations were performed on these rabbits.

*Qualitative and quantitative assays for infectious virus from tissues:* Rabbits were killed by cardiac injection of 4 ml of 5% pentobarbital 24 hr after the last iontophoretic treatment. The corneas and neural tissues were dissected under aseptic conditions and placed in ice baths (4°C or less). Tissues were washed three times in cold Eagle's minimum essential medium (E-MEM) immediately after removal. The corneas were minced and homogenized at 4°C with a polytron (setting 7 for 1 min) to make 10% homogenates (w/v) in E-MEM with 3% fetal calf serum (FCS). The neural tissues removed were the superior cervical ganglion (SCG), trigeminal ganglion (TG), 1 cm segment of the ophthalmic branch of the trigeminal nerve (TN), root entry zone of the trigeminal nerve (REZ), and cerebellum (CEB). CEB was divided nto two parts by cutting along the median line. SCG were placed in 1.0 ml cold E-MEM (approximately 1% [w/v] solution); TG and TN in 1.5 ml (approximately 5% [w/v] solution); REZ and CEB in 3.0 ml (approximately 10% [w/v] solution). After mincing, the neural samples at 4°C were sonicated (Branton Ultrasonic Corp.; Stamford, CT). The corneal and neural homogenates were centrifuged at 4°C at low speed and their supernatants (0.5 ml for each) inoculated on PRK monolayers in a 60-mm petri dish. The specimens were incubated for 2 hr at 37°C in a $CO_2$ incubator, and E-MEM with 7% FCS was added to the petri dishes. The appearance of HSV-induced cytopathic effect (CPE) was monitored for 7 days (90% were positive in 3–5 days).

The unused portion of all supernatants were stored at −75°C for later use in viral titration. Viral titers were determined for all positive supernatants. In the quantitative assay, 0.2 ml of undiluted cell-free homogenate, and triplicates of 1:10 and 1:100 dilutions were inoculated on CV-1 monolayers in a 12-well multi-well plate (area per well: 4.5 $cm^2$) and incubated for 2 hr at 37°C in a $CO_2$ incubator. Then, E-MEM with 20% FCS and 0.4% agar was added; after 96 hr, plates were fixed with 10% formaldehyde, and stained with methylene blue.

*Identification of viral isolates:* The specificity of HSV-1 in samples from ocular swabs and cell-free homogenates was identified by a plaque-reduction assay on CV-1 cells with a HSV-1 hyperimmune rabbit antiserum.

***Results.*** Table 1 shows the appearance of HSV-1 ocular shedding before and after epinephrine iontophoresis. On day 1, 24 hr after the first iontophoresis of epinephrine, no eyes (0/12) shed virus. On day 2, 24 hr after the second iontophoresis of epinephrine and 48 hr after the first iontophoresis of epinephrine, three of 12 eyes (25%) shed virus. On day 3, 24 hr after the third iontophoresis, 48 hr after the second iontophoresis, and 72 hr after the first iontophoresis, seven of 12 eyes (58%) shed virus. Eighty-three percent (10/12) of eyes shed virus within 72 hr after the initial iontophoresis (24 hr after the last iontophoresis). The total positive cultures per total cultures without contamination after the initial iontophoresis of epinephrine was 33% (10/33), whereas no viral shedding had been detected from 48 total cultures throughout 4 days before the initial iontophoresis of epinephrine. The difference between frequencies of positive cultures before and after the initial epinephrine reactivation

**Table 1.** HSV-1 ocular shedding pre- and postreactivation by epinephrine iontophoresis

| *Rabbit no.* | *PI** | *Eye* | *−3* | *−2* | *−1* | *0* | ↓‖ | *+1* | ↓ | *+2* | ↓ | *+3* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 220 | OD‖ | —† | — | — | — | | — | | — | | +‡ |
| | | OS** | — | — | — | — | | — | | + | | — |
| 2 | 220 | OD | — | — | — | — | | — | | — | | + |
| | | OS | — | — | Cξ | — | | — | | — | | — |
| 3 | 227 | OD | — | — | — | — | | — | | C | | + |
| | | OS | — | — | — | — | | — | | — | | + |
| 4 | 227 | OD | — | — | — | — | | — | | — | | + |
| | | OS | — | — | — | — | | C | | — | | + |
| 5 | 227 | OD | — | C | — | — | | — | | — | | + |
| | | OS | — | — | — | — | | — | | + | | — |
| 6 | 220 | OD | — | — | — | — | | — | | + | | — |
| | | OS | — | — | — | — | | — | | — | | C |
| Days pre- and posttreatment | | | −3 | −2 | −1 | 0 | ↑ | +1 | ↑ | +2 | ↑ | +3 |

All rabbit eyes had at least one positive spontaneous HSV-1 shedding prior to initiation of this experiment.

