Reactivation of Murine Latent HSV Infection by Epinephrine Iontophoresis

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Iontophoresis of epinephrine into the cornea of previously infected mice was used in an attempt to induce reactivation of latent herpes simplex virus (HSV) infection of the trigeminal ganglia. BALB/c mice infected with HSV-1 strain McKrae following corneal scarification developed a latent infection of the trigeminal ganglia within 15 days. At 28 days postinfection, mice were subjected to a 3-day cycle of iontophoresis of epinephrine (0.01%) into the cornea. Ocular shedding of HSV occurred in 16/23 (70%) of stimulated mice; these animals did not shed HSV in the 3-day period prior to iontophoresis. Spontaneous shedding of HSV, however, was noted in 3/97 (3%) mice not subjected to epinephrine iontophoresis. "Infectious" virus was isolated only from the trigeminal ganglia of stimulated mice, whereas "latent" virus was isolated from the trigeminal ganglia of both stimulated and nonstimulated mice. All virus isolates were verified to be HSV by neutralization with a known HSV-1 antiserum. This ocular system thus allows for the study of the full spectrum of latent HSV infections, including latency, ganglionic reactivation, and peripheral virus shedding. Invest Ophthalmol Vis Sci 25:945-950, 1984

Herpes simplex virus (HSV) causes an infection in man and animals with a characteristic acute, latent, and recurrent phase. 1-3 Following inoculation of experimental animals, the virus can be isolated from the external site of inoculation or recovered from homogenates of the local sensory or autonomic ganglia. 4-7 After approximately 14 days, the infection becomes latent and virus can no longer be recovered from the cell-free homogenates but can be recovered by explantation and cocultivation of the affected ganglia. 2,6-9 During the latent phase, spontaneous recurrent episodes of viral replication can result in the shedding of infectious virus at the external site of inoculation (ie, the eye, lip, or genital mucosa). Spontaneous reactivation of HSV latent infection has been shown to occur in man 1,2 and experimentally infected rabbits, 10 but in experimentally infected mice there are conflicting reports. 11-16

Intentional reactivation of latent ganglionic HSV infection has been accomplished in experimentally infected animals by several methods. Initially, Laibson and Kibrick 17 were able to induce HSV shedding in rabbits with intramuscular injection of epinephrine. Nesburn et al 18 were able to induce reactivation of HSV shedding into the rabbit eye by a single surgical manipulation of the trigeminal ganglion. Repeated electrical stimulation of the rabbit trigeminal ganglion with an implanted electrode also will induce reactivation and ocular HSV shedding. 19 Recently, Kwon et al 20 induced HSV shedding from latently infected rabbits following iontophoresis of epinephrine into the rabbit cornea. In mice, reactivation of latent HSV "infectious" virus from the cell-free homogenates of local sensory and autonomic ganglia has been demonstrated following bacterial infection, 21 immune suppression, 22,23 neurectomy, 24 and trauma to the cornea and trigeminal ganglia. 25 Hill et al 11 also isolated HSV from explants of the mouse cornea but not from the tear film. Latent HSV infection in mice following dermal inoculation has been reactivated with reappearance of skin involvement by using local trauma 12 and injection of prednisone. 16 In both reports, HSV was isolated from either explants of the ear or swabs of the skin; for the highest rate of HSV isolation, clinical signs had to be evident.

Although several techniques can be used to reactivate murine latent HSV as evidenced by infectious virus in the local ganglia and explants of peripheral tissue, there have been no reports of HSV ocular shedding after intentional reactivation. Unlike skin models,
Animals confirm that latent HSV infection established in mice where best isolation results require the presence of recurring episodes of ocular shedding.\(^{15}\) We report that subjecting mice to ocular HSV shedding without sacrifice of the involved tissue or inoculation allows detection of virus at the peripheral site of inoculation. We also confirm that latent HSV infection established in mice by corneal inoculation does produce spontaneously recurring episodes of ocular shedding.\(^{15}\)

**Materials and Methods**

**Animals**

Male BALB/c mice 4–6-weeks-old were purchased from Simenson Lab., Inc. (Gilroy, CA) and housed at the Estelle Doheny Eye Foundation (Los Angeles, CA) vivarium, which is fully accredited by the American Association of Laboratory Science. The use of animals conformed to the 1983 ARVO Resolution on the Use of Animals in Research.

**Cells and Virus**

Rabbit kidney (RK) cells were grown in minimal essential media (MEM) supplemented with 10% NCTC 135 and 10% fetal bovine serum (MEM 10-10) as described by Nesburn et al.\(^{26}\) RK cells were seeded into small glass bottles (20 mM x 30 mM) and maintained at 37°C in a humidified 5% CO\(_2\):95% air atmosphere. RK monolayers were used to detect the presence of ocular HSV shed at the tear film from the lacrimal gland, conjunctiva, and cornea. Stocks of HSV-1 McKrae strain were prepared in MRC-5 cells (Flow Labs; Inglewood, CA), aliquoted, and stored at -70°C.

