Timolol Induces HSV-1 Ocular Shedding in the Latently Infected Rabbit

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Timolol iontophoresis into the eye can induce herpes simplex virus type 1 (HSV-1) shedding in rabbits latently infected with HSV-1 strain McKrae. Anodal iontophoresis of 0.01% timolol was done at 0.8 mAmp for 8 min once a day for 3 consecutive days. Viral shedding was determined by the presence of HSV-1 in the preocular tear film obtained by eye swabs. In two experiments, iontophoresis of 0.01% timolol resulted in all eyes (18/18) shedding HSV-1 for an average duration of 4.3 days. When 5.0% timolol was applied topically to rabbit eyes supersensitized by iontophoresis of 6-hydroxydopamine (6-HD), all eyes (10/10) shed virus for an average duration of 2.9 days. All eyes (12/12) receiving iontophoresis of 6-HD, pre- and posttreatment with topical application of 5.0% timolol, and posttreatment with topical application of 1.0% epinephrine shed virus for an average duration of 3.6 days. Eyes treated with topical application of 5.0% timolol alone showed no difference in HSV-1 ocular shedding, compared with untreated eyes. We concluded that both iontophoresis of 0.01% timolol and topical application of 5.0% timolol to adrenergically supersensitized eyes induced HSV-1 shedding reliably and with a high frequency, and that topically applied timolol does not block the HSV-1 ocular shedding induced by epinephrine in adrenergically supersensitized eyes. Invest Ophthalmol Vis Sci 28:585–590, 1987

Previous investigations in our laboratory have demonstrated that epinephrine induces HSV-1 ocular shedding in the preocular tear film in New Zealand rabbits latently infected with HSV-1 strain McKrae.[1-9] These studies on adrenergically induced herpetic reactivation have been qualitative and quantitative for HSV-1 in the preocular tear film and associated neural tissues.[2,3,6,9] The development of this rabbit ocular model of herpetic reactivation by epinephrine provided a reliable method to study the mechanism of reactivation of latent HSV-1 infections. Of all the available animal models of HSV reactivation, epinephrine induction in the rabbit eye results in the highest frequency (90–100%) and longest duration (3–5 days) of viral shedding. We have presented pharmacological evidence that the mechanism of induction of HSV-1 ocular reactivation by epinephrine is correlated with the receptor potency of levo (–) epinephrine(s).[5] We have suggested that induction of HSV-1 ocular shedding by levo (–) epinephrine is a receptor-mediated event and is stereoselective.

Epinephrine activates both alpha and beta ocular receptors.[10-11] If reactivation of ocular herpes were induced exclusively by beta-adrenoreceptor activators, then the epinephrine response would be inhibited by a beta receptor antagonist. We assessed the ability of timolol, a nonspecific $beta_1$ and $beta_2$ receptor blocking agent, to inhibit or reduce the epinephrine induced herpetic reactivation. Zhang et al[12] reported that in rabbits, topically applied 0.5% timolol reduces the ocular HSV-1 shedding induced by topical application of epinephrine to eyes supersensitized to adrenergic agents by ocular iontophoresis of 6-hydroxydopamine (6-HD).[6,8,9] Harwick et al[13] reported that topical application of 0.5% timolol to mice latently infected with HSV-1 does not block ocular viral shedding induced by the 6-HD epinephrine procedure. We report here that, in rabbits, timolol does not block 6-HD-epinephrine-induced ocular shedding and that when timolol is given by ocular iontophoresis or applied topically to eyes supersensitized by 6-HD, it does induce HSV-1 ocular shedding.

Materials and Methods

Virus Strain

McKrae strain HSV-1 was propagated on primary rabbit kidney cell (PRK) monolayers and titered by

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plaque assay on green monkey kidney cell monolayers (CV-1). The virus was frozen in small aliquots at −70°C, and the same batch was used in all experiments.