* PI: postinoculation days; †—: no shedding; ‡+: HSV-1 ocular shedding; ξC: contamination; ‖↓: epinephrine 0.01% iontophoresis (0.8 mAmps, 8 min); ‖OD: right eye; **OS: left eye.

was statistically significant ($P < 0.002$) by the $\chi^2$ test. The four rabbits that received NaCl iontophoresis showed no positive cultures from 32 total cultures taken throughout the 4 days before the initial treatment (data not shown). The total positive cultures per total cultures after initial iontophoresis of NaCl was 4% (1/24). The difference between the frequencies of positive cultures after the initial epinephrine iontophoresis and after the initial NaCl iontophoresis was statistically significant ($P < 0.002$) by the $\chi^2$ test.

Table 2 shows cumulative data on ocular shedding induced by epinephrine iontophoresis and viral titers from the individual cell-free homogenates. Twelve samples of positive cultures in PRK monolayers did not show any plaques on CV-1 cells. Their titers were defined according to the aliquot used in the qualitative assay as 2 PFU for SCG, 3 PFU for TG and TN, and 6 PFU for REZ. If the cell cultures in PRK monolayers failed to induce CPE, the titers were reported as zero. No infectious HSV-1 was detected

**Table 2.** HSV-1 titration using cell-free homogenates of neural tissues following epinephrine iontophoresis to rabbit corneas

| *Rabbit no.* | *Eye* | *Cumulative ocular shedding* | *PFU/Neural Tissue* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *TG** | *SCG†* | *TN‡* | *REZξ* | *CEB‖* |
| 1 | OD‖ | + | 195 | 2 | 0 | 0 | 0 |
| | OS** | + | 190 | 75 | 480 | 0 | 0 |
| 2 | OD | + | 3 | 550 | 0 | 0 | 0 |
| | OS | − | 630 | 140 | 480 | 0 | 0 |
| 3 | OD | + | 3 | 0 | 0 | 0 | 0 |
| | OS | + | 3 | 10 | 3 | 6 | 0 |
| 4 | OD | + | 3 | 0 | 0 | 0 | 0 |
| | OS | + | 3 | 95 | 0 | 0 | 0 |
| 5 | OD | + | 30 | 12 | 12 | 0 | 0 |
| | OS | + | 48 | 40 | 0 | 0 | 0 |
| 6 | OD | + | 3 | 12 | 0 | 0 | 0 |
| | OS | − | 3 | 2 | 3 | 0 | 0 |
| % positive | | 83% | 100% | 83% | 42 | 8% | 0% |

Rabbits received epinephrine (0.01%) iontophoresis (0.8 mA for 8 min) once a day for 3 consecutive days. They were killed 24 hr after the last iontophoresis. The neural tissues were processed as described in *Materials and Methods.*

* TG: trigeminal ganglion; †SCG: superior cervical ganglion; ‡TN: a 1-cm segment of the ophthalmic branch of the trigeminal nerve; ξREZ: root entry zone of the trigeminal nerve at the brainstem; ‖CEB: cerebellum; ‖OD = right eye; **OS = left eye.

in the cell-free homogenates of 40 neural tissues and eight corneas from the four rabbits receiving NaCl iontophoresis.

The frequency of HSV-1 positive cultures from the homogenates after epinephrine iontophoresis was 0% of corneas (data not shown), 83% of SCG, 100% of TG, 42% of TN, 8% of REZ, and 0% of CEB. HSV was isolated from the tear film in 10 of 12 eyes. All 10 corresponding TG were positive for HSV, and eight of 10 corresponding SCG were positive for HSV. Two cultures of TG and SCG were positive, although no virus was detected in the tear film.

***Discussion.*** The present study corroborates our previous reports[1-3] that epinephrine iontophoresis can induce reactivation and ocular HSV shedding. Previously, epinephrine iontophoresis induced 100% ocular shedding in rabbits 60 days PI,[2] 90% ocular shedding in rabbits 90 days PI,[3] and 75% ocular shedding in rabbits 170–365 days PI (mean = 254).[1,2] Also, spontaneous shedding decreased with increased postinoculation time.[2] In the present study, epinephrine iontophoresis induced 83% ocular shedding in rabbits 220–227 days PI. All these results suggest that the frequency of both spontaneous and induced HSV-1 shedding decreases with increased postinoculation time.[1-3]

We detected infectious virus from the homogenates of neural tissues with a high frequency after epinephrine iontophoresis. Viral recovery from SCG (83%) suggests that SCG, as well as TG, have an important role in harboring HSV-1 during the latent infection and could participate in the appearance of HSV-1 in the tear film after the induced reactivation. Epinephrine is a neurohormone of the sympathetic nervous system and epinephrine iontophoresis to the cornea may trigger a reactivation of HSV-1 harbored in either or both ganglia. One possible pharmacologic explanation is that adrenergic activation by epinephrine may modulate neural transmission in corneal nerves. This could be either through adrenergic or sensory nerves to the cornea, or both.[10]

Martin et al[11] and Mintsioulis et al[12] have investigated the role of the superior cervical ganglia in acute and chronic (latent) HSV-1 infections in rabbits. Martin et al[11] used the HSV-1 strain RE and recovered virus in the trigeminal, superior cervical, and ciliary ganglia in the acute phase. The latently infected HSV-1 strain RE rabbits were not able to be induced to shed virus in their tear film after topical application of epinephrine. Mintsioulis et al[12] inoculated the superior cervical ganglia of rabbits with HSV-1 strain McKrae and observed eye disease and also detected the virus in the eye. Furthermore, Mintsioulis et al[12] suggested that autonomic mediators might trigger episodes of HSV-1 shedding in animals, which had viral latency in the superior cervical ganglia.