**Ocular Inoculation and Virus Isolation**

After one drop of 0.5% proparacaine hydrochloride (Alcon; Fort Worth, TX), both corneas of the mouse were abraded by gently rubbing a calcium alginate swab (Inolex; Glenwood, IL) over the cornea at least 10 times. Following corneal abrasion, HSV-1 was inoculated onto the cornea of both eyes in a volume of 5μl of 1 × 10^4 PFU/eye (approximately 1 LD\(_{50}\)). To confirm virus infection, the external eye was gently swabbed on day 3 postinoculation; to maximize the opportunity of virus, the same swab was used on both eyes. The inoculated RK monolayers were examined daily for 2 weeks for cytopathic effects (CPE). Suspected HSV isolates were confirmed by repeat passage in RK cells.

**Virus Neutralization**

Virus isolated during reactivation experiments was confirmed by a second passage in RK monolayers and using a microneutralization procedure with hyperimmune rabbit anti-HSV serum (serum neutralization titer >1:256) and normal rabbit serum (serum neutralization titer <1:2). Briefly, an equal volume (200 μl) of supernatant fluid from positive cultures was mixed with either immune serum or normal serum and incubated for 30 min at 37°C. After the incubation period, 10-fold dilutions were made and 100 μl of the supernatant fluid were inoculated onto RK cells grown in 96-well flat bottom plates (Falcon; Oxnard, CA); the plates were incubated at 37°C for 2–3 days. Virus isolates showing a greater than 100-fold dilution difference in CPE after incubation with normal serum in comparison with immune serum were considered to be positive HSV-1 isolates.

**Virus Detection in Neural Tissue**

Mice were killed by cervical dislocation; both trigeminal ganglia, including the proximal and distal

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**Reactivation of Latent HSV Infection**

Induction of virus reactivation with ocular shedding was attempted 28 days postinoculation on mice with confirmed acute infection by bilateral iontophoresis of epinephrine into the cornea using a modification of the procedure described by Kwon et al.\(^{20}\) Mice were anesthetized with 8% Avertin (5% 2,2,2-tribromoethanol in 1,2-propanediol) in 0.85% saline (0.1 ml/10 g body weight) administered by intraperitoneal injection. A beveled plastic eye cup (4 mM in diameter × 19 mM in length) containing 0.01% epinephrine in balanced salt solution was placed over the cornea. The anode was attached to the electrode within the eye cup; the cathode was attached to an aneurysm clip which was then placed on the ear. A direct current (6 mamp; 9V) was applied for 6 min. Between animals the eye cup was rinsed twice with 70% ethanol, twice with 0.85% saline, and then air-dried. This iontophoresis procedure was performed once a day on three consecutive days. The iontophoresis of epinephrine was performed on five separate groups of mice at 4-weeks postinoculation and one group of mice at 9-weeks postinoculation. Virus isolation procedures were performed beginning 3 days prior to iontophoresis and continuing for 7 days after iontophoresis was stopped. Eyes of mice not subjected to iontophoresis were cultured for at least 3 consecutive days beginning at 25-days postinoculation. Several of the 97 mice used as controls for the iontophoresis were cultured on consecutive days from day 25–40-postinoculation.
portions of the nerve, were dissected carefully and placed into MEM without serum (4°C). Both ganglia from the same mouse were minced with a scalpel into pieces less than 1 mM² and the minced tissue was divided into two aliquots. One aliquot, containing intact cells, was co-cultivated with RK monolayers at 37°C for 21 days and examined daily for CPE. The second aliquot was disrupted by one cycle of freeze-thawing and sonic oscillation in an ice bath for 2 min, centrifuged at 2000 RPM to pellet debris and then the cell-free supernatant fluid was inoculated onto RK monolayers. Inoculated RK cultures were incubated at 37°C for 14 days and examined daily for CPE. Isolates were passed into fresh RK monolayers and then confirmed by virus neutralization with reference anti-HSV antiserum, as previously described. Virus isolated by only co-cultivation of trigeminal ganglia tissue containing intact cell was designated “latent” virus; virus isolated from the same tissue by both co-cultivation and from cell-free supernatant was designated “infectious” virus.

Statistical Analysis

Differences in the rate of virus isolation from mice treated with epinephrine iontophoresis and from mice not treated by iontophoresis were compared by chi-square analysis. Data was considered significant with \( P < 0.05 \).

