Characterization of a Murine Model of Recurrent Herpes Simplex Viral Keratitis Induced by Ultraviolet B Radiation

Keith A. Laycock,* Steven F. Lee,* Robert H. Brady,* and Jay S. Pepose†

The authors characterized a murine model of herpes simplex virus (HSV) reactivation in which recurrent keratitis was obtained in up to 80% of animals. Five weeks after ganglionic latency was established in National Institutes of Health inbred mice after corneal inoculation, HSV type 1 (HSV-1) was reactivated by irradiating the previously inoculated eye with ultraviolet (UV) light. Comparison of different UV wavelengths showed UVB to be optimal for reactivation, with peak viral recurrence being induced by a total exposure of approximately 250 mJ/cm². Reactivated infectious virus generally began to appear in trigeminal ganglia 2 days postirradiation and was subsequently detectable in the cornea by both corneal swabbing and immunostaining for viral antigens. Two consecutive outbreaks of viral recurrence at the ocular surface were induced in selected animals by serial exposure to UVB. Advantages of this model over other models of recurrent keratitis are discussed. Invest Ophthalmol Vis Sci 32:2741-2746, 1991

Several animal models have been developed in an effort to reproduce different pathogenic aspects of recurrent herpes simplex virus (HSV) keratitis. Rabbits were used in many studies related to ocular herpetic disease and the subsequent latent infections established in the trigeminal ganglia. They were used in the first experimental production of recurrent herpetic eye disease in which ultraviolet (UV) light was used to induce HSV recurrence in the eye. The UV irradiation, combined with ocular and systemic corticosteroid treatment, resulted in recurrent herpes keratitis with viral shedding in three of four rabbits.

Subsequent experiments with rabbit models of herpetic eye disease used iontophoresis of epinephrine or other substances into the cornea to induce viral shedding, although recurrent ocular lesions did not accompany this shedding in all cases. Unfortunately, the rabbit model of herpetic disease is prone to spontaneous reactivation of latent virus; therefore it is not ideal for investigations requiring stable maintenance of the latent state between reactivation events.

The mouse is a more stable model of HSV latency, with an extremely low spontaneous reactivation rate. Until recently the murine model was not used widely in studies of recurrent ocular herpetic disease, although viral reactivation by iontophoresis of epinephrine into the cornea was reported. Other workers were unable to repeat this effect, but they observed reactivation by iontophoresis of 6-hydroxydopamine in combination with epinephrine and prednisolone phosphate eye drops. Viral shedding was increased by beta-adrenergic blockade with timolol eye drops used in addition to this regimen. Topical application of alpha-adrenergic blockers (thymoxamine and corynanthine) after iontophoresis of 6-hydroxydopamine significantly reduced the incidence and duration of viral shedding. Iontophoresis of vanadate into the cornea of latently infected mice also induced viral reactivation, although at a lower rate than that obtained with the combination of 6-hydroxydopamine and epinephrine. Other toxic, but nonphysiologic, stimuli such as the injection of distilled water or saline into the cornea, conjunctiva, or anterior chamber of latently infected rabbits also recently was shown to induce herpetic recurrence.

In comparison with immune suppression, iontophoresis, or physical trauma, exposure to UV light is an attractive mechanism for experimental viral reactivation because—unlike iontophoresis—it is a naturally occurring environmental phenomenon that was shown to cause reactivation of HSV in humans.
Recently, a murine model of recurrent herpetic eye disease was described that uses UV irradiation to induce reactivation of latent virus, with subsequent development of dendritic ulcers resembling the characteristic lesions seen in humans.\textsuperscript{10,11} In its initial form, the mice were inoculated with HSV by corneal scarification, and latent infection in the trigeminal and superior cervical ganglia was established. Subsequent treatment of these animals with UV irradiation in conjunction with cyclophosphamide and dexamethasone resulted in infectious virus being detected in the ophthalmic portion of the trigeminal ganglion and in eye washings. Signs of recurrent herpetic eye disease (dendritic lesions) were observed, but only in those eyes not permanently damaged during the primary ocular infection.

