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In Vivo Reactivation of Herpes Simplex Virus in Rabbit Trigeminal Ganglia: Electrode Model

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The rabbit provides an excellent model for the study of ocular herpes because herpetic keratitis in the rabbit eye resembles human disease in its clinical features and in its propensity for spontaneous recurrence. This paper presents a method for the electrical induction of multiple episodes of in vivo reactivation of latent HSV-1 infection with peripheral shedding of virus. Physiological levels of current delivered via an electrode implanted over the trigeminal ganglion of latently infected animals has enabled us to modify and synchronize virus shedding in preocular tear film and to cause multiple episodes of reactivation in a single animal. For this reason, the model is well suited for antiviral efficacy testing and provides an excellent opportunity for investigation of virus-host cell interactions in latent and recurring herpetic disease.

Herpes simplex keratitis is the most prevalent severe ocular infection in this country. The propensity of this infection to recur throughout life and to produce irreversible structural alterations of the cornea and intraocular structures results in considerable visual morbidity, medical expense, and loss of productivity of otherwise healthy individuals. Interruption of the recurrent herpes cycle depends on knowledge of the sequence by which latent infection of the nervous system is translated into active disease in the peripheral tissues. For this reason, a reliable and reproducible means of triggering recurrent ocular herpes has long been sought.

Historically, the rabbit has provided an excellent model for study of ocular herpes because herpetic keratitis in the rabbit eye resembles human disease in clinical features as well as in its propensity for spontaneous recurrence (5, 6, 8). Attempts to induce viral reactivation in other animals models have been only partially successful. The apparent problem has been the inability to detect the propagation of virus to peripheral tissues (1, 3, 8, 12-14). The most dependable animal model to date has been the manual manipulation of the rabbit trigeminal nerve (7). Although this model has provided insight as to the relationship between trigeminal nerve and recurrent ocular herpes in rabbits, its application is limited because animals rarely survive longer than 72 h. We recently determined that passage of current along the trigeminal nerve of latently infected animals via a small electrode implant elicits rapid release of virus at the eye (Green and Dunkel, Arvo abstr., p. 156, 1980). This paper presents a new method for induction of multiple episodes of in vivo reactivation of herpes simplex virus type 1 (HSV-1) infection with peripheral shedding of virus in latently infected animals.

MATERIALS AND METHODS

Electrode implantation. New Zealand albino rabbits (2 to 3 kg) were anesthetized with acepromazine maleate (5 mg/kg) and ketamine HCl (33 mg/kg), administered separately. Animals were then placed in a stereotaxic device (David Koff, Inc., Tujunga, Calif.). Both stereotaxic coordinates and morphological landmarks (the squamous portion of the temporal bone at the attachment of the zygomatic arch) were utilized to introduce a neurological electrode via a 5-mm craniotomy burr hole onto the surface of the tentorium overlying either the right or left trigeminal nerve ganglion. Each electrode consisted of a 22-mm, 22-gauge, epoxylite-coated, stainless-steel wire (Small Parts, Inc., Miami, Fla.) with a 0.5-mm exposed tip. Proper placement and function of the electrodes were demonstrated by reflex twitching of the eyelids and extraocular muscles on the implanted side in response to a continuous series of pulses of 1 ms duration at 10 Hz with voltages ranging from 0.5 to 4 V. Before removal from the stereotaxic device, electrodes were attached to the cranium with skull screws (1/8-inch [ca. 0.32-cm] furled screws) and dental adhesive (Getz Tru-Cure Dental Adhesive and Formix Denture Resin). The incision was closed with 4.0 silk sutures so that only the contact tip of the electrode remained exposed (Fig. 1A, B, and C).

Inoculation. Rabbits were anesthetized with 33 mg of ketamine HCl per kg. A 21-gauge needle was used

to administer four vertical and four horizontal lacerations to the corneal epithelium under direct vision, utilizing an operating microscope. A portion (50 μl) containing 10^5 plaque-forming units of McKrae strain HSV-1 was dropped onto each cornea. Eyes were closed and globes were massaged for 15 s (9).

