The Development of an Improved Murine Iontophoresis Reactivation Model For the Study of HSV-1 Latency

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The present study reviews the development of an effective murine iontophoresis reactivation model for the study of HSV-1 latency. In a series of experiments, Balb C mice latently infected with HSV-1 McKrae strain were iontophoresed with epinephrine × 3 days (EPI × 3/ION) or 6-hydroxydopamine × 1 day followed by topical epinephrine (6-HD ION/EPI). Reactivation and recovery of latent HSV-1 was determined by daily ocular swabs, titration, and neutralization. Additional studies determined the effect of topical ocular steroids on viral recovery rate. The results demonstrated no recovery of McKrae strain in Balb C (0%) with EPI × 3/ION, and no enhancement with topical steroids. 6-HD ION/EPI demonstrated a low recovery rate in mice (8%). However, the recovery rate was significantly increased to 50% by the addition of topical steroids to form the 6-HD ION/EPI/STEROID model, a useful experimental tool. The substitution of a clinical isolate, W strain, for McKrae strain further improved the model. The results demonstrated that, following the acute infection in mice, W strain was associated with a significantly higher (P = .001) survival rate than McKrae strain (81% vs. 52%). There was no statistically significant difference between the two strains, W vs McKrae, in Balb C mice comparing keratitis, establishment of latency (by co-cultivation), spontaneous shedding rate, or induced ocular shedding following iontophoresis. The development of an effective murine iontophoresis model offers an economical method which is uniquely suited for immunological and genetic studies of HSV-1 latency. Invest Ophthalmol Vis Sci 27:1230-1234, 1986

In the past, several animal models have been developed to study HSV-1 latency in vivo. Successful reactivation of latent HSV-1 in vivo has been accomplished by: a) physical manipulation of the nerve, i.e., neurectomy,1 b) mechanical2 or electrical stimulation3 of the trigeminal ganglia, c) mechanical,4 ultraviolet light or chemical stimulation of the epithelial surface,5 and d) systemic immunosuppression with prednisone6 or cytotoxic agents.7 Unfortunately, many of these methods were limited by a low reactivation rate and/or technical difficulty. Recently, iontophoresis has been proposed as a simple effective method to reliably induce reactivation of latent HSV-1. Studies in the rabbit demonstrated that epinephrine8 or 6-hydroxydopamine iontophoresis9 efficiently induced reactivation, and promoted ocular shedding of latent HSV-1 McKrae strain in 100% of animals. Subsequently, epinephrine iontophoresis was reported to promote ocular shedding of HSV-1 McKrae strain in 70% of latently infected Balb C mice.10 The present study evaluated the efficacy of the iontophoresis reactivation model, and suggests modifications to improve its usefulness, including the use of a less virulent clinical isolate, HSV-1 W strain, in place of the more virulent standard laboratory strain, HSV-1 McKrae.

Materials and Methods

Viruses

HSV-1 McKrae, a laboratory strain, was obtained courtesy of A. B. Nesburn and M. D. Trousdale. W strain was isolated from a 56-yr-old black male with recurrent dendritis keratitis. Both strains were grown in secondary rabbit kidney (RK-2) monolayers with Eagles MEM plus 10% calf serum, titered, divided into 1.5 ml aliquots, and frozen at −70°C prior to use.

Virus Characterization

The thymidine kinase phenotype was determined by BUDR/autoradiography,11 and temperature sensi-
Activity studies included the determination of virus titers in RK-2 cells at 72 hr following incubation at 33°C, 37°C, and 39°C, respectively. Keratitis was evaluated 2 days after corneal inoculation with .025 ml of 10^7 pfu/ml virus suspension. Mice infected with each virus strain were graded (scale 0-4) (Table 1) in a masked fashion using a microscope, 0.12% fluorescein eye drops, and a cobalt blue filtered light source. One month post-inoculation, the survival rates were determined. Table 2 compares the biochemical and clinical characteristics of the two virus strains.

**Animals**

Four- to 6-week-old male Balb C mice (Charles River Laboratories, Wilmington, MA) were used in all experiments. All studies presented adhered to the ARVO Resolution on the Use of Animals in Research.

