A Fast, Simple Reactivation Method for the Study of HSV-1 Latency in the Rabbit Ocular Model

Y. Jerold Gordon, Eric Romanowski, and Trinita Araullo-Cruz

We developed a simple, fast, economical, and versatile reactivation method for the study of herpes simplex virus type 1 (HSV-1) latency in the rabbit ocular model. Intrastromal injection of sterile, deionized water induced reactivation and ocular shedding of latent HSV-1 in 21 of 27 eyes (78%) in 93% of New Zealand rabbits. Other control groups (eg, anterior chamber injection of sterile, deionized water; topical administration of sterile, deionized water; intrastromal injection of air; and intrastromal needle track), were less efficient in reactivating latent HSV-1. Although the mechanism of reactivation in this model is unknown, the reactivation signal may be related to the relative amount of corneal trauma. Invest Ophthalmol Vis Sci 31:921–924, 1990

Over the past 44 yr, investigators have sought to develop a simple, efficient reactivation method for the study of herpes simplex virus type 1 (HSV-1) latency in an animal model, but most methods were limited by low reactivation rates, expense, and technical difficulty. These methods included: 1) direct stimulation of sensory ganglia by mechanical or electric means; 2) sectioning of the nerve; 3) stimulation of the epithelial surface at the peripheral inoculation site by mechanical trauma; 4) ultraviolet light or chemicals; and 4) manipulation of the host immune system by generalized anaphylaxis, localized corneal Arthus reaction, and systemic immunosuppression with prednisone or cytotoxic agents.

An efficient reactivation method was reported by Kwon et al in which iontophoresis of epinephrine (EPI) into rabbit eyes for 3 consecutive days induced ocular shedding of latent HSV-1 in 100% of rabbits. This method was further simplified by iontophoresis of 6-hydroxydopamine (6-HD) once, and the subsequent daily administration of topical epinephrine. Because of the high reactivation rate, the latter method became the standard in rabbits, and both methods were successfully adapted to the mouse.

Although the iontophoresis methods are very efficient in the reactivation of latent HSV-1, they are also time-consuming, labor-intensive, and expensive, and require special equipment and the use of chemicals which may interfere with the design and interpretation of experiments to study the pharmacology of reactivation. A simpler, faster, more economical, drug-free method in an animal model was desirable, and this became the primary goal of the current study.

Materials and Methods

Animals

Female New Zealand (NZ) albino rabbits, weighing 1.5–2.0 kg, were purchased from Green Meadows Rabbitry, Murrysville, PA, and were housed in the Animal Care Facility of The Eye and Ear Institute of Pittsburgh. These studies strictly adhered to the ARVO Resolution on the Use of Animals in Research.

Virus Inoculation

Following topical anesthesia with 0.5% proparacaine HCl eye drops, each unscarified rabbit eye was inoculated by placing 50 μl viral suspension (5 × 10^4 pfu/eye) of HSV-1 W strain into the lower fornix and closing the lids and massaging the eye for 30 sec. HSV-1 W strain has been described previously in the mouse model as a thymidine-kinase-positive strain which establishes latency, and reactivates in the ocular model in a manner similar to HSV-1 McKrae strain. This is the first report of HSV-1 W in New Zealand rabbits. Successful inoculation of 100% of eyes was documented on day 7 after inoculation by the presence of typical herpetic dendritic and geo-
graphic ulcers, and significant HSV-1 viral titers (10⁴ pfu/ml).

**Preparation of Sterile, Deionized Water**

The water used in the experiment was College of American Pathologists (CAP) Grade 2 laboratory-quality water that is provided by tap to all laboratories in The Eye and Ear Institute of Pittsburgh. It was prepared by passing through a system consisting of a fan filter, carbon filter, several weak base cation exchange resins, and a reverse-osmosis Millipore filter. Prior to sterilization, the water was cultured, and found to contain 140 bacterial colonies/ml. The water was sterilized by autoclaving (50’, 121 °C, 15 psi), and was then found to have 0 bacterial colonies/ml. After sterilization, a sample of the water used for intracorneal injection was tested by the Associates of Cape Cod, Woods Hole, MA, and found to contain 0.125 endotoxin units/ml using the Gel-Clot Limulus Amebocyte Lysate (LAL) Assay.

**Experimental Design**

In a series of experiments, latently infected rabbits were used up to 181 days postinoculation. Rabbits were randomly selected and divided into five treatment groups. After satisfactory anesthesia with separate intramuscular injections of ketamine HCl (33 mg/kg), and acepromazine maleate (1.1 mg/kg), and topical 0.5% proparacaine eye drops, the globe was propptosed with a wooden cotton applicator, and stabilized in that position by an assistant. An operating microscope was used to facilitate all surgical manipulations.