Ocular culture. Virus shedding was monitored by the culture of the preocular tear film. Sterile cotton-tipped applicators (Schering Medical Scientific, Inc., Carson, Calif.) were passed over the upper and lower conjunctival cul-de-sac, lightly rolled over the cornea, and retained in the nasal fornix for 5 to 10 s for maximum tear film absorption (11). Swabs were eluted in 0.5 ml of Hanks balanced salt solution (Gibco Laboratories, Grand Island, N.Y.) for 15 s with agitation. Portions (50 μl) of the eluate were absorbed onto confluent 16-mm Vero cell monolayers (CCL 81, Flow Laboratories Inc., Rockville, Md.) for 10 min at 37°C in 5% CO_2. Cultures were reconstituted with 0.5 ml of minimal essential medium (GIBCO) plus 5% fetal bo-

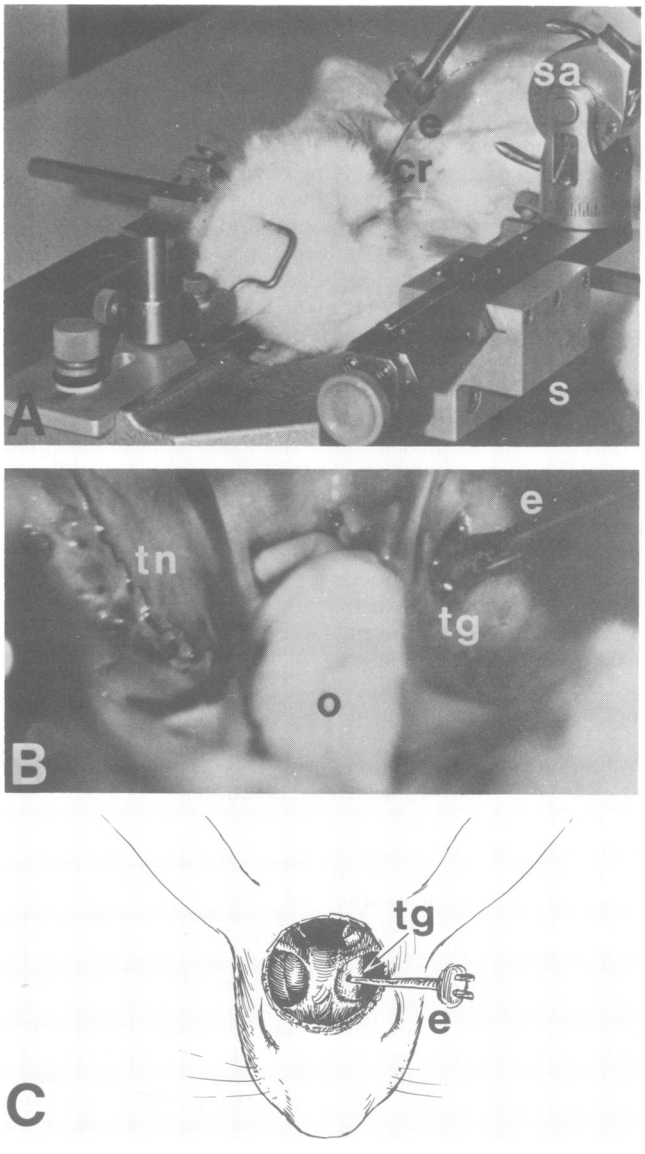

FIG. 1. *(A) Rabbit placement in stereotaxic apparatus. (B) Postmortem autopsy of rabbit demonstrating electrode placement over the left trigeminal ganglion. Tentorium intact during experimental period but disrupted during dissection; tg, tentorium overlying trigeminal nerve ganglion: tn, tentorium overlying trigeminal nerve. (C) Diagrammatic representation of electrode placement: e, electrode; sa, adjustable side arm; s, stereotaxic apparatus; cr, exposed cranium; o, optic nerve and chiasm.*

vine serum (GIBCO). Cultures were monitored daily for 10 days for the development of cytopathology consistent with HSV-1 infection.

Trigeminal nerve ganglion stimulation via electrode implant. All rabbits were stimulated using a Grass model S9 stimulator. Stimulation consisted of a continuous series of pulses of 1 ms duration at 10 Hz, with voltages ranging from 0.5 to 4 V for 15-s intervals. The physiological endpoint of extraocular muscle and lid twitch was used to delineate the delivery of appropriate amounts of electrical stimulation to the trigeminal nerve.

