Timolol Promotes Reactivation of Latent HSV-1 in the Mouse Iontophoresis Model

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The present study examined the effect of topical timolol, a nonspecific beta 1 and beta 2 blocker on reactivation and ocular shedding of latent HSV-1 in an improved mouse iontophoresis model. Latent trigeminal ganglionic infection was established in Balb/C mice following inoculation by corneal scarification with HSV-1 W strain, a clinical isolate, and confirmed by co-cultivation. On day 30, postinfection (pi), the mice were divided into two groups, and treatment begun with coded eye drops (timolol 0.5% or placebo) BID OU for 5 days. On day 31 pi, iontophoresis with 1% 6-hydroxydopamine was performed, and daily treatment with topical epinephrine and 1% prednisolone was administered. Reactivation and recovery of latent HSV-1 was determined by daily ocular swabs, and characteristic HSV-1 cytopathic effect in Vero cells. Results demonstrated that the timolol-treated group had a significantly greater number of positive eyes, multiple shedding episodes, and total shedding days compared to the control group. We conclude that beta blockade promotes recurrent ocular shedding induced by epinephrine in the mouse iontophoresis latency model. Invest Ophthalmol Vis Sci 28:580-584, 1987

The study of the establishment, maintenance, and reactivation of latent herpes simplex virus type-1 (HSV-1) infections has been greatly advanced in the last 10 yr following the development of reproducible animal models. Induction of latent HSV-1 ocular shedding in rabbits has been accomplished by trauma to the corneal epithelium,1 by direct mechanical2 or electrical3 stimulation of the trigeminal ganglia, and by the administration of epinephrine intramuscularly4 or topically via iontophoresis.5-9 In the rabbit model, supersensitization of the adrenergic system with 6-hydroxydopamine iontophoresis followed by topical application of epinephrine induced ocular shedding of HSV-1 virus in 100% of latently infected rabbits.10

Recently, a mouse model for reactivation of latent HSV-1 infections has been developed using epinephrine iontophoresis11, and improved upon by employing 6-hydroxydopamine iontophoresis followed by topical epinephrine to the eye.12 Similarly, iontophoresis with 6-hydroxydopamine produced a chemical sympathectomy13 and increased sensitivity of mouse ocular tissues to epinephrine. In mice, we have reported that reactivation of latent HSV-1 virus by 6-hydroxydopamine iontophoresis and topical epinephrine and prednisolone occurred in 50% of animals.14

The mechanism of action of epinephrine-mediated reactivation of latent HSV-1 can be studied with adrenergic agonists and antagonists. Dunkel et al demonstrated that propranolol, a beta blocker, decreased the reactivation of suppressed HSV-1 in a tissue culture system.15 In the present study, we examined the effect of topical timolol, a nonspecific beta 1, and beta 2 adrenergic antagonist on reactivation and ocular shedding of latent HSV-1 in an improved mouse iontophoresis model.

Materials and Methods

Animals

Male BALB/c mice 4–6 weeks old were purchased from Charles River Laboratories and housed at the Graduate School of Public Health (Pittsburgh, PA), which is fully accredited by the Association of Laboratory Science. The use of animals conformed to the ARVO Resolution on the Use of Animals in Research.

Virus Inoculation

Male BALB/c mice weighing 20 to 28 grams were anesthetized with Nembutal (0.32 cc of 2.84 mg/ml concentration) prior to scarification and inoculation. Scarification of each cornea consisted of producing six linear cuts in the corneal stroma with a 25 gauge needle. Following scarification the corneas were inoculated with HSV-1 "W" strain, a clinical isolate (0.025 ml of
10^7 PFU/ml/eye) that had been cultivated on primary rabbit kidney cell monolayers and titrated on African green monkey kidney cell cultures.

**Determination of Viral Shedding**

On day 30 postinoculation, all surviving latently infected mice were randomly assigned to either a timolol 0.5% treatment group or placebo control group. The experimental protocol is summarized in Table 1. The ocular tear film was cultured in each eye for 2 days prior to iontophoresis to differentiate between spontaneous shedding and induced shedding. Swabbing was continued for an additional 10 days after iontophoresis to determine reactivation and recovery of latent HSV-1. Each eye was swabbed individually using a sterile forceps and sterile cotton miniballs measuring 2 mm in diameter. The swabs were gently manipulated over the entire ocular surface and then immersed in 0.15 cc of Modified Eagles Medium + 10% newborn calf serum + 1% streptomycin and penicillin (10^4 units/ml). The inoculated fluid was then plated on Vero cells and incubated in 5% CO2 at 37°C. The cells were examined daily for 7 consecutive days for the appearance of cytopathic effect characteristic of HSV-1, and random samples confirmed HSV-1 by microneutralization.