Rabbits and Virus Inoculation

The unscarified corneas of New Zealand albino rabbits (1.5–2.5 kg) were inoculated with 25 μl of suspension of the HSV-1 McKrae strain (2–4 × 10^6 PFU/ml). Prior to inoculation, the eyes were normal as judged by slit-lamp biomicroscopy. The closed eye with the viral suspension was massaged for 20–40 sec with care taken to avoid leakage of the suspension. Primary corneal infection, mostly epithelial keratitis, was verified by slit-lamp biomicroscopic examinations on days 4–8 postinoculation (PI). Spontaneous shedding during PI days 20–39 was determined by the eye swab procedure described by Berman and Hill.[14] The care and maintenance of the rabbits used in all these experiments conformed to the ARVO resolution on the use of animals in research.

Tear Film Swabs

Tear film was collected from the rabbit eyes on sterile Dacron-tipped swabs by gentle rotation of the swab in the upper cul-de-sac, across the cornea, and then into the lower cul-de-sac, where the swab was allowed to absorb tear film in the fornix for 5 sec. The swabs were immediately placed in tissue culture tubes containing confluent PRK monolayers and incubated for 18–24 hr at 37°C in a CO_2 incubator. Subsequently, the swabs were squeezed against the side of the tubes to remove excess medium and removed. Eagles' Minimum Essential Medium with 2% fetal bovine serum (1 ml) was added for nutrition and pH adjustment. The tubes were monitored daily for 7–9 days for the appearance of cytopathic effects indicative of HSV-1.

Identification of Viral Isolates

Isolates from ocular swab cultures were identified by a plaque-reduction assay on CV-1 monolayers using HSV-1 McKrae specific rabbit antiserum, essentially as described by Knotts et al.[15] In all cases, the virus shed was identified as McKrae strain HSV-1.

Adrenergic Agents

Epinephrine (l-epinephrine hydrochloride, Epifrin®; Allergan Pharmaceuticals, Irvine, CA) at 1.0% was used for topical application. Solutions (1.0 or 0.1%) of 6-hydroxydopamine (6-HD) were prepared immediately prior to iontophoresis. The 6-HD was dissolved in sterile, deoxygenated distilled water with pH adjustment to 6.0–6.5 using sodium hydroxide. Paredrine® (hydroxyamphetamine hydrobromide, 1%; Smith Kline & French, Philadelphia, PA) and Timoptic® (timolol maleate, 0.5%; Merck, Sharp and Dohme, West Point, PA) were used. Other concentrations of timolol (0.01% or 5.0%) were prepared using the powder (STK #95642) supplied by Merck, Sharp and Dohme Research Laboratories through the courtesy of Dr. Clement A. Stone. Also, on one occasion, 0.01% timolol was prepared by dilution of commercial 0.5% timolol. Sterile, deoxygenated water was used in the preparation of all solutions.

Iontophoresis

Rabbits that shed HSV-1 spontaneously at least once in at least one eye were used in these experiments. Spontaneous shedding was determined by the procedure described by Berman and Hill.[14] Rabbits were used only once in these experiments. The rabbits were anesthetized by separate intramuscular injection of xylazine (4 mg/kg) and ketamine (20 mg/kg). An eye cup was centered within the limits of the corneoscleral limbus. The anode (+) made contact with the solution for ocular iontophoresis to the eye. The cathode (−) was attached to the ear over a saline-saturated cotton pad. The iontophoretic conditions (mAmps and time) and the solutions used are given in the tables. After iontophoresis of 6-HD, successful drug delivery was verified 24 hr later by the inability of two drops of 1.0% hydroxyamphetamine to dilate the pupil. The normal (untreated) rabbit eye will dilate since 1.0% hydroxyamphetamine causes a release of stored norepinephrine from sympathetic nerve terminals. After iontophoresis of 6-HD, the majority of sympathetic nerve terminals of the iris are depleted of norepinephrine.