**Intracorneal injection**: A no. 30 short bevel needle attached to a 0.25-ml tuberculin syringe was carefully inserted into the central corneal stroma, and advanced until the bevel was beyond the entry wound. For one group, sterile, deionized water (100 μl) was slowly injected, and appeared as a greyish-white circular bleb, which slowly increased in size. For another group, air (100 μl) was rapidly injected, and appeared as a crystalline, circular corneal opacity. For a third group (needle track), no injection was made, and for all three groups, the needle was carefully withdrawn, and the propptosed globe gently returned to the orbit by gentle digital pressure.

**Anterior chamber injection**: A no. 30 short bevel needle on a tuberculin syringe was introduced at the limbus, and inserted into the anterior chamber parallel to the iris plane. Sterile, deionized water (100 μl) was injected, and no obvious changes were seen. The needle was carefully withdrawn, and the insertion site was pressed with a cotton swab for 30 sec, to avoid aqueous loss. This pressure also returned the propptosed globe to its proper place in the orbit.

**Topical administration**: Using a Gilson Pipetman, 100 μl sterile, deionized water was dropped onto the cornea of the propptosed globe. The globe was returned to the orbit by gentle digital pressure.

**Determination of Viral Shedding**

The detection of latent HSV-1 after reactivation and induced shedding into the tear film was determined by swabbing the eyes 2 days prior to treatment and for 7 consecutive days after treatment. Eyes determined to be shedding virus immediately prior to treatment were not included in the results. Each eye swab was placed into a tube containing 0.3 ml outgrowth media (modified Eagle’s medium with Earle’s salt, 10% newborn calf serum (NBCS), 1% penicillin-streptomycin, 1% Fungizone). The tube was vortexed, and the eluant plated onto a Vero cell monolayer in a single well of a 12-well plate. After a 1-hr adsorption period, an additional 1.5 ml outgrowth media was added to the well, and the plate was examined daily for 7 days for the progressive cytopathic effect characteristic of HSV-1. Random isolates were confirmed as HSV-1 by neutralization.¹⁷

**Statistical Analysis**

After the completion of all experiments, the data were pooled and analyzed statistically with the chi square, Fisher Exact Test, and one-way analysis of variance (ANOVA).

**Results**

Table 1 summarizes the pooled data of multiple trials in which different methods were used to induce reactivation and ocular shedding of HSV-1 W strain in latently infected NZ albino rabbits. In general, over 90% of ocular shedding occurred between days 3 and 7, and by day 7, 82% of shedding eyes had ceased to shed. Anterior chamber injection of the sterile, deionized water induced reactivation and ocular shedding of latent HSV-1 W in 21 of 27 eyes (78%) in 13 of 14 animals (93%). Reactivation and shedding occurred more often after the administration of sterile, deionized water by intrastromal injection than by anterior chamber injection (7/20 positive eyes in 5/10 animals, P < 0.005), or by topical administration (4/17 positive eyes in 3/9 animals, P < 0.005). There was no statistically significant difference in the number of reactivation events between anterior chamber injection and topical administration of sterile, deionized water.

The percentage of shedding days/total days was also significantly greater (P < 0.0001) after intrastromal injection of sterile, deionized water than after anterior chamber injection or topical administration.
Induced shedding of HSV-1 W strain in latently infected NZ rabbits

Table 1. Induced shedding of HSV-1 W strain in latently infected NZ rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Trials</th>
<th>Eyes: positive (% with group)</th>
<th>Rabbits: positive (% with group)</th>
<th>Days shedding total (days 1–7) (77/189) (%)</th>
<th>Mean duration of shedding (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrastromal H₂O</td>
<td>3</td>
<td>21/27* (78)</td>
<td>13/14* (93)</td>
<td>77/189† (41)</td>
<td>3.6 ± 1.9†</td>
</tr>
<tr>
<td>Intrastromal air</td>
<td>2</td>
<td>7/16§ (44)</td>
<td>5/8 (63)</td>
<td>15/112 (13)§</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Intrastromal needle track**</td>
<td>2</td>
<td>1/16 (6)</td>
<td>1/8 (12)</td>
<td>1/112 (0.9)</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Anterior chamber H₂O</td>
<td>2</td>
<td>7/20 (35)</td>
<td>5/10 (50)</td>
<td>22/140 (16)†</td>
<td>3.1 ± 2.1</td>
</tr>
<tr>
<td>Topical H₂O</td>
<td>2</td>
<td>4/17 (24)</td>
<td>3/9 (33)</td>
<td>6/119 (5)</td>
<td>1.5 ± 0.6</td>
</tr>
</tbody>
</table>

* P < 0.05 compared to anterior chamber H₂O and topical H₂O.
† P < 0.0001 compared to anterior chamber H₂O and topical H₂O.
§ P < 0.05 compared to topical H₂O.
** P < 0.0001 compared to intrastromal H₂O.