**Iontophoresis**

Details of the iontophoresis method have been previously described. Briefly, bilateral iontophoresis with 1% 6-hydroxydopamine was performed on day 31 postinoculation. Following anesthesia with ketamine (33 mg/kg) and acepromazine (1.1 mg/kg) administered by intraperitoneal injections, the eyes of the mice were propised and individual eye cups containing cotton saturated with 1% 6-hydroxydopamine were applied to the corneas. The anode was connected to the eye cups through which a current of 0.6 mamp was applied for 6 min, while the cathode was attached to the ear by use of an aneurysm clip.

**Treatment**

Following iontophoresis, the mice received topical epinephrine 0.1% twice a day to each eye for a total of 4 consecutive days. Topical timolol 0.5% or placebo, which was begun 1 day prior to iontophoresis was delivered in a masked fashion twice a day for a total of 5 consecutive days. Starting the day of iontophoresis and continuing for 6 consecutive days, prednisolone phosphate 1% was delivered to each eye four times daily. A total of five experimental trials were carried out.

**Table 1. Experimental protocol**

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<td>T 0.5%/Control</td>
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</table>

**Co-cultivation of Trigeminal Ganglia**

Thirty days after iontophoresis, the animals were sacrificed. Each trigeminal ganglion was excised, minced, and planted on Vero cells. The cultures were incubated at 37°C, 5% CO2 and examined daily for 30 consecutive days for the cytopathic effect characteristic of HSV-1. Random samples confirmed HSV-1 by neutralization.

**Statistical Analysis**

Chi-square analysis was performed on all pooled data. Statistical significance was considered to be P = 0.05. Each eye was treated as an individual observation because the BALB/c mice were genetically identical and kept under identical controlled environmental conditions.

**Results**

Table 2 summarizes the frequency of induced ocular shedding of HSV-1 “W” strain in the timolol and control groups in five experimental trials. As previously reported in the mouse iontophoresis model, there was considerable variation between trials in the reactivation rates of mice in both the timolol (38% to 100%) and control groups (12% to 50%). However in each of the five trials, the timolol-treated group always had a higher reactivation rate than its respective control. Also, the pooled data of five trials, representing a large sample size for both the timolol and control groups, yielded highly significant differences between the groups. Virus was isolated from the ocular tear film following iontophoresis in 40% of eyes of 64% of mice treated with timolol compared to 19% of eyes of 35% of the mice in the placebo treatment group. This was significant at P = 0.005 comparing eyes, and P = 0.02 comparing mice by Chi-square analysis. Spontaneous shedding was not observed in any of the experimental trials prior to iontophoresis.

Table 2 compares the timolol and control groups with respect to multiple viral shedding episodes in the same eye. The timolol-treated group demonstrated significantly more (P = 0.03) eyes with multiple shedding episodes than the control group (28% vs 14%).
Table 2. Induced ocular shedding of HSV-1 W in Balb/c mice (five trials)

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive eyes</th>
<th>Positive mice</th>
<th>Multiple shedding eyes</th>
<th>Shedding days</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Group (%)</td>
<td>Group (%)</td>
<td>Group (%)</td>
<td>Total (Day 3–10) (%)</td>
</tr>
<tr>
<td>Timolol 0.5%</td>
<td>29/72 (40)</td>
<td>23/36 (64)</td>
<td>20/72 (28)</td>
<td>78/568 (14)</td>
</tr>
<tr>
<td>Control</td>
<td>15/80 (19)*</td>
<td>14/40 (35)*</td>
<td>11/80 (14)*</td>
<td>36/640 (6)*</td>
</tr>
</tbody>
</table>

* P = 0.005 compared to the timolol group.
* P = 0.02 compared to the timolol group.

Table 2 also compares total days of virus shedding for the two groups. Virus was shed a total of 78 days out of a possible 568 days (14%) in the timolol treatment group while the control group shed 36 days out of 640 (6%) possible days. It should be noted that virus was never recovered before day 3 following iontophoresis in the mouse model. In this study, timolol significantly (P = 0.0001) promoted the number of days of viral shedding.

Table 3 demonstrates comparable trigeminal ganglionic latency rates in the timolol and control groups (97% versus 95%). It was important to show that the differences in reactivation rates following iontophoresis were due to treatment and not due to differences in the number of latently-infected animals per group.

Discussion

This is the first published report that a topical beta 1, beta 2 adrenergic antagonist, timolol, promotes the reactivation of latent HSV-1 in the mouse iontophoresis model. In order to clarify the mechanism of action of timolol, it is first necessary to review how 6-HD iontophoresis and topical epinephrine induce reactivation of latent HSV-1. There are three current theories that explain the reactivation of latent HSV-1: (1) the ganglion trigger theory, (2) the skin trigger theory, and (3) the combined ganglion and skin trigger theory. The ganglion trigger theory asserts that a stimulus switches on virus replication in the latently-infected ganglia. Virus then travels down the neuron by retrograde axoplasmic flow to the peripheral site, and infects epithelial cells to produce a clinical lesion. The skin trigger theory asserts that a peripheral stimulus produces local changes at the peripheral site, ie depression of local immune mechanisms, which allow microfoci of HSV infection already present to produce a clinical lesion. The ganglion and skin trigger theory combines (1) and (2) and contends that the peripheral stimulus first induces reactivation of the latent infection in the ganglion and produces local changes at the peripheral site which favors virus replication at the peripheral site. The original report of the rabbit iontophoresis model proposed that epinephrine was the trigger that acted centrally (ganglion trigger theory) or peripherally (skin trigger theory). Convincing experimental evidence that supported the combined ganglion and skin trigger theory was later demonstrated in the rabbit and mouse when epinephrine iontophoresis led to the direct recovery of infectious virus from the trigeminal and superior cervical ganglia, as well as from the external ocular surface.