This model subsequently was improved by administering antiserum against HSV-1 before inoculation of the mouse corneas to protect them from permanent damage during primary infection. Mice whose eyes appeared normal after primary infection abated then received the same combination of UV irradiation and immunosuppressive drugs as before or UV irradiation alone. Of mice receiving combined treatment, 52% shed virus or had recurrent disease; the incidence in animals exposed to UV alone was 59%. Recurrent disease was more severe in animals receiving immunosuppressive treatment.

In our laboratory, we adapted and characterized this model of UV-induced viral reactivation using two different commercially available UV transilluminators, both of which were easily obtainable in the United States. The model originally described\textsuperscript{10,11} depended on a specific ultraviolet lamp of limited availability emitting radiation at a peak of 4.03 mW/cm\textsuperscript{2} at 320 nm. Using our modifications, a higher incidence of viral reactivation was obtained. We also demonstrated the feasibility of inducing several reactivation events by serial exposure to UVB resulting in many bouts of recurrent infection at the ocular surface in these animals. Finally, the anatomic and temporal features of UV light-induced reactivation were characterized using our reactivation protocol.

**Materials and Methods**

**Virus and Cells**

In all experiments, the virus used was HSV-1 McKrae strain. A plaque-purified virus stock was grown and assayed on Vero cells in modified Eagle's medium with Earle's balanced salts (MEM-EBS) containing 5% fetal bovine serum. Material from eye swabs was cultured similarly on Vero cells. Cells were cultured at 36°C in a humidified incubator containing 5% CO\textsubscript{2}.

**Mice and Inoculation Procedures**

The National Institutes of Health (NIH) inbred strain of mouse was selected because of its susceptibility to induced reactivation of latent HSV-1.\textsuperscript{12} Only female mice were used; they are easier to maintain for long periods. The mice were obtained at 4–6 weeks of age from Harlan Olac Limited (Bicester, Oxford, England). The eyes of all animals were examined before inoculation; only mice with no ocular abnormalities were used in the experiments. All investigations conformed to the ARVO Resolution on the Use of Animals in Research.

The mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) followed by ketamine (70 mg/kg). Then the surface of the right cornea was scarified in a grid pattern with a no. 15 scalpel blade. A drop of virus suspension containing 10\textsuperscript{6} plaque-forming units of HSV-1 McKrae strain in 5 µL MEM-EBS was placed on the scarified cornea, and a drop of Hank's balanced saline solution was placed on the uninfected eye to prevent drying of the cornea while the animal was unconscious.

Concomitant with viral inoculation, 0.5 ml of pooled human serum (Chemicon International, Temecula, California) containing antibodies to HSV-1 (effective dose for 50% viral neutralization, 1:640) was injected intraperitoneally to protect the ocular tissues from damage during the acute phase of infection. On days 3 and 4 postinoculation, the infected eyes of the mice were swabbed with Dacron surgical sponges from damage during the primary infection. Mice whose eyes appeared normal after primary infection abated then received the same combination of UV irradiation and immunosuppressive drugs as before or UV irradiation alone. Of mice receiving combined treatment, 52% shed virus or had recurrent disease; the incidence in animals exposed to UV alone was 59%. Recurrent disease was more severe in animals receiving immunosuppressive treatment.

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**UV Sources and Irradiation Procedure**

The two UVB sources used were a Haake-Buchler UVT transilluminator (Saddle Brook, NJ) and a UVP TM 20 transilluminator (San Gabriel, CA). Both emit UVB light at a peak wavelength of 302 nm. In addition UVA and UVC radiation were obtained using a Spectrolite model ENF-280C lamp (Spectronics, Westbury, NY) that emits long wavelength UVA at 365 nm or short wavelength UVC at 254 nm. The intensity of the emitted radiation was measured using a UVX digital radiometer (UVP). To irradiate the animals, the mice were anesthetized as described and placed on the UV sources on top of a card screen perforated with holes so that only the eye was exposed to the radiation.