(41%, 16%, and 5%, respectively). Also the mean duration of each shedding episode was significantly longer (P < 0.05) following intrastromal injection of sterile, deionized water than after topical administration (3.6 ± 1.9 days vs 1.5 ± 0.6 days).

Table 1 also compares the intrastromal injection of sterile, deionized water to intrastromal injection of air and to intrastromal needle tract alone with respect to their capacity to reactivate latent HSV-1 and induce ocular shedding. Needle tract alone was significantly less effective (P < 0.0001) than intrastromal injection of sterile, deionized water for all parameters of induced reactivation measured: positive eyes (1/16 vs 21/27), positive animals (1/8 vs 13/14), percentage of shedding days/total days (0.9% vs 41%), and mean duration of each shedding episode (1.0 day vs 3.6 ± 1.9 days). In contrast, there were no statistically significant differences between intrastromal air and sterile, deionized water with respect to reactivation as measured by the number of positive animals (5/8 vs 13/14), or mean duration of each shedding episode (2.1 ± 1.2 days vs 3.6 ± 1.9 days). However, intrastromal air was less effective than intrastromal sterile, deionized water as measured by the number of positive eyes (7/16 vs 21/27, P < 0.05), and the percentage of shedding days/total days (13% vs 41%, P < 0.0001).

Discussion

The current study presents intrastromal injection of sterile, deionized water as a new, rapid, economical method for the study of herpetic latency and reactivation in an animal model. We recovered HSV-1 in 78% of eyes of 93% of latently infected NZ albino rabbits (Table 1). The current method compares favorably with the standard reactivation method, ie, 6-HD iontophoresis followed by topical epinephrine (6-HD + EPI). When all experimental animals are considered, both methods efficiently reactivated latent HSV-1 in the rabbit eye. There were no statistically significant differences in the number of HSV-1-positive eyes after intrastromal injection in the current study, compared to 6-HD + EPI reported in a previous study14 (21/27 positive eyes vs 19/26 positive eyes, P = 0.47, not significant).

The methods differ in that intrastromal injection of sterile, deionized water is simpler, faster, more economical, and more versatile than 6-HD + EPI. Intrastromal injection requires only a single intracorneal injection that can be completed in less than 1 min in the anesthetized animal, whereas 6-HD + EPI requires at least 5–10 min per animal for iontophoresis, and additional time for the administration of eye drops twice per day for the duration of the experiment. For experiments requiring large numbers of animals, the potential saving of technician time is significant. Intrastromal injection is also more economical in that it requires only simple, inexpensive equipment (syringe, needle, sterile, deionized water) as compared to 6-HD + EPI, which requires an electromedicator, current splitter, and expensive chemicals (6-HD, EPI). Intrastromal injection is also more versatile than 6-HD + EPI in that it allows for “cleaner” pharmacologic studies on reactivation without introducing possible interference by exogenous drugs (6-HD, EPI).

The current study offers an empirical method which successfully induced ocular shedding of HSV-1 into the tear film of latently infected rabbits. The mechanism of reactivation of latent HSV-1 in the current model is unknown, but may be related to the relative amount of corneal trauma. Specifically, the forceful mechanical separation of the collagen lamellae after intrastromal injection of sterile deionized water or air may directly stretch the corneal branches of the trigeminal or sympathetic adrenergic nerves. This mechanical stretching of nerves may generate the reactivation signal from the peripheral site. Reduced corneal trauma as represented by an intrastromal needle tract alone, or the anterior chamber injection or topical administration of sterile, deionized water were less effective in inducing reactivation. A potential role for very small amounts of endotoxin...
contributing to the reactivation process cannot be totally excluded. However, endotoxin is unlikely to be a major factor, since 1) the amount injected was very low (0.125 endotoxin unit [EU]/ml). United States Pharmaceutical standard for pyrogen-free is defined as <0.250 EU/ml; 2) anterior chamber injection of the sterile, deionized water containing the same amount of endotoxin did not yield a number of reaction events comparable to that observed after intrastromal injection; and 3) intrastromal injection of air (presumably without any endotoxin) was associated with a considerable number of reactivation events: 7 of 16 eyes (44%) in 5 of 8 rabbits (63%). Additional studies to clarify the mechanism of reactivation are indicated.

Key words: herpes simplex virus, HSV-1, latency, intrastromal injection, iontophoresis

References