Experimental design. (i) Part A. Study A was designed to determine whether there is a positive correlation between passage of low-dose electrical current along the trigeminal nerve of latently infected animals and release of virus in preocular tear film. Monopolar electrodes were implanted over the left trigeminal ganglion of 19 rabbits. Five to ten days after implantation, animals were stimulated and monitored by ocular swab culture to assess probe function and placement and to insure the absence of innate virus. After a negative "control stimulation," animals were bilaterally infected with 10^5 plaque-forming units of McKrae strain HSV-1. After resolution of active infection and after a minimum of 4 days of negative bilateral ocular cultures, animals were "test stimulated" and monitored by daily ocular swab cultures for the shedding of virus. After several days of negative bilateral ocular cultures, animals were again stimulated and monitored. To determine the relative quantity of virus shed in preocular tear film in response to electrical current stimulation, eyes of a separate group of 10 rabbits were cultured after known quantities of virus were dropped into the cul-de-sacs. Each concentration (10^4, 10^3, 10^2, 10, and 5 plaque-forming units) of HSV-1 in 50 μl of Hanks balanced salt solution was dropped into the cul-de-sac of four eyes. The eyes were cultured as previously described after 5 min, to allow for some interaction between virus and host factors in the tear film, while minimizing the loss of infectious virus particles due to uptake by host cells. Cell monolayers were observed for 5 days for appearance of cytopathology.

(ii) Part B. Study B was designed (i) to confirm the relationship between administration of low-dose electrical current and the elicitation of virus shedding in latently infected animals; (ii) to determine whether the acute stage of infection could be prolonged as evidenced by continuous shedding of virus for >14 days; (iii) to determine whether a shift in the pattern of induced virus shedding would occur with time; and (iv) to determine whether the ability to shed virus could be exhausted with continuous long-term stimulation. Six test and six control animals were stimulated every other day for 60 days beginning on day 7 postinfection. Stimulus consisted of a series of pulses of 1 ms duration at 10 Hz ranging from 0.1 to 0.5 mA. Four of the test group animals were stimulated every third day for an additional 140 days and monitored via ocular culture.

(iii) Part C. Study C was designed to determine the optimal interval of stimulation with respect to induction of virus shedding in preocular tear film. Five animals were stimulated daily for 60 days beginning 3 days postinoculation; five animals were stimulated every other day for 60 days beginning 7 days postinfection; and five animals were stimulated every fifth day for 60 days beginning 7 days postinfection. Data on stimulation every third day beginning day 7 postinoculation was obtained from study B.

RESULTS

Study A. Twenty-three biologically definitive unilateral stimuli resulted in 19 ipsilateral virus recoveries in 15 animals (rabbit no. 1–4, 6–8, 11, 14–19) (Fig. 2). Three animals responded after each of two separate stimulations (rabbit no. 2, 4, and 7). Virus shedding limited to the contralateral side was elicited in five animals after stimulation (rabbit no. 1, 12, 14, 15, 18). Virus shedding occurred on both the ipsilateral and contralateral side in 13 animals (rabbit no. 5–14, 16, 18, 19). Increased voltage (7 to 10 V) was administered to all animals exhibiting contralateral shedding resulting in a bilateral biological response (lip and eye twitch on the contralateral as well as the ipsilateral side). Seven unilateral stimuli failed to induce virus shedding on either the ipsilateral or contralateral side. All ipsilateral viral responses were initiated within 48 h of stimulation. A single contralateral response was initiated 72 h poststimulation.

In a separate study, known virus concentrations were instilled into the conjunctival cul-de-sac of 20 eyes. Cytopathology was evident in 20 of 28 total positive cultures within 24 h of swab inoculation. Because only those eyes receiving known portions $\geq 10^3$ plaque-forming units produced positive cultures within 24 h of inoculation, it was inferred that 71% of the positive cultures represented shedding of a minimum of 10^3 plaque-forming units in the preocular tear film.