**Detection of Viral Shedding**

To detect infectious virus at the ocular surface, the cornea was swabbed with a Weck-Cel Dacron surgical
spear (Edward Weck) previously soaked in 0.5 ml MEM-EBS. The swab material was transferred to 12 × 75-mm culture tubes, and 0.2-ml aliquots were placed on confluent monolayers of Vero cells in 24-well titer plates. If infectious virus was present, cytopathic effects were visible after 2–5 days.

**Detection of Latent Virus**

To detect latent virus in ganglionic tissues, ganglia were dissected from mice and divided into small pieces. These were placed on confluent Vero cell monolayers and incubated for up to 2 weeks to detect reactivating virus. Cytopathic effects usually were detectable after 4–12 days.

**Detection of Viral Antigens**

Sections of formalin-fixed tissues were deparaffinized by immersion in xylene and a series of graded alcohols. Immunostaining for antigens was done using a polyclonal serum against HSV-1 raised in the rabbit (Accurate Chemical and Scientific, Westbury, NY), in conjunction with the Vectastain avidin-biotin complex (ABC) kit (Vector, Burlingame, CA). Tissue sections on slides were treated for 30 min with normal goat serum (Vector) to block nonspecific binding sites. The polyclonal serum then was placed on the sections for 30 min. The slides were rinsed in phosphate-buffered saline (PBS) and exposed to the secondary, biotinylated goat anti-rabbit antibody for a further 30 min. After rinsing the slides in PBS, ABC was placed on each slide for 45 min. All incubations were done at room temperature. After exposure to ABC, the slides were rinsed in PBS and then soaked in 3-amino-9-ethyl carbazole solution for 9 min at room temperature. Then the slides were rinsed in distilled water and counterstained in Mayer's hematoxylin for 3 min.

**Results**

**Determining the Optimum Wavelength of UV**

Groups of ten NIH inbred mice, latently infected with HSV-1 strain McKrae after inoculation of the right cornea, were exposed to 360 mJ/cm² UV radiation at different wavelengths. The wavelengths used were UVA (peaking at 350 nm), UVB (peaking at 302 nm), and UVC (peaking at 265 nm). Corneas of irradiated eyes then were swabbed daily for 10 days, with topical ocular administration of prednisolone phosphate from day 2 onward. Viral shedding was detected in two of ten animals irradiated with UVC and in none of those irradiated with UVA or UVB. The UVC radiation therefore appeared to be the most effective for reactivating latent HSV-1.

**Determining the Optimum Exposure to UVB**

The optimum exposure to UVB light was determined using two separate UVB sources: a Haake-Buchler UVT and a UVP TM20 transilluminator, both of which emit UVB at a peak wavelength of 302 nm. In the initial determination, the Haake-Buchler device was used. Seven groups of ten NIH inbred mice, latently infected with HSV-1 McKrae, were irradiated with UVB at 1.4 mW/cm² for periods of 1, 1.5, 2, 2.5, 3, 3.5, or 4 min. The mice received prednisolone drops commencing 2 days after exposure to UVB, and their eyes were swabbed daily for 7 days after irradiation. Results from culture of the swab material are presented in Table 1. Peak recovery of virus at the ocular surface was obtained from mice exposed for 3.0 min (ie, 252 mJ/cm²); seven of ten mice shed virus at the ocular surface.

Additional experiments were done to determine the optimum exposure period using the UVP TM20 transilluminator. This emits radiation at a greater intensity than the Haake-Buchler model: 4.5 mW/cm² compared with 1.4 mW/cm². Therefore the exposure times tested were shorter. Groups of eight NIH inbred mice latently infected with HSV-1 McKrae were exposed to UVB from this device for periods of 30, 35, 40, 45, 55 or 65 sec, then swabbed daily for 9 days, with prednisolone phosphate being administered from day 2 onward as before. The results from culturing the swab material are shown in Table 2. Of the time exposures investigated, the highest incidence of viral shedding was achieved with a 55-sec exposure (ie, 248 mJ/cm²); seven of eight mice shed virus at the ocular surface. Thus, for both transilluminators tested, an exposure of approximately 250 mJ/cm² was optimal in inducing reactivation of latent HSV-1.

