# Reports

## *Herpes Simplex Virus Recovery in Neural Tissues after Ocular HSV Shedding Induced by Epinephrine Iontophoresis to the Rabbit Cornea*

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**Ocular HSV-1 shedding from latently infected rabbits was induced by iontophoresis of 0.01% epinephrine into the eye. Anodal Iontophoresis of epinephrine was performed at 0.8 mAmps for 8 min once a day for 3 consecutive days. Shedding was determined by the presence of HSV-1 in the preocular tear film obtained via eye swabs. Bilateral epinephrine iontophoresis performed on selected days during 220–280 days after inoculation resulted in HSV-1 shedding in 75% of the eyes (30/40) and 100% of the rabbits (20/20). Following the induction of ocular HSV-1 shedding, rabbits were killed and selected neural tissues were homogenized. Cell-free preparations were assayed for the presence of infectious virions using primary rabbit kidney cell monolayers. When the tissues were homogenized immediately after death, virus was detected in only one neural tissue, the trigeminal ganglia. However, when the tissues were incubated in vitro for 18–24 hours prior to the homogenization, infectious HSV-1 was recovered from homogenates of the trigeminal ganglion, superior cervical ganglion, the ophthalmic branch of the trigeminal nerve, and the root entry zone of the trigeminal nerve. A relationship was noted between the time of the last ocular shedding and recovery of infectious HSV from the tissue homogenates. Furthermore, a positive correlation in 11 eyes between the recovery of HSV-1 from the perocular tear film and HSV-1 recovery from one or more corresponding neural tissues was found. These results suggested that epinephrine iontophoresis to the cornea triggered an "alteration" in the state of the virus in the neural tissues of the latently infected rabbits and that the change can be related to the induced ocular shedding. Invest Ophthalmol Vis Sci 24:243–247, 1983**

We have previously reported a rabbit model for induced ocular shedding of HSV-1.[1] Epinephrine iontophoresis to the rabbit cornea induces HSV-1 shedding into the preocular tear film reliably and with a high frequency during the latent phase of the infection. In our previous report, HSV-1 was detected in one or both co-cultivated explants of the trigeminal and the superior cervical ganglia for every eye in all experimental groups.[1] These groups included epinephrine iontophoresis, NaCl iontophoresis, and intramuscular injections of epinephrine. In these experiments, we noted that the neural tissues from the rabbit receiving epinephrine iontophoresis had the highest frequency of detection of HSV-1 in the explants. Also the time for development of cytopathic effects (CPE) on the indicator cells (primary rabbit kidney cell monolayers) was the shortest for the rabbits receiving epinephrine iontophoresis. These results suggested that infectious HSV might be present in these neural tissues prior to cocultivation. The present study was performed to determine if infectious HSV-1 could be detected in cell-free homogenates of neural and ocular tissues after epinephrine iontophoresis to the cornea.

***Materials and methods.*** *Virus inoculation*: New Zealand albino rabbits (1.5–2.0 kg) were inoculated in both corneas with 50 μl of HSV-1 McKrae strain ($10^6$ PFU/ml) that had been propagated on primary rabbit kidney cell (PRK) monolayers and titrated using CV-1 cells. The disease process, ocular scoring, and disease severity of the acute infection were reported previously.[1]

*Determination of viral shedding*: Eye swabs were taken from rabbits with a sterile, dacron-tipped applicator that was rotated gently in the upper cul-de-sac, then across the cornea and into the lower cul-de-sac. The swabs were placed onto PRK monolayers in culture tubes and incubated at 37 C in a $CO_2$ incubator. The appearance of CPE was monitored daily for 7 days.

*Iontophoresis*: Rabbits were anesthetized with xylazine (4 mg/kg, IM) and ketamine (20 mg/kg, IM). An eye cup was inserted with its periphery within the limit of the corneal limbus. Epinephrine (0.01%) or NaCl (0.01%) was added to the eye cup. The cathode (−) was attached to a shaved area of trunk, and the anode (+) made a wet contact with the ocular solution through a cotton wick. A direct current (0.8 mAmps; 7 volts) was applied for 8 min. Iontophoresis was performed once a day for 3 consecutive days.