Results

Six rabbits latently infected with HSV-1 strain McKrae were treated twice daily for 3 days with topical application of 5.0% timolol prior to iontophoresis of 6-HD (Table 1). Timolol was administered 60–90 min prior to iontophoresis of 6-HD. After iontophoresis of 6-HD, topical 5.0% timolol was given 60–90 min before topical application of 1.0% epinephrine; this was 4–6 hr after iontophoresis. The topical application of timolol and epinephrine was continued for the next few days, each drug given twice a day. Tear film swabs were taken for 11 consecutive days beginning 3 days prior to the day of iontophoresis and for 7 days after iontophoresis. Three rabbits had ocular iontophoresis of 1.0% 6-HD at 0.75 mAmps for 3 min; three other rabbits had iontophoresis of 0.1% 6-HD at 0.5 mAmps for 8 min. The average duration of shedding was 3.3 days and 4.0 days for the 1.0% and 0.1% 6-HD groups, respectively. The ratios of total positive swabs per total swabs for the 7 days after iontophoresis were 24/42

Table 1. Ocular shedding of HSV-1 in rabbit eyes after treatment with adrenergic agents

Conditions of iontophoresis	*Day PI*	*Rabbit number*	*Eye*	*Days pre/post-iontophoresis of 6-HD*										
				-3^{tt}	-2^{tt}	-1^{tt}	0^{tet}	1^{tete}	2^{tete}	3^{tete}	4^{tete}	*5*	*6*	*7*
1% 6-HD														
0.75 mAmp														
3 min	167	A-23	OD§	+	–	–	–	–	C	+	+	–	–	–
			OS§	–	–	–	–	–	–	+	+	+	+	–
	167	A-24	OD	–	–	–	–	–	+	+	+	C	–	–
			OS§	–	–	–	–	–	+	+	+	+	+	–
	82	A-57	OD§	–	–	–	–	–	+	+	–	–	–	–
			OS	–	–	–	–	–	+	–	–	+	+	+
Eyes—positive (%)				17	0	0	0	0	67	83	67	50	50	17
Rabbits—positive (%)				33	0	0	0	0	67	100	67	100	100	33
0.1% 6-HD														
0.5 mAmp														
8 min	157	A-26	OD§	–	–	–	–	–	+	–	–	–	–	–
			OS§	–	–	–	–	–	–	+	+	–	–	–
	157	A-30	OD	–	–	–	–	–	+	+	+	+	+	+
			OS§	–	–	C	+	–	–	+	+	+	+	+
	157	A-32	OD§	–	–	–	–	–	+	+	+	–	–	–
			OS§	–	–	–	–	–	+	+	+	+	+	+
Eyes—positive (%)				0	0	0	17	0	83	83	83	50	50	50
Rabbits—positive (%)				0	0	0	33	0	100	100	100	67	67	67

6-HD = 6 hydroxydopamine. 5% timolol maleate was given 60 min prior to 1% epinephrine. This was done twice a day for 5 consecutive days.
+ = eye swab positive for HSV-1; – = negative; C = contaminated.
§ These eyes spontaneously shed HSV-1 at least once during PI days 20–39.
Timolol (5.0%) was started three days prior to iontophoresis and given topically twice a day for 8 consecutive days for a total of 16 times. 1% epi was given for 5 consecutive days: once on the day of iontophoresis of 6-HD and twice a day for the next 4 days for a total of nine times.

(57%) and 20/40 (50%) for the 0.1% and 1.0% 6-HD groups, respectively.

Table 2 shows HSV-1 ocular shedding after topical application of 5.0% timolol to eyes supersensitized to adrenergic agents by iontophoresis of 6-HD. All 10 eyes shed HSV-1 at least once during the 7 days after iontophoresis of 6-HD. Topical application of 5.0% timolol was done once on the day of iontophoresis and twice a day for the next 4 days. Total positive HSV-1 swabs per total swabs was 29/67 (43%) and the average duration of shedding was 2.9 days.