Study B. All inoculated animals developed acute herpetic keratitis as evidenced by the presence of corneal dendritic figures (by day 3 postinoculation) and virus shedding in preocular tear film for a minimum of 12 to 14 consecutive days. During the period of acute infection, there was no significant difference in virus shedding in preocular tear film between test and control animals. By day 18 post-inoculation, acute infection had cleared as evidenced by methylene blue staining of intact corneal epithelium and negative tear film cultures. After establishment of latency (18 days postinfection), a significant difference in tear film virus shedding was observed between stimulated and nonstimulated animals (Table 1). Virus shedding typical of acute disease was not significantly prolonged by consistent electrode stimulation at 3-day intervals. Test animals became refractile to induction of virus shedding in preocular tear film for periods of 5

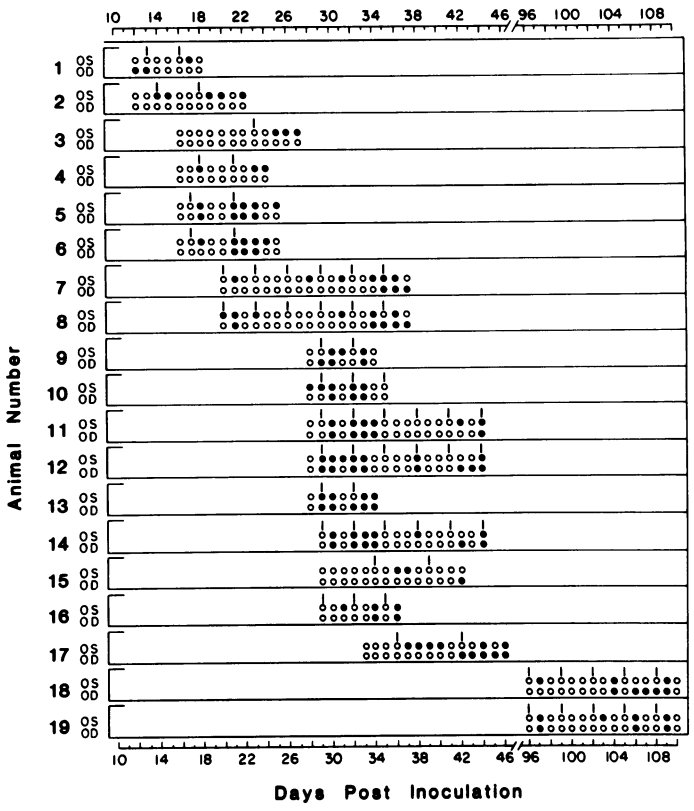

FIG. 2. *Virus recovery from preocular tear film of rabbits after induction by electrical stimulation. Symbols: ●, positive virus cultures; ○, negative virus cultures; vertical line above dot indicates stimulation. Each dot represents one culture.*

TABLE 1. *Virus shedding in induced and control rabbits*

Determinant	No. of positive cultures/total cultures	
	Control	Induced
Primary infection	25/108 (23)[a]	27/108 (25)
Recurrent shedding[b]	34/492 (6)	71/492 (15)
Eyes represented	12/12 (100)	12/12 (100)

[a] Numbers in parentheses indicate percentages.
[b] $P < 0.005$.

to 12 days. Although the ability to shed virus could not be exhausted, the refractile period increased toward 30 days, with increasing time postinfection resulting in decreased shedding of virus in preocular tear film (Table 2). Although electrical induction caused viral recurrence at the eye, clinical symptoms (conjunctivitis, iritis, and corneal lesions) were not marked after cessation of acute disease.

Study C. Stimulation every third day resulted in exacerbation of virus shedding more frequently than stimulation every second or fifth day. Daily stimulation resulted in reduction of ocular shedding of virus to levels below that observed in nonstimulated animals (Table 3).

Combined observations (from all groups of electrode-stimulated animals) revealed a periodicity with regard to virus shedding; 2 to 3 days of positive tear film cultures were followed by 7 to 10 days of negative tear film cultures. All animals subjected to a particular interval of stimulation appeared to become synchronized with respect to virus shedding. For example, in a single group of 10 rabbits stimulated every third day beginning 3 days postinoculation: (i) 9 out of 10 rabbits had negative tear film cultures by day 14 postinfection; (ii) 10 out of 10 rabbits had positive tear film cultures between day 19 and day 21 postinfection; (iii) 10 out of 10 rabbits had negative tear film cultures day 22 through day 30 postinfection; (iv) 8 out of 10 rabbits had positive tear film cultures from day 31 through 34. This pattern of positive ocular cultures, interrupted by 8 to 12 days of negative tear film cultures, was observed throughout early latency (~80 days postinoculation).