Results

Acute Ocular HSV-1 Infection of Mice

The LD\(_{50}\) for BALB/c mice inoculated by the ocular route with HSV-1 strain McKrae was found to be \( 10^4 \) PFU/eye. Virus was isolated at the external eye from 95% of the mice from day 3 through day 7. The infection became latent at approximately 15 days; after this time period, virus could not be isolated from the external eye nor could “infectious” virus be isolated from the neural tissue. However, virus was isolated from the trigeminal ganglia by co-cultivation of minced neural tissue.

Following inoculation with the McKrae strain of HSV-1, mice showed very few clinical signs of infection, an observation previously noted by Davis et al.\(^{28}\) Superficial punctate keratitis, dendritic lesions or geographic ulcers were observed rarely. The signs most commonly observed were conjunctivitis and lid edema.

During studies in which latently infected mice were cultured starting 25 days after inoculation, HSV was detected in the tear film swabs of 3/97 (3%) mice (Table 1). Since these mice had not been subjected to any intentional reactivation procedure, we consider these shedding episodes to be spontaneous reactivation events. From these studies and others,\(^{15}\) spontaneous reactivation apparently occurs at a very low rate in nonstimulated mice. The percent of HSV positive cultures (number of positive cultures/total number of cultures \( \times 100 \)) was 0.9% (5/531) for the 97 mice.

Reactivation of Latent HSV Infection by Epinephrine Iontophoresis

Mice surviving bilateral inoculation with \( 1 \times 10^4 \) PFU/eye and having confirmed ocular HSV infection were subjected to iontophoresis beginning at either 4 or 9 weeks after inoculation. Following iontophoresis of epinephrine into the cornea at 28 days postinoculation, a total of 16/23 (70%) stimulated mice shed HSV (Table 1). There was wide variation in the success of reactivation (Table 1) for the five individual iontophoresis stimulation trials. However, comparing the number of mice with ocular shedding in the stimulated (16/23) and nonstimulated (3/97) groups demonstrates that iontophoresis with epinephrine significantly \( (P < 0.001) \) increases the incidence of ocular shedding. In addition, if one compares the percent of HSV positive cultures in mice subjected to iontophoresis (11.3%; 26/223) with the percent which is obtained from nonstimulated mice (0.9%; 5/531), the effect of epinephrine iontophoresis is very significant \( (P < 0.001) \).

The sequence of HSV shedding during and following iontophoresis for all groups is shown in Figure 1. No mice shed virus for 3 days prior to initiating ionto-
Mouse Days of Culture

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Fig. 1. Ocular HSV shedding associated with epinephrine iontophoresis initiated at 4-weeks postinoculation. Epinephrine iontophoresis performed on days indicated by a superscript a. Each point (•) denotes a positive HSV ocular shedding event. Horizontal dotted lines separate the groups of mice subjected to iontophoresis.

phoresis. However, four of the stimulated mice shed virus 8–12 hr after the first iontophoresis stimulus.

Reactivation of HSV shedding from latently infected mice also was attempted at 9 weeks postinoculation. Of eight mice subjected to iontophoresis, five mice (63%) shed virus within the 10-day period of culturing (Table 1); the percent of positive cultures was 8.8% (7/80) for this group. As shown in Figure 2, the shedding episodes were after the 3-day iontophoresis cycle; two mice shed virus more than once during the culture period. There is no significant difference (P > 0.05) between either the number of mice shedding virus or the percent of positive cultures after epinephrine iontophoresis at either 4 weeks or 9 weeks postinoculation. There is, however, a significant (P < 0.001) increase in the percent of positive cultures from mice stimulated at 9 weeks when compared with nonstimulated mice.

Isolation of Virus from Neural Tissue

At the end of the culture period (40-days postinoculation), the mice from the five groups were killed in order to determine the presence of virus in the trigeminal ganglia. As stated before, virus isolated only from intact cells of neural tissue was considered to be in the "latent" state; virus isolated from cell-free supernatant fluid and intact cells of neural tissue was considered to be "infectious" virus. As shown in Table 2, all HSV isolates from nonstimulated mouse trigeminal ganglia were "latent" virus. In contrast, in stimulated mice, 58% (7/12) of the trigeminal ganglia isolates were "infectious" virus. There was no significant difference (P > 0.05) in the ability to isolate virus from the trigeminal ganglia of stimulated and nonstimulated mice.