**Procedures**

In the first series of experiments (Table 3), 4-6-week-old male anesthesized Balb C mice were inoculated in both eyes following corneal epithelial scarification (six vertical strokes with a #25 needle) with .025 ml of 10^7 pfu/ml suspension of HSV-1 McKrae strain. One month post-inoculation, surviving latently-infected mice were divided into six groups. Group 1 (CONTROL) consisted of mice who received no treatment. Virus recovered from this group represented spontaneous shedding. Group 2 (TOPICAL STEROID) consisted of mice whose eyes were treated beginning Day 0 with 1% prednisolone phosphate eye drops four times a day for 7 days. Virus recovered from this group represented steroid-induced shedding without iontophoresis. Group 3 (EPI X 3/ION) consisted of mice whose eyes were iontophoresed beginning Day 0 with .01% epinephrine (EPI) for 6 min at 0.6 mA for 3 days. Group 4 (EPI X 3/ION/STEROID) was the same as Group 3 except that prednisolone phosphate was administered topically as in Group 2. The iontophoresis apparatus (Fig. 1) consisted of an iontophoresis unit (Medtherm Electromedicator, Huntsville, Ala), a current splitter, a tube holder, modified Eppendorf tubes, platinum electrodes, cotton wicks, and the iontophoresis solution. Iontophoresis was performed under ketamine (33 mg/kg) and acepromazine (1.1 mg/kg) anesthesia, and successful iontophoresis was monitored by pupillary dilatation following each run. Ocular shedding of latent HSV-1 was determined daily for 12 days beginning 1 day prior to iontophoresis and continuing for 11 consecutive days. Each mouse eye was swabbed individually with media-moistened miniature cotton balls which were placed in tubes containing .15 ml of Hanks MEM, and vortexed. The media was withdrawn and plated on Vero cell monolayers which were examined for 7 days. HSV-1 was identified by characteristic cytopathic effect, and micro-neutralization of random samples. Following the completion of each experiment, HSV-1 latency was confirmed by

**Table 1. Keratitis score**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No keratitis.</td>
</tr>
<tr>
<td>1</td>
<td>Superficial punctate keratitis with at least one small branching dendrite.</td>
</tr>
<tr>
<td>2</td>
<td>Multiple small branching dendrites.</td>
</tr>
<tr>
<td>3</td>
<td>Intermediate-sized dendrites with or without an associated small geographical ulcer involving less than one-half of the corneal surface.</td>
</tr>
<tr>
<td>4</td>
<td>Geographical ulcer involving more than one-half of the corneal surface.</td>
</tr>
</tbody>
</table>

**Table 2. Virus characterization**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>TK phenotype</th>
<th>Virus Titers in RK-2 at 72 hr (Log_{10} pfu/ml)</th>
<th>Keratitis Day 2</th>
<th>Survival Day 30 (Co-cultivation)</th>
<th>Latency Day 30 (Co-cultivation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Group (%)</td>
<td>Positive mice (%)</td>
</tr>
<tr>
<td>HSV-1 McKrae</td>
<td>Positive</td>
<td>33°C 7.209</td>
<td>2.15 ± .63*</td>
<td>80/152 (52) b</td>
<td>17/18* (94)</td>
</tr>
<tr>
<td>(Laboratory strain)</td>
<td></td>
<td>36°C 7.127</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>39°C 2.875</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV-1 W</td>
<td>Positive</td>
<td>33°C 7.274</td>
<td>2.10 ± .52</td>
<td>118/141 (81)</td>
<td>25/28 (89)</td>
</tr>
<tr>
<td>(Clinical isolate)</td>
<td></td>
<td>36°C 7.260</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>39°C 2.966</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P = NS compared to HSV-1 W

b P = .001 compared to HSV-1 W
Table 3. Induced ocular shedding of HSV-1 McKrae in Balb C mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive eyes</th>
<th>Positive mice</th>
<th>Multiple shedding eyes</th>
<th>Shedding days (days 3–10) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CONTROL</td>
<td>1/22e (5)</td>
<td>1/12 (8)</td>
<td>0/22 (0)</td>
<td>1/176 (.6)</td>
</tr>
<tr>
<td>2. TOPICAL STEROID</td>
<td>1/22e (5)</td>
<td>1/12 (8)</td>
<td>0/22 (0)</td>
<td>1/176 (.6)</td>
</tr>
<tr>
<td>3. EPIx3/ION</td>
<td>0/16 (0)</td>
<td>0/8 (0)</td>
<td>0/16 (0)</td>
<td>0/128 (0)</td>
</tr>
<tr>
<td>4. EPIx3/ION/STEROID</td>
<td>0/16 (0)</td>
<td>0/8 (0)</td>
<td>0/16 (0)</td>
<td>0/128 (0)</td>
</tr>
<tr>
<td>5. 6-HD ION/EPI</td>
<td>1/24 (4)</td>
<td>1/12 (8)</td>
<td>0/24 (0)</td>
<td>1/126* (.8)</td>
</tr>
<tr>
<td>6. 6-HD ION/EPI/STEROID</td>
<td>9/24 (38)a</td>
<td>6/12 (50)a</td>
<td>5/24 (20)a</td>
<td>22/192 (11)d</td>
</tr>
</tbody>
</table>