*Assay for HSV-1 in cell-free homogenates*: The eyes were dissected into cornea, iris, sclera, and lacrimal gland. The neural tissues taken were the trigeminal ganglia (TG) and the superior cervical ganglia (SCG), a 1-cm segment of the ophthalmic branch of the trigeminal nerve (TN) and the root entry zone of the

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**Table 1.** Recovery of infectious HSV-1 from neural and ocular tissue homogenates

| *Treatment of eyes and neural tissues* | *Eye swabs* | *Number of eyes* | *Neural tissues** | *Cornea* | *Other ocular tissues†* |
|---|---|---|---|---|---|
| Epinephrine‡ iontophoresis to the cornea | Positive§ | 15 | 20/60#¶ | 2/15 | 0/60 |
| (18–24 hr incubation of neural tissues) | Negative‖ | 5 | 1/20 | 0/5 | 0/20 |
| Epinephrine‡ iontophoresis to the cornea | Positive§ | 15 | 1/60 | 3/15 | 0/60 |
| (immediate homogenization of neural tissues) | Negative‖ | 5 | 0/20 | 0/50 | 0/20 |
| Untreated Rabbit eyes (18–24 hr | Positive§ | 3 | 1/12 | 0/3 | 0/12 |
| of incubation of neural tissues) | Negative‖ | 17 | 0/68 | 0/17 | 0/68 |
| NaCl** iontophoresis to the cornea | Positive§ | 1 | 0/4 | 0/1 | 0/4 |
| (18–24 hr incubation of neural tissues) | Negative‖ | 9 | 0/36 | 0/9 | 0/36 |

‡ Epinephrine (0.01%) iontophoresis (0.8 mAmps for 8 min) was once daily for 3 consecutive days. Rabbits were killed 4 days after the last treatment.

** NaCl (0.01%) iontophoresis (0.8 mAmps for 8 min) was once daily for three consecutive days. Rabbits were killed 4 days after the last treatment.

* The neural tissues were the trigeminal and superior cervical ganglia, a 1-cm segment of the ophthalmic branch of the trigeminal nerve, and the root entry zone of the trigeminal nerve at the brain stem.

† The other ocular tissues were the aqueous humor, iris, sclera, and lacrimal gland.

§ Positive are eyes that had at least one HSV-1 positive eye swab in the last 7 days before death.

‖ Negative are eyes that were not positive by the eye swab procedure during the last 7 days before death.

# The number of tissues positive for HSV-1/the number of tissues tested.

¶ Significantly different ($P < 0.009$) from all seven other groups of positive and negative eyes (Fisher's exact test).

trigeminal nerve (REZ). The ocular tissues were minced and homogenized with a polytron (setting 7 for 1 min) to make 10% homogenates (w/v) in Eagle's minimum essential medium (E-MEM) containing 3% fetal calf serum (FCS). The neural tissues were weighed, washed in E-MEM, minced, and sonicated with a Sonifier (Branson Ultrasonic Corp., Stamford, CT). SCG were prepared in 1.0 ml E-MEM (about 1% solution), TG and TN, in 1.5 ml E-MEM (about 5% solution) and REZ, in 3.0 ml E-MEM (about 10% solution). Cell debris was removed by centrifugation at 5000 × g for 10 min at 4 C. A 0.5-ml aliquot of the supernatant of the tissue homogenates, or 0.2 ml of aqueous humor obtained by anterior chamber paracentesis, was inoculated onto a PRK monolayer in a 60-mm petri dish. The specimens were incubated for 2 hours at 37 C in a $CO_2$ incubator, and E-MEM-7% FCS was added to the petri dishes for further incubation. The appearance of CPE was monitored for 7 days, adding E-MEM-7% FCS as needed.

*Identification of viral isolates*: Randomly selected HSV-1 positive cultures from ocular swabs and cell-free homogenates were identified by a plaque-reduction assay on CV-1 cells using an HSV-1 specific rabbit antiserum.

*Experiment design*: Thirty-five HSV-1 latently infected rabbits were used in these experiments. All rabbits had at least one positive spontaneous HSV-1 ocular shedding prior to initiation of the experiments. The rabbits were divided randomly into four groups and used between days 220–280 postinoculation (PI). Twenty-five rabbits (two groups of ten; one group of five) had eye swabs on 14 consecutive days: 7 days before and 4 days after iontophoresis. Ten rabbits were untreated but had eye swabs taken daily for 14 consecutive days.

