Corneal Langerhans Cell Dynamics After Herpes Simplex Virus Reactivation

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Purpose. The authors investigated the progressive changes in the distribution of corneal Langerhans cells (LC) after reactivation of latent herpes simplex virus type 1 (HSV-1) in mice.

Methods. After corneal inoculation of National Institutes of Health inbred mice with HSV-1 and the establishment of latency, viral reactivation was induced by irradiating the ocular surface with 250 mJ/cm² of ultraviolet B (UV-B) light.

Results. Subsequent viral replication in the cornea was followed by the migration of the LC toward the paracentral and central corneal epithelium. These areas are normally devoid of LC. The number of LC in the paracentral and central regions of the eye reached a peak at day 14 post-UV-B irradiation. After UV-B irradiation of mice latently infected with HSV-1, the development of corneal stromal opacification and neovascularization closely followed the migration of LC toward the central cornea and paralleled the influx of T-cells into the corneal stroma. This pattern was not observed in irradiated uninfected mice.

Conclusions. LC migrate centrally in the corneal epithelium after viral reactivation. There is a direct correlation between the number of LC in the cornea and the degree of persistent stromal opacification.


In many tissues, such as the skin and mucous membranes, Langerhans cells (LC) appear to be an integral component of the regional immune response.1-6 The antigen-presenting capabilities7-10 of these migratory, bone marrow-derived,11 Ia-expressing cells12-14 place them at a critical outpost to generate local immunity against microbial pathogens.

The distribution of LC appears to be compartmentally localized within specific regions of the ocular surface. This localization may contribute to some of the unique immunologic features of the eye. Ocular LC generally reside in the conjunctiva and corneal limbus.13,14 Although the central cornea is normally devoid of LC, they can be detected in large numbers in all regions of the corneal epithelium after a variety of insults, including acute infection with herpes simplex virus 1 (HSV-1).15-20

The exquisite antigen-presenting properties of LC implicate them as central in the activation of T-lymphocytes.121-24 In the eye, which depends on transparent media for visual function, a side effect of the T-cell activation, proliferation, and cytokine release may be the opacification of clear corneal tissue.25-30 Damage from the inflammatory reactions in the corneal stroma induced by recurrent HSV-1 exposure can lead to irreversible tissue necrosis, scarring, and neovascularization.31 The ocular damage observed during HSV-1 reactivation appears to arise predominantly from the response of the immune system to the presence of the reactivated virus, rather than from viral cytolysis itself.28,29,32

Whereas previous studies have quantified corneal LC after primary ocular infection with HSV-1, we initiated experiments to investigate the progressive changes in the distribution of LC after reactivation of latent HSV-1. Our interest in reactivation (as opposed
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to primary infection) reflects our effort to mimic the relevant clinical situation in which the majority of sight-threatening ocular disease is the result of viral recrudescence not subclinical primary infection. Understanding the influence of LC on the manifestations and severity of recurrent disease will help us explore ways to modulate the immune system to provide a defense against HSV-1 while ameliorating the destructive consequences of its responses.

For these studies, we modified a murine ocular model of herpetic reactivation recently characterized in our laboratory, in which HSV-1 is isolated from the ocular surface of infected National Institutes of Health inbred mice after ultraviolet B (UV-B) irradiation. In our previous studies, topical corticosteroids were applied to the ocular surface, starting 2 days after irradiation, to increase the yield and detection of infectious virus during the postirradiation period. Steroid treatment was excluded from the current study to avoid any complicating effects that the steroids might have on the LC. As a consequence, the amount of viral shedding at the ocular surface was reduced and might be below detectable levels in some animals, as assessed by once-a-day swabbing. This has been evidenced in our previous work by the detection of viral antigens in the basal corneal epithelium, but not in the superficial corneal cells, in selected animals that had negative swab cultures at the time they were killed. Because of potential limitations in relying on swab culture to identify all mice with recurrent keratitis, we present a population study in which we compared corneal LC migration in an uninfected group and a latently HSV-1-infected group of mice after exposure of both groups to UV-B light. Data from the latter population were stratified, and the results from mice with positive eye swabs are also presented separately.