The importance of the action of epinephrine on the ocular adrenergic nervous system was conclusively shown in the rabbit iontophoresis model as supersensitization by chemical sympathectomy (6-hydroxydopamine) produced the highest levels of reactivation of latent HSV-1 to date. Shimomura et al proposed that the action of epinephrine on the adrenergic receptors at the peripheral site might be the trigger directly involved in reactivation. Furthermore, adrenergic activation suggested that the sympathetic superior cervical ganglion (SCG) might be a source of latent HSV-1, that corneal sympathetic nerves might stimulate sensory nerves (ie trigeminal N.), and that both sympathetic and sensory ganglia might be triggered by an adrenergic mechanism. Shimomura et al proposed that the adrenergic antagonists should be studied further. The pharmacological response of cells and tissues to direct, local application of epinephrine depends on the net result of epinephrine interaction with alpha 1, alpha 2, beta 1, and beta 2 receptors. Dunkel et al reported preliminary data that the adrenergic antagonist propranolol, a beta blocker, decreased reactivation of suppressed HSV-1 in a tissue culture system. In the current study, another adrenergic antagonist, timolol, a non-specific beta 1, beta 2 blocker, significantly promoted the reactivation of latent HSV-1 W strain in Balb/c mice following 6-HD iontophoresis and topical epi-

Table 3. Latency by co-cultivation (day 30 after IONTO)

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive mice/group (%)</th>
</tr>
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<tbody>
<tr>
<td>Timolol 0.5%</td>
<td>34/35 (97)</td>
</tr>
<tr>
<td>Placebo</td>
<td>37/39 (95)</td>
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neurphrine administration. We propose that the trigger for reactivation may be the direct action of epinephrine on supersensitized alpha 1 and alpha 2 receptors of post-ganglionic neurons. Santos et al.\textsuperscript{22} reported preliminary data in the rabbit model that pretreatment alone with timolol decreased the reactivation rate of latent HSV-1, while pretreatment and treatment with timolol for 5 consecutive days postiontophoresis increased the reactivation rate. The differing results and apparent contradictions between our work and the other studies may be explained by the lack of comparability between in vitro and in vivo systems, differences in animal species, and major differences in experimental protocols. Further studies are needed to reconcile these differences.

The discussion thus far has interpreted the reactivation of latent HSV-1 in the 6-HD iontophoresis and topical epinephrine model in terms of the combined ganglionic and skin trigger theory which assumes a direct, local action of epinephrine on the sensory and/or sympathetic nerves of the cornea, conjunctiva, and ocular adnexae. However, a second possible explanation is that epinephrine acts systemically through presently unknown mechanisms to promote reactivation. Evidence that supports this interpretation includes the known clinical triggers of herpetic infections such as emotional stress, fever, sunlight, hormonal factors,\textsuperscript{23} and systemic immunosuppression,\textsuperscript{24} as well as the systemic experimental triggers of bacterial infection,\textsuperscript{25} anaphylaxis,\textsuperscript{26} and immunosuppression.\textsuperscript{27} Furthermore, earlier studies by Laibson and Kibrick\textsuperscript{4,5} demonstrated that systemic administration of epinephrine (intramuscular injection of hind thigh) was associated with an increased incidence of ocular shedding, ie, a local ocular effect induced by a stimulus at a distant site.

A third possible explanation is that both local and systemic effects are operative in epinephrine-induced reactivation of latent HSV-1. Topically administered 0.5% timolol is known to produce profound systemic effects in a 70 kg man (inhibition of exercise tachycardia, reduced forced expiratory volume, status asthmaticus, impotence).\textsuperscript{28} Therefore, in the 20 mg Balb/c mouse, 0.5% timolol may also promote reactivation of latent HSV-1 through some unknown systemic effect. Future investigations are necessary to clarify the mechanism of action of adrenergic agonists and antagonists. However, the acquired data must be interpreted with extreme caution, as the vertebrate animal is a complex, multivariate system whose responses may not be comprehensible in the same terms as a simple, isolated pharmacological muscle preparation.

**Key words:** timolol, herpes simplex, latency, iontophoresis, 6-hydroxydopamine

**Acknowledgment**

The authors wish to thank Mrs. Dolores Markowitz for her assistance in the preparation of this manuscript.

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