*P = .001 compared to Groups 1–3.
**P = .005 compared to Groups 1–3.
***P = .01 compared to Groups 1–3.

* Two eyes lost due to ruptured globe.

expplantation of trigeminal ganglia (TG) and co-cultivation of minced TG on Vero cells which were followed for 28 days for HSV-1 cytopathic effect.

In the second series of experiments (Table 4), 4–6-week-old anesthetized male Balb C mice were inoculated in both eyes following corneal scarification with .025 ml of 10^7 pfu/ml suspension of a clinical isolate, W strain. At 1 month, all surviving latently-infected mice were randomly assigned to one of four treatment groups: (A) no treatment, to determine the spontaneous shedding rate, (B) topical steroid alone, (C) 6-hydroxydopamine iontophoresis with topical epinephrine (6-HD ION/EPI), and (D) 6-HD ION/EPI with topical steroid (6-HD ION/EPI/STEROID). The protocols for iontophoresis, virus recovery, and co-cultivation were the same as those followed in the first series of experiments.

**Statistical Analysis**

Following the completion of each series of experiments, the data for that series were pooled, and analyzed statistically using an analogue of analysis of variance and the chi square analysis.

**Results**

Table 2 compares the two virus strains. Both strains were thymidine kinase positive, and demonstrated similar growth patterns at different temperatures (36°C, 33°C, 39°C). On day 2 post-inoculation, the mean
keratitis score for HSV-1 McKrae (2.15) was comparable to HSV-1 W (2.10), as was the latency rate (94% vs. 89%) following explantation and co-cultivation on day 30. The major significant difference between the two strains (P = .001) was the higher survival rate of mice inoculated with W strain (81%) when compared to McKrae strain (52%).

Table 3 summarizes ocular shedding of HSV-1 McKrae in Balb C mice. The CONTROL group demonstrated minimal spontaneous shedding in 1 of 22 eyes. Topical steroid alone without iontophoresis did not induce ocular shedding above the spontaneous shedding rate, 1 of 22 eyes. Following iontophoresis, the EPI X 3/ION method (Group 3) failed to induce ocular shedding of latent HSV-1 in 16 eyes of 8 mice. The addition of topical steroid (Group 4) did not result in a detectable virus in 16 eyes of 8 other mice. The 6-HD ION/IPI method (Group 5) demonstrated a marginal effect with 1 of 24 eyes positive (4%) in 12 mice. However, the 6-HD ION/IPI/STEROID method (Group 6), which was formed by the addition of topical steroid to Group 5, resulted in a significant increase (P = .0001) in virus-positive eyes, 9 of 24 (38%), in 6 of 12 mice (50%). Five of 24 eyes (20%) in Group 6 demonstrated eyes with multiple days of shedding compared to none in the other groups (P = .01). The 22 total shedding days in the 6-HD ION/IPI/STEROID group was significantly higher (P = .0001) than 1 or 0 days in the other groups.