MATERIALS AND METHODS

Experimental Design

Mice. Female National Institutes of Health inbred mice (Harlan Sprague Dawley, Indianapolis, IN) were inoculated with virus at an average age of 4 weeks. The following four groups were involved in this study:

1. The test group consisted of 90 mice that were latently infected with virus and subsequently exposed to UV-B light.
2. The UV-B control group consisted of 43 mice that were not infected with virus but were exposed to UV-B light.
3. The latent control group consisted of 11 mice that were latently infected with virus but were not exposed to UV-B light.
4. The untreated control group consisted of 16 mice that were neither infected with virus nor exposed to UV-B light.

The experiments conformed to the ARVO Resolution on the Use of Animals in Research.

Virus. HSV-1, the McKrae strain, was used for the inoculations. The virus was from a plaque-purified stock that had been grown and assayed on African green monkey kidney (Vero) cells in minimum essential medium with Earle's salts and L-glutamine (catalog no. 320-1095PK, Gibco, Grand Island, NY) containing 5% fetal bovine serum (Biocell, Carson, CA), 100 units/ml of penicillin, and 100 µg/ml of streptomycin.

Anesthesia. The mice were anesthetized with a combination of 10 mg/kg of xylazine and 70 mg/kg of ketamine HCl.

Inoculation. After scarification of the cornea of the right eye in a grid pattern with a No. 15 scalpel blade, a 5-µl drop of medium containing 1 × 10^6 plaque-forming units of HSV-1 was placed onto the eye. While the animal was anesthetized, the uninoculated left eye was kept moist with a 5-µl drop of Hanks balanced salt solution (catalog no. 21-022-LM, Mediatech, Washington, DC).

Antiserum. To limit the extent of primary HSV-1 infection, the mice were inoculated intraperitoneally at the time of viral inoculation with 1 ml of pooled human serum (catalog no. S1, Chemicon, Temecula, CA) containing antibodies to HSV-1 (50% effective dose, = 1/640).

Establishment of Latency. The mice were housed (five to ten animals per cage) and fed and watered ad libitum for 46 days after the viral inoculation.

UV-B Irradiation. Forty-six days after the viral inoculation, the right eye of each mouse in which HSV-1 was to be reactivated and that of each mouse that served as an uninfected UV-B-irradiated control was exposed to 250 mJ/cm² of UV-B light (55 sec of 4.5 mW/cm² at 302 nm on a UVP Chromato-Vue transilluminator (model TM-20, San Gabriel, CA).

Data Acquisition

Shedding of Virus. Surgical spears (catalog no. 8680, Xomed-Treace, Jacksonville, FL) saturated with minimum essential medium with Earle's salts and L-glutamine were used to swab the inoculated eyes of all but those of the untreated control group on day 3 postinoculation and to swab the test mice and UV-B controls once daily on days 1–11 post-UV-B exposure. The swab material was cultured on Vero cells, which were examined for virally induced cytopathic effects. Neither topical nor systemic steroids were used in this reactivation model.

Clinical Observations. The eyes were observed through a binocular dissecting microscope and a slit-
Jeweler’s forceps were used to peel and tease the epithelium at 4°C overnight (16 hr).

Dilution in PBS of catalog no. 1199293, Boehringer with the primary antibody, rat anti-mouse Ig (a 1:100 dilution in PBS of catalog no. BA4000, Vector, Burlington, CA) was applied. The slides were then incubated for 2 hr at room temp. The secondary antibody was then washed off, and the slides were soaked 4 min.

The slides were then processed with the avidin–biotin complex procedure of the Vectastain Peroxidase Standard PK4000 kit (Vector). This incubation was performed at room temperature for 45 min and, after washing, was followed by a 13-min room temperature incubation in 3-aminophenylcarbazole solution (17.8 mmol/l sodium acetate, pH 5.1, 0.02% H2O2, 0.85 mol/l 3-aminophenylcarbazole [catalog no. A5754, Sigma, St. Louis, MO], made as 7.94 mol/l in dimethyl sulfoxide [catalog no. D5875, Sigma]). After removal of the 3-aminophenylcarbazole, Mayer’s hematoxylin stain (catalog no. MHS-32, Sigma) was applied for 1 min at room temperature. The counterstain was washed off, and the slides were allowed to dry. A drop of Gelvatol (20 g of polyvinyl alcohol [catalog no. P8136, Sigma] in 80 ml of PBS and 40 ml of glycerol) was placed on top of the epithelium, and a cover slip was placed over the Gelvatol.