**Time Course of Viral Reactivation in Ganglia**

Six groups of 13 latently infected mice were exposed to 252 mJ/cm² of UVB. One group of mice each was killed on days 0, 1, 2, 3, 4, and 5 postirradiation. The right (inoculated) eye, trigeminal ganglion,
Table 2. Reactivation data for UVP Inc. transilluminator

<table>
<thead>
<tr>
<th>UV-B exposure time (seconds)</th>
<th>Exposure energy (mJ/cm²)</th>
<th>Total number of mice shedding virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>135</td>
<td>2/8</td>
</tr>
<tr>
<td>35</td>
<td>158</td>
<td>1/8</td>
</tr>
<tr>
<td>40</td>
<td>180</td>
<td>4/8</td>
</tr>
<tr>
<td>45</td>
<td>203</td>
<td>4/8</td>
</tr>
<tr>
<td>55</td>
<td>248</td>
<td>7/8</td>
</tr>
<tr>
<td>65</td>
<td>293</td>
<td>6/8</td>
</tr>
</tbody>
</table>

Table 3. Viral recovery from tissue homogenates following exposure to UV-B

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of mice from which virus recovered (total = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>D0 D1 D2 D3 D4 D5</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>0 0 4 6 4 3</td>
</tr>
<tr>
<td>Superior cervical ganglion</td>
<td>0 1 4 4 0 1</td>
</tr>
</tbody>
</table>

*D = day postirradiation.*

and superior cervical ganglion were dissected from each mouse, homogenized, and then plated onto Vero cell monolayers to detect infectious virus present in the tissues. Virus recovery from each tissue is shown in Table 3. Viral recovery peaked on day 3 postreactivation for ocular tissues and on days 2 and 3, for trigeminal ganglia. Only one trigeminal ganglion contained infectious virus before day 2, and only one superior cervical ganglion contained infectious virus (day 4). No infectious virus was recovered from homogenates of corresponding tissues from latently infected control animals not irradiated with UVB.

Subsequently, a batch of ten latently infected mice were killed, and their trigeminal and superior cervical ganglia were cultured in explant. Infectious virus was reactivated from all of the trigeminal ganglia and from none of the superior cervical ganglia. This suggests that, in this model, there is little or no reactivatable viral latency in the superior cervical ganglia. There is, therefore, effectively a 2-day "window" between the reactivation-inducing event and the appearance of infectious virus in the trigeminal ganglia.

Localization of Herpetic Antigens

A group of ten NIH female mice latently infected with HSV-1 were irradiated with 250 mJ/cm² with UVB, and the right (inoculated) eyes were swabbed daily. On day 3 postirradiation, the animals were killed, and their right eyes were embedded in paraffin. Subsequent culture of eye-swab material revealed that six of ten animals were shedding infectious virus at the time of death. Sections were cut from these six eyes and stained for HSV-1 antigens using the rabbit polyclonal serum. The HSV antigens generally were localized in focal corneal epithelial ulcers with patches of antigen-stained cells in all layers of the epithelium, surrounded by normal-appearing unstained epithelium, in all six eyes. On occasion (Fig. 1), antigens were restricted to the basal epithelium and wing cells with superficial layers devoid of staining, suggesting that cells in the deeper layers were the first infected after reactivation. No viral antigens were found in the ocular tissues of control animals. Therefore, by day 3 postirradiation, viral recurrence detected by eye swabbing was confirmed by immunostaining for viral antigens. However, the restricted localization of viral antigens in the basal epithelium of selected mice with recurrences suggested that, at some earlier time, ocular shedding may be detected by corneal homogenization but not by swabbing. The infected basal cells may not be accessible to the Dacron swab. Comparative culture data between ocular swabs and corneal homogenates at various times confirmed this on several occasions.

Role of Prednisolone in the Model

A group of ten latently infected animals received no UV exposure, but prednisolone drops were applied to their eyes daily for 7 days, with swabbing of the inoculated eye daily. No infectious virus was recovered from the ocular surfaces of these animals, showing that neither the ocular administration of prednisolone nor the physical effects of eye swabbing was sufficient to induce viral reactivation in these animals.