Correlation Between Virus in Neural Tissue and External Site

A total of 18 of the 23 mice treated by epinephrine iontophoresis had cultures taken from both tear film and trigeminal nerve. Table 3 shows the breakdown of the virus isolation from mice treated by epinephrine iontophoresis. Ten of 18 stimulated mice (56%) had virus isolated from the trigeminal ganglia and also shed virus in the tear film. Virus was isolated only in the

| Table 2. Effect of epinephrine iontophoresis on HSV isolation from trigeminal ganglia of latently infected BALB/c mice at 40-days postinoculation |
|-------------------------|-------------------------|-------------------------|
| Percent of mice with HSV isolation from trigeminal ganglia | Percent of isolates as: |
| Iontophoresis \(\dagger\) | 67 (12/18) | 58 (7/12) |
| No iontophoresis \(\ddagger\) | 43 (7/16) | 0 (0/7) |

\* "Infectious" virus is defined by the isolation of HSV from cell-free homogenates and from co-cultivation of minced tissue from mouse trigeminal ganglia with indicator monolayers.

\(\dagger\) "Latent" virus is defined by the isolation of HSV only from co-cultivation of minced tissue from mouse trigeminal ganglia with indicator monolayers.

\(\ddagger\) Trigeminal ganglia were removed from each mouse at 10 days after initiating epinephrine iontophoresis and were minced and split into two aliquots for culturing of cell-free homogenates and co-cultivation.

Table 3. Correlation of HSV isolation from ocular and trigeminal ganglia of latently infected BALB/c mice subjected to epinephrine iontophoresis at 4-weeks postinoculation

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<th>HSV positive ocular culture</th>
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<td>HSV positive ganglia culture</td>
<td>10/18 (56%)</td>
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<td>HSV negative ganglia culture</td>
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tissue from 4 of 18 mice (22%), and 2 of 18 (11%) mice had virus isolated only from the trigeminal ganglia. Virus was not cultured from either site in the remaining two mice.

Discussion

That spontaneous ocular HSV shedding occurs in mice is an important finding. This observation, which has been reported previously for humans and rabbits, indicates that experimental murine HSV infection is similar to the infection in other animals. Therefore, BALB/c mice can be used as a model for in vivo studies of recurrent HSV infections. Our finding supports the observation of Hill et al., who detected spontaneous shedding in mice after ear inoculation and Tullo et al., who detected spontaneous shedding after ocular inoculation.

Our experiments indicate that iontophoresis of epinephrine is a useful procedure to induce reactivation of latent herpes virus infection in mouse trigeminal ganglia. It is significant, as well as experimentally advantageous, that this reactivation of the ganglionic infection is associated with a high rate of HSV shedding at the site of peripheral infection (i.e., eye). Though variable, the average rate of ocular shedding (70%) following epinephrine iontophoresis is substantially higher than has been reported following other reactivation procedures in mice. Recently Hill et al. demonstrated a consistently higher rate of shedding after iontophoresis when rabbits were preselected for the ability to shed HSV spontaneously. This may be a factor contributing to the variability observed in virus shedding after iontophoresis in our unselected, latently infected mouse population. In addition, we have demonstrated the presence of “infectious” virus in the trigeminal ganglia following epinephrine iontophoresis which was absent in nonstimulated mice. We were able to demonstrate a correlation between the presence of virus in the ganglia and the shedding at the external site, which has also been reported by Hill et al.

Many investigators have reported the rate of latent ganglionic infection following HSV infection in mice to be as high as 90–100%. In this study, we were able to verify “latent” infection in 43% of the mice. Because we wanted to examine the state of ganglionic infection at the time of killing, we chose to use the immediate mincing technique, even though this procedure has been reported to reduce the rate of virus recovery. Using other techniques, we have been able to verify “latent” ganglionic infection in 90–100% of the mice infected by the ocular route with 10^6 PFU of HSV-1 (data not included).

This report demonstrates that iontophoresis of epinephrine into the mouse cornea is associated with reactivation of latent ganglionic infection and with a high incidence of ocular HSV shedding. This mouse ocular system should thus be a valuable tool for studying recurrent HSV-1 infections since: (1) the latent HSV ganglionic infection can be reactivated, (2) ocular shedding is associated with reactivation, (3) the genetic backgrounds of inbred mice are defined, (4) the immune response to HSV is studied more easily and manipulated in the mouse, and (5) the cost per experiment is reduced with mice. This murine ocular model can be used to study and manipulate factors involved in the whole spectrum of acute and recurrent HSV infections, being especially helpful for in vivo studies on latency, ganglionic reactivation, spontaneous shedding, and induced shedding. Further investigations should help unveil some of the crucial virus-host interactions involved in establishing, maintaining, and modulating latent HSV infection and shedding.

Key words: ocular HSV, mice, reactivation, epinephrine iontophoresis, trigeminal ganglia, latency

References

15. Tullo AB, Shimeld C, Blyth WA, Hill TJ, and Easty DL: Spread