Detection and Quantitation of LC. The slides were viewed through a Zeiss (Thornwood, NY) binocular microscope. Cells identified morphologically as LC were counted with the aid of a 100-square grid that encompassed 0.16 mm² at 250X power. The cornea was partitioned into three concentric circles by dividing the overall radius of the corneal flat mount into thirds. The number of LC per 100-square grid were counted in the entire center region and at four points around the paracentral and peripheral regions. The data were then converted to the average number of cells per millimeter squared for a given region of the epithelium.

Immunostaining for Macrophages, T-Cells, and HSV-1 Antigens. Cryostat-cut 8-μm sections of corneal tissue were fixed in chilled acetone and stained with monoclonal antibodies against macrophage (a 1:30 dilution in PBS of Anti-Mac-1, catalog no. 1118 129, Boehringer Mannheim) or T-cells (a 1:30 dilution in PBS of Anti-Thy 1.2, catalog no. 1331, Becton Dickinson, San Jose, CA). Formaldehyde-fixed, paraffin-embedded tissues were cut to an 8-jam thickness and analyzed with rabbit polyclonal antibodies against HSV antigens (a 1:50 dilution, catalog no. AXL-237, Accurate Chemical and Scientific, Westbury, NY). The avidin–biotin complex kit described earlier was used for these analyses.

Photography. Photographs were taken through a Zeiss microscope with an MC100 attachment. ASA 400, 35-mm black-and-white film (XP1, ILFORD, Chicago, IL) or ASA 200, 35-mm color film (Kodacolor Gold, Eastman Kodak, Rochester, NY) was used for the original LC photographs. Technical Pan black-and-white film (catalog no. 129 7563, Eastman Kodak), shot at
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FIGURE 1. Photomicrographs illustrating the morphologic characteristics of LC detected in the corneal epithelium with antibodies to Ia antigen. (A) This corneal epithelium is from the left eye of a latent control mouse (HSV-1 infected by the right cornea, non-UV-B irradiated) and was taken at a time in our experiment equivalent to 43 days post-UV-B-irradiation (X250). (B) This corneal epithelium is from a test mouse (HSV-1 infected, UV-B irradiated) and was taken 11 days post-UV-B irradiation (X250).

ASA 200, was used for the macrophage and T-cell photographs. Filters, times, and f-stops were varied to optimize the contrast in each sample.

Statistics. The averages, standard errors of the means, two-tailed t-tests, and regression analyses were calculated on an Apple Macintosh (Cupertino, CA) with Microsoft Excel (Bellevue, WA) and StatView II and plotted with Cricket Graph 1.2 and Apple MacDraw II (Malvern, PA).

Data Presentation. The data in Figures 2, 3, and 5 are presented as the means ± the standard errors of the means.

RESULTS

Identification of LC

LC were easily identified by the reddish-brown reaction product formed after incubation with the Ia monoclonal antibody and immunoperoxidase development. The cells contained scanty amounts of cytoplasm and had many delicate dendritic processes that were seen interdigitating between neighboring epithelial cells (Fig. 1).

Control Animals: Uninfected, UV-B Irradiated

The changes observed in the distribution of corneal LC after exposure to the UV-B used to initiate HSV-1 reactivation in our murine model were transient (Fig. 2). The initial trend in the uninfected mice was a slight decrease in the number of LC in the periphery of the corneas. LC were virtually absent in the paracentral and central regions of the cornea during this same period. After the early decrease in the number of LC in the peripheral cornea, the number of LC increased between days 7–21 in the periphery, paracentral region, and center of the corneas of these uninfected UV-B-irradiated animals, but to a lesser degree than in the infected animals (Figs. 2, 3). The number of LC in the central and paracentral regions decreased after day 21, approaching baseline levels. The number of LC in the periphery on day 35 was greater than that on day 0.