Hematoxylin-and-eosin- and toluidine blue-stained sections of trigeminal nerve ganglion

TABLE 2. *Effect of long-term induction on virus shedding*

Animals	Positive ocular cultures/total no. of cultures on days postinfection				
	20–59	60–119	120–179	180–239	240–300
Stimulated	81/432 (19)[a]	20/264 (8)	26/612 (4)	33/480 (7)	28/480 (6)
Control	18/240 (7)	43/720 (5)	49/600 (8)	20/360 (5)	23/360 (6)

[a] Numbers in parentheses indicate percentages.

TABLE 3. *Optimal pattern of stimulation: effect of induced virus shedding*

Periodicity of stimulation	Episodes of virus shedding[a]
Daily	10 episodes/10 eyes/60 days[b, c]
Every 2nd day	36 episodes/10 eyes/60 days
Every 3rd day	64 episodes/10 eyes/60 days
Every 5th day	47 episodes/10 eyes/60 days
Nonstimulated	23 episodes/10 eyes/60 days

[a] An episode of virus shedding consisted of an isolated series of positive ocular cultures obtained on two or more consecutive days.

[b] The 60-day time period for both test and control animals began on day 20 postinoculation (after 4 consecutive days of negative bilateral tear film cultures) and continued through day 80 postinoculation.

[c] 600 cultures were obtained from each group during the 60-day test period.

from animals with long-term electrode implants showed no histological evidence of cellular damage. Corneal sensation was retained after multiple episodes of virus shedding in preocular tear film.

DISCUSSION

Physiological levels of current delivered via an electrode implanted over the trigeminal ganglion of latently infected animals has enabled us to modify virus shedding in preocular tear film and to cause multiple episodes of reactivation in a single animal. Although this method of electrical induction does not prevent the natural progression from active infection to latency, it has allowed us to accelerate and synchronize recurrent episodes of virus shedding after resolution of acute disease. The number of episodes of spontaneous recurrence normally occurring in a 12-month period can be observed in 3 months under experimental conditions. Because it is efficient and can be standardized (induction of multiple episodes of virus shedding in the same eye), the electrode model is well suited for antiviral efficacy testing. In addition, this model provides an excellent opportunity for investigation of alterations in virus host cell interactions in latent and in recurring herpetic disease.

In this report, latency is defined as the stage of HSV infection when infectious virus can no longer be isolated from cell-free ganglion homogenates (10), although reactivation can be induced by organ cocultivation. Reactivation is measured by shedding of infectious virus in preocular tear film. All latently infected animals in the present study (39 total test animals) have been induced to shed virus. Virus reactivation is more consistently accomplished with a single induction stimulus within the first 40 days postinoculation; we have not been able to exhaust the potential for reactivation after multiple induction stimuli as late as 300 days postinfection. It is not known whether reactivation and subsequent infection with endogenous virus (via centripetal and centrifugal intraaxonal transport) play a role in perpetuation of latency.

Refractile periods are time intervals during which virus is not released in preocular tear film in the presence of inducing stimuli. Of interest is the fact that these refractile periods increase in duration with increasing time after primary infection. The decrease in ocular shedding of infectious virus observed in animals undergoing long-term (285 days) maximal induction (stimulus interval every third day) is consistent with clinical observations of the natural history of spontaneous reactivation of herpetic disease in humans. The mechanism for this decrease in activity is puzzling, since multiple recurrent episodes do not lead to anesthesia in humans or in the experimental rabbit.

Use of physiological levels of electrical current has allowed us to either induce or prevent HSV-1 reactivation in latently infected animals. The outcome of stimulation appears to depend on the interval between stimuli. According to classical physiological concepts, application of current to nerve cell membranes causes permeability alterations resulting in reversal of cellular potential and generation of an action potential (2, 4). Although the mechanism of viral reactivation in response to passage of current through the trigeminal nerve and ganglion is unknown, it is possible that slight changes in membrane permeability in response to an electrical current might initiate a chain of events culminating either in viral reactivation or the prevention of reactivation.

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