Tables 3 and 4 show ocular shedding of HSV-1 after iontophoresis of 0.01% timolol at 0.8 mAmps for 8 min for 3 consecutive days. Timoptic® (0.5%) was used to prepare the 0.01% timolol for iontophoresis (Table 3). The purified powder of timolol maleate was used to prepare the 0.01% timolol used in Table 4. The data from Tables 3 and 4 show an average duration of shedding of 5.0 and 3.4 days and total positive HSV-1 swabs per total swabs of 50/68 (74%) and 27/52 (52%). Table 5 is a summary of Tables 1–4 plus five other experiments (individual data not shown). The experiments

Table 2. Ocular shedding of HSV-1 before and after iontophoresis of 6-HD followed by topical 5.0% timolol

Rabbit number	*Eye*	*Days pre/post iontophoresis of 6-HD**											*Total positive on days 1–7*
		–3	*–2*	*–1*	*0'*	*1''*	*2''*	*3''*	*4''*	*5*	*6*	*7*	
K-2	OD	–	–	–	–	–	–	C	–	–	+	–	1
	OS	–	–	C	–	–	–	–	–	–	+	–	1
K-4	OD	–	–	–	–	–	+	+	+	–	C	–	3
	OS	–	–	–	–	–	–	–	–	+	+	–	2
K-5	OD	–	C	–	C	+	–	+	+	+	+	–	5
	OS	–	–	C	–	–	–	+	+	–	+	–	3
K-8	OD	–	–	–	–	–	–	C	+	+	+	+	4
	OS	–	–	–	–	–	–	+	+	+	+	+	5
K-10	OD	–	C	–	–	–	–	+	+	–	–	–	2
	OS	–	–	–	–	–	–	+	–	+	+	–	3
Daily (%)													
Eyes		0	0	0	0	10	10	60	60	50	80	20	
Rabbits		0	0	0	0	20	20	80	80	80	100	20	

* Iontophoresis of 1.0% 6-HD for 0.75 mAmp for 3 min. The day of iontophoresis was PI day 49.
t = topical 5.0% timolol; C = contaminated; + = eye swab positive for HSV-1; – = negative.
All eyes positive for HSV-1 at least once during PI days 20–39.

Table 3. Ocular shedding of HSV-1 before and after iontophoresis with 0.01% timolol

Rabbit number	Eye	Days pre/post iontophoresis of timolol											Total positive on days 1–7
		−3	−2	−1	0*	1*	2*	3	4	5	6	7	
H53	OD†	−	−	−	−	−	−	+	+	+	−	−	3
	OS†	−	−	−	−	+	+	+	+	+	+	+	7
H54	OD†	−	−	−	−	+	+	+	+	+	+	+	7
	OS†	−	−	−	−	+	+	C	+	+	+	+	6
H56	OD†	−	−	−	−	−	−	+	+	+	+	+	5
	OS†	+	−	−	−	+	+	+	+	+	+	−	6
H58	OD†	−	−	−	−	−	−	+	+	+	−	−	3
	OS†	−	−	−	−	+	+	+	+	−	−	−	4
H59	OD	−	+	−	−	+	+	+	+	−	−	+	5
	OS†	−	C	−	−	−	−	C	+	+	+	+	4
Total (%)													
Eyes		10	10	0	0	60	60	80	100	80	60	60	
Rabbits		20	20	0	0	100	100	100	100	100	80	80	

The first day of iontophoresis was PI day 89 for all rabbits.
* 0.01% timolol iontophoresis at 0.8 mAmp for 8 min.
C = contaminated; + = eye swab positive for HSV-1; − = eye swab negative.
† These eyes shed HSV-1 at least once during PI days 20–39.

are numbered 1 through 10. This summary includes the average PI day on which each experiment began for each group. The ratios of total positive HSV-1 swabs per total swabs were calculated only for the last 7 days of the experiment. As seen in Tables 1–4, only a few eye swabs were positive prior to administration of the adrenergic agents.

Rabbits that received no treatment demonstrated 4/68 (5.8%) total positive swabs per total swabs (experiment 1). Iontophoresis of NaCl followed for 5 days by topical application of NaCl produced the same percent of positive eye swabs as in the untreated rabbits (experiment 2). Topical application of 5% timolol given twice a day for 5 days resulted in 7 of ten eyes shedding virus, with a total positive swabs per total swabs ratio of 8/69 (12%) (experiment 3). When the topical application was increased to four times per day for 5 days, 4 of 12 eyes shed virus, with a total positive swabs per total swabs ratio of 6/83 (7.2%) (experiment 4). Iontophoresis of 0.35% NaCl followed by topical application of 5.0% timolol resulted in seven of ten eyes shedding virus during a 7 day period after NaCl iontophoresis, and the shedding was for only one day (experiment 5). The average duration of shedding for the five groups ranged from 0.4 to 0.8 days.