The neural tissues from the untreated rabbits were incubated for 18–24 hours prior to homogenization. The 20 rabbits that received epinephrine iontophoresis were divided into two groups. One group of neural tissues from ten rabbits were homogenized immediately after they were killed. The other group of neural tissues from ten rabbits were incubated individually in E-MEM-7% FCS with no other cells present at 37 C in a $CO_2$ incubator for 18–24 hours prior to homogenization. The neural tissues from the five rabbits that received NaCl iontophoresis were individually incubated in E-MEM + 7% FCS with no other cells present at 37 C in a $CO_2$-incubator for 18–24 hours prior to homogenization. This in vitro incubation to allow viral amplification has been described and discussed by Openshaw et al.[2]

***Results.*** Table 1 shows a summary of the recovery of HSV-1 from eye swabs and from neural and ocular tissue homogenates. Both groups of rabbits receiving epinephrine iontophoresis has a shedding frequency of 75% (15/20). This ocular shedding frequency is essentially the same as reported previously. The untreated group had a 15% (3/20) shedding rate with a duration of 1 day, whereas the epinephrine-treated eyes showed shedding for an average duration of 3.7 days. In these four groups of rabbits, all their ocular tissues were homogenized immediately after killing them. The corneas that had recent ocular shedding (data not shown) had some positive HSV-1 tissue homogenates, but all the other tissues (iris, aqueous humor, sclera, and lacrimal gland) were negative for HSV-1.

From the group of rabbits that received epinephrine iontophoresis and that had their neural tissues immediately homogenized, HSV-1 was recovered from only one TG. In the same group, there were

**Table 2.** HSV-1 recovery from eye swabs and cell-free homogenates of neural tissues following epinephrine iontophoresis to rabbit corneas

| *Rabbit no.* | *Eye* | *Days postinoculation* | *Ocular shedding* | *Virus recovery** | | | |
|---|---|---|---|---|---|---|---|
| | | | | *TG* | *SCG* | *TN* | *REZ* |
| 1 | OD | 278 | + | − | − | − | − |
| | OS | | + | − | + | + | − |
| 2 | OD | 278 | + | − | − | − | − |
| | OS | | − | + | − | − | − |
| 3 | OD | 260 | + | + | + | + | − |
| | OS | | + | + | + | − | + |
| 4 | OD | 220 | + | − | − | − | − |
| | OS | | − | − | − | − | − |
| 5 | OD | 232 | + | + | + | + | − |
| | OS | | + | + | + | + | − |
| 6 | OD | 225 | − | − | − | − | − |
| | OS | | + | − | − | − | + |
| 7 | OD | 220 | + | − | − | − | + |
| | OS | | + | − | − | − | + |
| 8 | OD | 225 | + | + | − | − | − |
| | OS | | + | + | − | − | − |
| 9 | OD | 244 | − | − | − | − | − |
| | OS | | + | − | − | − | − |
| 10 | OD | 225 | − | − | − | − | − |
| | OS | | + | − | − | − | + |
| Totals | 20 eyes | | 15/20 (75%) | 7/20 (35%) | 5/20 (25%) | 4/20 (20%) | 5/20 (25%) |

Key OD = right eye; OS = left eye; + = HSV-1 present; − = no virus present. These results are more details of the first group of rabbits reported in Table 1.

* HSV-1 was recovered from the cell-free homogenates. After the rabbits were sacrificed, the neural tissues were individually incubated for 18–24 hr and then the free-cell homogenates were prepared.

Abbreviations: TG, trigeminal ganglia; SCG, superior cervical ganglia; TN, a segment of the ophthalmic branch of the trigeminal nerve; REZ, root entry zone of the trigeminal nerve at the brain stem.

three corneas positive for HSV-1 that were obtained from three rabbits that had ocular shedding of HSV during the last 3 days prior to killing them (data not shown). The one positive TG was the ipsilateral ganglion from one of the positive corneas.