Table 4 summarizes ocular shedding of HSV-1 W strain, a clinical isolate, in Balb C mice. No spontaneous shedding was noted in the control group during the 12 days of the experiment. The TOPICAL STEROID group alone was associated with ocular shedding in 4 of 26 eyes. The 6-HD ION/IPI method demonstrated a marginal effect with the recovery of virus in 2 of 28 eyes. However, the addition of topical steroids, to form the 6-HD ION/IPI/STEROID group, again demonstrated a significant increase (P = .002) in virus-positive eyes, 11 of 32 eyes (34%) in 8 of 16 mice (50%). Statistically significant results were also demonstrated for multiple shedding eyes/group (P = .02), and number of shedding days/total days (P = .0001). When the data from Table 3 were compared to Table 4 for the 6-HD ION/IPI/STEROID method, there were no statistically significant differences between HSV-1 McKrae and HSV-1 W strains comparing positive eyes/group, positive mice/group, multiple shedding eyes/group, and total shedding days. However, for both virus strains, induced ocular shedding in the 6-HD ION/IPI/STEROID method was statistically significant between HSV-1 McKrae (2.0 ± 1.7 days) and HSV-1 W (2.38 ± 1.8 days). It should be noted that no ocular shedding was ever demonstrated earlier than 72 hr after iontophoresis for either virus strain.

Following the completion of all studies, the mice were sacrificed, and comparable rates of latency (50–100%) were confirmed for both McKrae and W strains in all groups by explantation and co-cultivation of trigeminal ganglia.

Discussion

The most successful animal model for the study of HSV-1 latency in vivo is the rabbit iontophoresis model, because of its simplicity and high rate (75–100%) of induced reactivation of latent HSV-1. The initial rabbit model, which required 3 days of iontophoresis with epinephrine, was significantly improved by substituting only 1 day of iontophoresis with 6-hydroxydopamine followed by topical administration of epinephrine for 5 days.

Despite the success in the rabbit, it appeared desirable to adapt this method to the mouse. The mouse is clearly superior to the rabbit for reasons of economics and statistics, as well as for immunological and genetic studies of HSV-1 in inbred strains. Therefore, the first reported successful adaptation of iontophoresis to the mouse employed the EPI X 3/ION model with a 70% success rate.

However, this model was characterized by an extremely variable reactivation rate (25–100%).
In the present study, we found significant disadvantages to this method. Three days of iontophoresis produced a higher anesthetic mortality rate, increased trauma to mouse eyes, and did not yield a satisfactory reactivation rate in our laboratory. Our failure to reproduce the 70% success rate with the EPI × 3/ION model may be due to differences in suppliers of inbred mice, pooled vs. individual eyes cultures, swabbing technique (dacricon swabs vs. cotton pledgets), etc. The addition of topical steroid to the EPI × 3/ION model failed to enhance reactivation or promote detection of reactivated virus by amplification. We, then, applied the 6-HD ION/EPI method to the mouse, which was less traumatic, but only marginally successful (Tables 3, 4). The addition of topical steroids to the 6-HD ION/EPI method finally resulted in a method which reliably and reproducibly reactivated latent HSV-1.

We believe that the topical steroids acted to promote detection of virus at the ocular surface by allowing increased virus replication due to impaired immune clearance mediated by the application of topical steroids. However, we cannot rule out that topical steroids also induced reactivation of latent HSV-1. Studies are currently underway to clarify the mechanism. The current mouse model differs from the rabbit model in that it is technically more difficult, as it requires working with a smaller animal, and the reactivation rates show greater variability (25–67%) from experiment to experiment. However, in our opinion, for certain types of investigations, the advantages previously cited outweigh the disadvantages. The mouse model also offers an additional advantage over the rabbit model in that the reactivation rate is 50% vs. 100% for rabbits. Pharmacological studies can demonstrate drugs which promote reactivation when compared to a control with a 50% reactivation rate. Our preliminary studies suggested that the reactivation rate in the current model may be further increased by the addition of topical timolol. Such pharmacological studies cannot be done in the rabbit model with a 100% reactivation rate.

Previous studies in viral pathogenesis have demonstrated that neurovirulence is virus strain dependent for HSV-1. Prior to this communication, a standard laboratory strain, HSV-1 McKrae, was the most effective virus strain found to be capable of reactivation following iontophoresis in rabbits and mice. Preliminary studies suggested that the Ota strain may also be effective in the iontophoresis model. The present study demonstrated for the first time that a clinical isolate, W strain, cultured from a patient with recurrent herpetic keratitis, was capable of establishing latency in the rabbit model with a 100% reactivation rate. The reactivation model offers an important new tool to investigate the immunology and genetics of HSV-1 latency. Together with the rabbit model, the biochemistry, physiology, and pharmacology of HSV-1 latency may be further explored.

Key words: HSV-1 McKrae, HSV-1 W, iontophoresis, herpetic latency, 6-hydroxydopamine

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References