The Fisher Exact test was used to compare the untreated group and the group receiving topical and iontophoretic NaCl with the groups receiving topical timolol alone or in combination with iontophoresis of NaCl (controls); no statistically significant differences were obtained (all $P > 0.2$). Comparison of the untreated group and the topical and iontophoretic NaCl group with the groups receiving the experimental protocols (6-HD or timolol iontophoresis with or without topical timolol and/or epinephrine; Tables 1–4) demonstrated a significant increase in total positive swabs per total swabs, ie, an increase in shedding, in all experimental groups ($P < 0.001$).

Table 4. Ocular shedding of HSV-1 before and after iontophoresis of 0.01% timolol

Rabbit number	Eye	Days pre/post iontophoresis								Total positive on days 1–7
		0*	1*	2*	3	4	5	6	7	
K-41	OD	−	−	C	−	+	+	+	+	4
K-43	OD	−	−	+	+	+	+	+	+	6
K-47	OD	−	−	−	−	+	+	−	−	2
	OS	−	−	−	−	+	+	−	−	2
K-48	OD	−	−	−	−	+	C	−	−	1
	OS	−	−	C	+	+	+	+	+	5
K-50	OD	−	−	C	−	+	+	+	−	3
	OS	−	−	−	+	+	+	+	−	4
Daily (%)										
Eyes		0	0	13	38	100	88	63	38	

The first day of iontophoresis was PI day 59 for all rabbits.
* Iontophoresis 8 min 0.8 mAmp with 0.01% (0.22 mM) of timolol prepared from pure powder with sterile distilled water.
C = contaminated; + = eye swab positive for HSV-1; − = negative. All eyes positive for HSV-1 at least once during PI days 20–39.

Table 5. HSV-1 ocular shedding in latently infected rabbits

Experiment number	Treatment: Iontophoresis	Treatment: Topical application	Average PI day	Positive rabbits/total rabbits	Positive eyes/total eyes	Positive swabs/total swabs	Average duration of shedding (days)	P value*
1	None	None	49	3/5	4/10	4/69 (5.8%)	0.4	
2	NaCl	NaCl	136	3/5	4/10	4/69 (5.8%)	0.4	
3	None	5% timolol†	59	4/5	7/10	8/69 (12%)	0.8	0.22
4	None	5% timolol††	133	3/6	4/12	6/83 (7.2%)	0.5	0.72
5	NaCl	5% timolol	59	4/5	7/10	7/68 (10%)	0.7	0.33
6	1.0% 6-HD§	5% timolol 1% epinephrine	139	3/3	6/6	20/40 (50%)	3.3	<0.001
7	0.1% 6-HD§	5% timolol 1% epinephrine	157	3/3	6/6	24/42 (57%)	4.0	<0.001
8	1.0% 6-HD¶	5% timolol	49	5/5	10/10	29/67 (43%)	2.9	<0.001
9	0.01% timolol (×3)**	None	59	5/5	8/8	27/52 (52%)	3.4	<0.001
10	0.01% timolol (×3)§§	None	89	5/5	10/10	50/68 (74%)	5.0	<0.001

* Compared with untreated rabbit or NaCl ocular iontophoresis plus NaCl topical application to eyes. Fisher Exact test for *P* values.

† Twice a day for 5 consecutive days.

†† Four times a day for 5 consecutive days.

§ See Table 1 for iontophoretic conditions and timing of topical applications of timolol and epinephrine.

¶ See Table 2 for iontophoretic conditions and timing of topical application of timolol.

** Prepared from pure powdered form in sterile distilled water.

§§ Prepared by dilution of the commercially available formulation (Timoptic®).