In the untreated, latently infected rabbits, the neural tissues were individually incubated for 18–24 hours prior to homogenization. The cell-free homogenate from only one TG was positive for HSV-1. During the 14 days of consecutive ocular swabbing of the 20 eyes, five positive eye cultures were obtained: two positives occurred in the first 7 days (data not shown) and three positives occurred in the last 7 days. The rabbit with the positive TG also had an ipsilateral positive eye swab that occurred 5 days prior to killing it.

In the group of rabbits that received NaCl iontophoresis, the neural tissues were incubated individually for 18–24 hours prior to homogenization. All the cell-free homogenates from neural and ocular tissues were negative for HSV-1. During the 14 days of consecutive ocular swabbing of the ten eyes, three positive eye cultures were obtained in the first 7 days (data not shown). One positive eye swab culture was detected in the last 7 days (Table 1).

Table 2 shows data on shedding induced by epinephrine iontophoresis and the results of virus recovery from the individual neural tissue homogenates. Shedding occurred in 75% of the eyes (15/20 eyes) and in 100% of the rabbits (10/10 rabbits). The frequency of HSV-1 recovery from the homogenates of neural tissues after epinephrine iontophoresis was 35% of TG, 25% of SCG, and REZ and 20% of TN. The data in Table 2 shows a positive correlation in 11 eyes between the recovery of HSV-1 in eye swabs and the recovery of HSV-1 from one or more corresponding neural tissues.

Table 3 shows the relationship between the time of the last shedding and HSV recovery from the tissue homogenates. The shedders (15 eyes) were grouped according to the interval between the day of the last shedding and day of killing them. Seven eyes were assayed within 3 days from the last shedding and eight eyes were assayed 4–6 days from the last shedding. The frequency of HSV recovery when assayed within 3 days after the viral shedding was 71% of TG, 57% of SCG, and 43% of TN and REZ. For these seven eyes the neural tissues showed a frequency of 54% (15/28) positive for HSV-1. In the eight eyes that shed virus 4–6 days prior to killing, their corresponding neural tissues had a frequency of only 16% (5/32) positive for infectious HSV-1. In the nonshedding eyes, only one neural tissue (the TG) was positive. From the homogenates of the ocular tissues, 29% of

**Table 3.** Relationship between the time of the last ocular shedding and HSV-1 recovery from tissue homogenates

| Eye group | Days between last shedding and death | Recovery of infectious virus from tissue homogenates | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | TG | SCG | TN | REZ | Total neural tissues | Cornea | Other ocular tissues |
| Shedder | 0–3 | 5/7 (71%) | 4/7 (57%) | 3/7 (43%) | 3/7 (43%) | 15/28 (54%) | 2/7 (29%) | 0/28 (0%) |
| Shedder | 4–6 | 1/8 (13%) | 1/8 (13%) | 1/8 (13%) | 2/8 (25%) | 5/32 (16%) | 0/8 (0%) | 0/32 (0%) |
| Nonshedder | >14 | 1/5 (20%) | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 1/20 (5%) | 0/5 (0%) | 0/20 (0%) |

These results are more details of the rabbits reported in Table 2. The abbreviations and notations are also the same as noted in Table 2.

Among shedders (15 eyes), seven eyes were assayed within 3 days from the last shedding and eight eyes were assayed 4–6 days from the last shedding.

Nonshedders are the eyes that did not shed virus after epinephrine iontophoresis and did not shed virus during the 7 days before iontophoresis.

the corneas (2/7) were HSV-1 positive only during or immediately after the viral shedding. None of the other ocular tissues (0/80) showed virus.

***Discussion.*** Iontophoresis of epinephrine to the cornea induces a significantly higher frequency of HSV-1 shedding than reported for either topical or systemic applications of epinephrine.[1,3,4] Our current results strongly suggest that epinephrine iontophoresis alters the state of the virus in the latently infected neural tissues and ultimately results in HSV-1 ocular shedding. One critical consideration is the definition of terms. The functional definition of latency is that infectious virus cannot be detected in a cell-free homogenate but can be detected by an explant coculitivation system. In the HSV-1 latently infected rabbit, investigators have reported spontaneous ocular shedding and episodes of spontaneous recurrent ocular lesions.[1,3–10] Induced ocular shedding of HSV-1 in the rabbit has been reported.[1,3–5,8–10] In these reports no one has detected infectious HSV from direct cell-free homogenates of neural tissues.