Some investigators have determined the presence of infectious virus in the homogenates of neural tissues after reactivation.[7,8,9,13,14] However, the frequencies of viral recovery were lower (14–70%) than those after epinephrine iontophoresis (83%–100%); thus, epinephrine iontophoresis appears to be the most reliable and reproducible method for reactivation of HSV-1 in the TG and SCG.

To the best of our knowledge, only one report[9] quantified HSV-1 from the reactivated neural tissues. Price et al[9] detected infectious virus, with a mean of greater than 168 PFU per SCG after cyclophosphamide treatment following postganglionic neurectomy. However, the ratio of positive detection of infectious virus was only 17/45 (38%).

In a previous study,[1] epinephrine iontophoresis performed during 220–280 days PI resulted in 75% viral shedding of the eyes (30/40) within 4 days after the last iontophoresis. However, infectious virus was detected in only one neural homogenate from the tissues homogenized immediately after killing. These results,[1] with the present data, indicate that the frequency of detecting infectious virus in neural tissue homogenates decreased with time from the termination of iontophoresis when comparing 4 days after the last iontophoresis to 1 day after the last iontophoresis.

One rabbit (PI day 175) not included in the data in Table 1 showed an unusual response. The protocol was identical to the other animals. This rabbit did not shed virus into the tear film and had either none or very low levels of HSV-1 in the TG, SCG, and TN (3/6 were positive). This rabbit was unusual in that the two REZ had titers of 1,710 and 1,500. We have never observed a titer this high in the REZ in any previous studies and can offer no explanation. The CEB was negative for HSV-1 in this rabbit.

In conclusion, these results demonstrated that epinephrine iontophoresis to the cornea reactivated HSV-1 in the neural tissues of the latently infected rabbits. We are continuing our studies of this rabbit model in an attempt to elucidate the mechanisms of HSV latency and reactivation. This model appears to yield valuable information about latency and reactivation and may eventually aid in our understanding of the mechanisms of these processes.

**Key words:** HSV-1, rabbit, neural tissues, eye, epinephrine, iontophoresis

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# *Fibrinolytic Activity of the Retinae in Streptozotocin-Diabetic Rats*

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**Fibrinolytic activity of the retinae in control and diabetic rats was assayed quantitatively in twenty male rats made diabetic by giving a single injection of streptozotocin. All these rats were killed at either 3 months or 12 months. Ten saline-injected rats and five rats treated with 3-0-methylglucose and streptozotocin served as controls. As the plasminogen activator activity in diabetic rats maintained for 12 months was significantly lower than that in controls, we postulate that there may be a poor defense mechanism against microthrombus formation in the retinal vasculature of diabetics, which may contribute to the development of diabetic retinopathy. Invest Ophthalmol Vis Sci 26:125–127, 1985**

Clinically, the occlusion of retinal vascular beds is an important phenomenon in the development of diabetic retinopathy. We previously suggested that the initial vascular occlusion was due to microthrombus formation.[1]

The plasminogen activator is localized mainly in the vascular endothelium and converts plasminogen to plasmin, which in turn acts on fibrin or fibrinogen.[2] In physiologic states, fibrinolysis in vascular beds plays an important role in the resolution of thrombi, and patency of the vasculature is maintained.

We now have studied the fibrinolytic activity of the retinae in control and diabetic rats to determine whether plasminogen activator activity of the retinae would be influenced by diabetes.

***Materials and Methods.*** Twenty male Wistar-King A rats, weighing approximately 200 g, were given streptozotocin intravenously, 65 mg/kg body weight, dissolved in 0.3 ml citrate buffer (pH 4.5), to induce diabetes. Ten control rats of similar body weight were given a corresponding volume of a solution of 0.9% NaCl. Five rats of the same body weight were pretreated with 3-0-methylglucose (3-OMG) before administration of streptozotocin, thus precluding direct toxicity of streptozotocin; 3-OMG (1.1 mmole/200 g body weight), dissolved in 1 ml 0.9% NaCl was given intravenously over 60 sec, followed by streptozotocin injection.[3] The animals were examined every 3 weeks for body weight, blood glucose, and glycosuria. The blood glucose was determined with Dextrostix and glycosuria checked with Tes-Tape. A diagnosis of diabetes was established by persistent hyperglycemia (>300 mg/100 ml), glycosuria, and impaired growth.

All rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (9 mg/100 g body