Discussion

We have quantitated HSV-1 in the preocular tear film and ocular-associated neural tissues after epinephrine-induced herpetic reactivation.[2,3,6,9] The HSV-1 titer from the preocular tear film of adrenergically induced eyes showed peak values of 10^4 and 10^5 PFU/eye.[2,9] We have reported quantitation of HSV-1 recovered from ocular-associated neural tissues after epinephrine-induced reactivation. This was the first report to quantify infectious HSV-1 with a high frequency from neural tissues following induced ocular reactivation and to demonstrate a relationship between viral recovery at a peripheral site and neuronal reactivation.[6]

Although most evidence suggests that the source of recurrent ocular HSV-1 is the neuronal cell body from sensory or autonomic ganglia, a few reports have suggested the eye as a possible source. In humans, Shimeld et al[16] have isolated HSV-1 from the corneas of patients with chronic stromal keratitis. There have been two reports of HSV-1 recovery in mice after explantation of whole eyes. Openshaw[17] has recovered HSV-1 strain F from female BALB/c mouse eyes 5–7 months after corneal inoculations; he suggested that the site of latency may be the mouse retina. Shimeld et al[18] recovered HSV-1 strain KOS from outbred Swiss white mice 30–36 days after ocular inoculation. They reported no evidence of retinal involvement. Furthermore, they raise the possibility that the ciliary ganglia could be a neuronal source of the HSV-1, since this tissue is in close proximity to the back of the eye.

Zhang et al[12] reported that HSV-1 ocular shedding could be induced by 6-HD plus epinephrine[4,8,9] in rabbits from which both superior cervical ganglia had been removed. The average duration of shedding was 1.5 days, and the ratio of total positive swabs per total swabs was 12/40 (30%). Four of eight eyes never shed HSV during the induction period. In sham-operated rabbits, the average duration of shedding was 2.3 days; the ratio of total positive swabs per total swabs was 23/50 (46%). Two of ten sham-operated eyes never shed HSV-1 during the induction period. Zhang et al[12] also reported that timolol reduces ocular shedding induced by the 6-HD plus epinephrine procedure.[4,8,9] They suggested that the adrenergic induction is the result, in part, of pharmacologic effects on peripheral cells, for example, post-synaptic cells. Furthermore, they suggested that while peripheral neurons are a source of HSV-1, perhaps not all the HSV in the tear film is released from the nerve endings. However, Hill, Blyth, and Harbour[19] reported that in mice, an intact nerve supply from the ganglion to the skin is necessary for HSV skin reactivation and recurrence. After neurectomy, infectious HSV was isolated from the skin very rarely and no recurrent disease was seen.[19]

In addition to studies of rabbit ocular HSV-1 reactivation described above, Willey et al,[20] Harwick,[13] and Romanowski et al[21] reported epinephrine induced ocular shedding in the tear film of mice. Willey et al[20] reported 70% of mice shed HSV-1 after 3 consecutive days of a once daily iontophoresis of epinephrine. The procedure was the same as the one we described in rabbits.[1–3,5,6] Romanowski et al[21] reported 50% of mice shedding HSV-1, induced by corticosteroids topically applied to the eye in combination with the 6-HD plus epinephrine procedure.[4,8,9] Furthermore, Harwick[13] showed that topical application of timolol alone or in combination with topical application of epinephrine

promotes HSV-1 shedding in the mouse eye supersensitized by iontophoresis of 6-HD.

The mechanism by which ocular reactivation is induced by timolol, a beta antagonist, and epinephrine, an alpha and beta agonist, is unknown. Klyce et al[22] reported that in vitro, timolol blocks the effect of epinephrine in the cornea. However Cyrlin et al[23] reported that in humans, epinephrine and timolol demonstrate an additive effect in the reduction of intraocular pressure. The exact in vivo mechanisms that result in decreased intraocular pressure are unknown. The apparent paradox is that an agonist (epinephrine) and an antagonist (timolol) can, alone or in combination, produce the same in vivo response, ie, lower intraocular pressure.[23] We report another relationship common to timolol (antagonist) and epinephrine (agonist). Both these adrenergic agents, alone or in combination, can induce ocular HSV-1 reactivation in the rabbit latently infected with HSV-1 strain McKrae.

Key words: epinephrine, 6-hydroxydopamine, ocular HSV-1, rabbits, reactivation, tear film, timolol

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