Two reports,[2,11] concerning the HSV-1 latently infected mouse, cite the recovery of HSV from *direct* cell-free homogenates of neural tissues. Using a differentiated cell culture system derived from *fetal* mouse dorsal root ganglia, Schwartz et al[11] detected infectious HSV from direct cell-free homogenates of the *adult* mouse dorsal root ganglia. The infectious HSV could be detected in direct cell-free homogenates for up to 8 months postinoculation. Openshaw et al,[2] using cyclophosphamide-treated mice, have found infectious HSV-1 in the trigeminal ganglia of mice previously inoculated in the eye. Using an in vitro, 24-hour amplification system they found that 80–90% of the trigeminal ganglia contained infectious HSV. When the eye globes were asayed for HSV, only 10–22% were positive, and in only one mouse was there a positive correlation between recovery of HSV in the eye and the ipsilateral ganglia. In Table 2 we show a positive correlation between the recovery of infectious HSV-1 in the eye and the corresponding neural tissues in 7 rabbits and 11 eyes. We believe this relationship is the most significant aspect of this report, because it is the first to show a high correlation between the induced appearance of HSV at a body surface and the recovery of infectious HSV from one or more corresponding neural tissues.

We are currently assessing the state of the virus during and immediately following epinephrine iontophoresis. Also, we have recently determined the kinetics of viral shedding during the induction period.[12] This new information will be used to assess the state of HSV during the early phases of the epinephrine-induced ocular shedding. We hope to develop further the rabbit model of induced viral shedding so that we can induce recurrent HSV-1 ocular lesions and detect infectious HSV-1 in neural tissues without an in vitro amplification procedure.

**Key words:** HSV-1, rabbit, eye, induction, shedding, reactivation, neural tissues, epinephrine, iontophoresis.

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# *Evidence for Corneal Endothelial Cell Hypertrophy during Postnatal Growth of the Cat Cornea*

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**Endothelial cell counts made from specular micrographs of 1-month-old kitten and adult cat corneas demonstrate that a progressive increase in endothelial cell size and a reduced endothelial cell density occurs during the postnatal development of the cat cornea. In addition to confirming the difference in cell size, scanning electron micrographs show that kitten endothelial cells are much more pleomorphic than those of the adult. When the number of corneal endothelial cells/mm$^2$ and the size of the whole cornea are calculated for the kitten and adult, hypertrophy rather than mitosis appears to be the principal mechanism responsible for maintaining a confluent endothelial cell monolayer during the postnatal development of the feline cornea. Hypertrophy also appears to play a role in establishing the adult corneal endothelial cell population of the rabbit when the previously published data of others are treated in a similar manner to those of the kitten and adult cat. Thus, endothelial cell hypertrophy plays a role in establishing an "adult" endothelial cell monolayer in species that have a widely divergent corneal endothelial cell mitotic capacity. Invest Ophthalmol Vis Sci 24:247–250, 1983**

The corneal endothelium of the adult cat, like that of the human, has little regenerative potential.[1,2] In addition, the cat appears to be a valuable model for corneal transplantation because surgically associated endothelial cell loss and the incidence of spontaneous graft rejection are similar to those observed in the human following penetrating keratoplasty.[3] The cat is also an excellent model in which to study induced homograft rejection.[4] In order to characterize the development of the corneal endothelium in this species, we have studied the changes in endothelial shape and size that occur concomitantly with corneal growth.

***Materials and methods.*** A total of 16, 1-month-old kitten and ten adult cat corneas were studied. Corneas were removed and placed in buffered tissue culture medium that contained 5% dextran (mw 40,000). A laboratory specular microscope was used to photograph the central corneal endothelial surface.[5]

The number of endothelial cells/mm$^2$ was determined by counting a minimum of three separate frames per specimen. Cell counts were made by an experienced observer who did not know the source of the photographs. The variability in the cell count was found to be less than 3% when previously counted photomicrographs were included among those of the present animal series. The number of endothelial cells per cornea was estimated by calculating the surface area of the cornea using the formula, Area = $2\pi r$, h. (Calculations treating the adult cat cornea as a sphere

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