Conversely, in a group of ten latently infected animals irradiated with UVB for the optimum exposure period but not subsequently treated with topical prednisolone, viral shedding at the ocular surface was detectable by corneal swabbing in three of ten mice compared with seven of ten in the group receiving prednisolone. In addition, prednisolone administration did not commence until the second day after UV irradiation. As shown in Table 3, reactivated infectious virus can be recovered from ganglionic tissues on days 1 and 2 postexposure to UVB. Thus, steroid administration does not begin until after the virus in the ganglia is committed to reactivate. This showed that topical prednisolone was not necessary for viral reactivation to occur, but it enhanced viral recovery at the ocular surface.

Repeated Reactivation of Latent Virus

The possibility of inducing several cycles of recurrent keratitis in experimental animals by UV irradiation was investigated using a batch of 16 NIH female
mice. In these animals ocular recurrence of HSV-1, as detected by eye swabbing, already had been induced successfully once by UV. After cessation of viral shedding from this first reactivation event, the animals were not tested for an additional 4 weeks to permit the virus to reestablish latency in the ganglia. The animals then were irradiated for a second time, with daily swabbing and prednisolone administration as before. Cultures of swab material showed that none of the animals were shedding infectious virus immediately before the second UV exposure, but 6 of the 16 mice (38%) subsequently shed infectious virus at the ocular surface by the 8th day postirradiation. Observation of the eyes of these animals showed that ocular disease, in some cases, was significantly worse during the second bout of recurrent disease. This generally manifested as corneal stromal opacification and, in severe cases, corneal neovascularization. Because not all the animals with exacerbated ocular disease actually shed detectable virus, it is possible that the true number of mice in which virus reactivates at the ganglion or travels to corneal tissues have detectable viral shedding at the ocular surface. Therefore the true incidence of viral reactivation in this UVB model may be greater than the swab data alone suggest.

An important advantage of this model is its relative simplicity and lack of direct corneal trauma compared with the commonly used iontophoresis procedure; apart from the UV transilluminators described (unmodified, commercially available machines), no special equipment is required, and large numbers of mice may be irradiated simultaneously. Also, UV radiation unlike iontophoresis, is a naturally occurring environmental phenomenon and more likely to induce viral reactivation by a mechanism analogous to that found in naturally occurring herpes keratitis. There is no requirement for immunosuppression to induce reactivation; we showed that the prednisolone drops administered postirradiation were not necessary or able to induce viral reactivation alone, but they enhance viral detection by swabbing of the ocular surface. The lack of a requirement for administration of pharmacologic agents makes the UVB model...
ideal for investigating the effects of such agents on HSV reactivation and recurrent disease.

The mouse appears to be a more stable model of HSV latency than the rabbit; it has a very low incidence of spontaneous reactivation. Data from culturing of tissue homogenates from irradiated latently infected mice uncovered a time window of approximately 2 days between exposure to UV and appearance of infectious virus in the trigeminal ganglia. That infectious virus appears in the trigeminal ganglia earlier than in other tissues supports the hypothesis that these ganglia are the source of the recurrent virus detected in this model. The low incidence of viral latency detected in the superior cervical ganglia confirms that it is unlikely to be the source of recurrent virus at the ocular surface. The 2-day window, coupled with the inherent stability of the viral latent state in mice, presents an excellent opportunity for investigating molecular events surrounding the reactivation of latent HSV-1 in ganglionic neurons. Studies currently are being done using in situ hybridization to determine changes in viral transcription patterns during reactivation in this model.

Additionally, we demonstrated the feasibility of inducing multiple consecutive bouts of HSV recurrence in the same experimental animals. Although not every mouse receiving a second exposure to UVB subsequently shed virus a second time, there was a significant proportion of mice in which viral shedding was reinduced. This, coupled with the observed pathologic changes in the corneas of other animals from which no shedding was detected, showed that the model might be valuable for reproducing the progressive, cumulative corneal tissue damage observed in human HSV keratitis patients. Finally, the model provides a powerful means of testing new drugs that may inhibit viral replication and others that may prevent viral reactivation.

Key words: herpes simplex, keratitis, viral recurrence, ultraviolet animal models

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References