FIGURE 2. Uninfected, UV-B-irradiated mice. These data illustrate the progressive changes in the number of LC in the peripheral, paracentral, and central regions of the corneal epithelium as the time post-UV-B irradiation increases. They are presented as the means accompanied by the standard errors of the means.
LATENTLY INFECTED MICE

LANGERHANS CELLS, \( \frac{\text{PER mm}^2}{\text{DAY POST-UV IRRADIATION}} \)

**FIGURE 3.** Infected, UV-B-irradiated mice. These data illustrate progressive changes in the number of LC in the peripheral, paracentral, and central regions of the corneal epithelium as the time post-UV-B irradiation increases. They are presented as the means accompanied by the standard errors of the means.

A temporary clouding of the cornea was detected with the binocular dissecting microscope in the eyes exposed to the UV-B irradiation we used to reactivate HSV-1 in our model. Examination with the slit-lamp biomicroscope revealed that this photokeratitis manifested itself as corneal epithelial stippling and edema, stromal edema, and a fibrinoid anterior chamber reaction. These signs dissipated substantially after reaching a peak on the 4th day after UV-B exposure. The majority of uninfected animals were left with clear or relatively clear corneas.

**Additional Control Animals: Latent and Uninfected, Non-UV-B irradiated**

The corneal epithelia of untreated controls and of non-UV-B-treated latent controls did not display a significant migration of LC toward the paracentral and central regions at the times examined (days equivalent to days 0, 4, 7, 11, 14, 21, and 35 post-UV-B irradiation for the untreated controls; days equivalent to days 35 and 43 post-UV-B irradiation for the latent controls (Fig. 1A, data not shown). These animals displayed no corneal opacification.

**Test Animals: Infected, UV-B Irradiated**

Both the number of corneal LC and the degree of stromal opacity increased after UV-B-induced HSV-1 reactivation (Fig. 3). On the basis of single daily swabs, the virus was detected at the ocular surface of 60% of the mice between days 2-9 after UV-B exposure. In addition, viral antigens were detected in corneal epithelium during this period, as demonstrated by immunoperoxidase staining (Fig. 4). During this period, the initial trend was toward a decrease in the number of LC detected in the periphery of the cornea (Figs. 3; 5A, B). During this same period, the paracentral and central regions of the cornea were virtually devoid of LC (Figs. 3; 5A, B, F, G). After the early decrease in the number of LC in the peripheral cornea, the number of LC increased substantially between days 7-14 post-UV-B irradiation in all regions of the corneal epithelium of mice harboring latent HSV-1 (Figs. 3; 5C, D, H, I). A further increase in LC was observed between days 21-35. The results from viral shedders alone are presented in Table 1.

As in uninfected animals, UV-B irradiation of latently infected mice produced a transient edema of the corneal epithelium and stroma that peaked at 4 days, then dropped precipitously. Conversely, in latently infected, UV-B-irradiated animals, a stromal opacification began to manifest at day 7 and rose to a plateau by day 21 post-UV-B exposure (Fig. 6). Using the scoring system outlined earlier, the degree of stromal opacification in the UV-B-treated latently infected animals differed significantly at days 21 and 35 from that seen in UV-B-irradiated uninfected controls at the 99.9% confidence level \((P < 0.001)\). Corneal neovascularization occurred in 85% of the infected UV-B-exposed animals that were scored as 3 or 4 (degree of opacification) by day 7 or later post-UV-B exposure; 50% of those scored 1 or 2; and 1% of those scored 0.

Regression analyses were performed to determine the extent of correlation between corneal opacification and the presence of LC. The coefficients of correlation \((r)\) tended to increase with time post-UV-B irradiation, reaching \(r\) values of 0.95, 0.98, and 0.67 for the periphery, paracentral, and central regions, respectively, by day 35 after UV-B exposure.

**DISCUSSION**

In this murine model of recurrent HSV-1 keratitis, we have shown that LC migrate centrally in the corneal

**FIGURE 4.** HSV-1 antigens (arrows) as demonstrated by immunoperoxidase staining in the corneal epithelium 5 days after ocular exposure to UV-B (X200).
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FIGURE 5. Photomicrographs illustrating the progressive changes in the number of LC in the corneal epithelium after the UV-B irradiation of the corneas of mice harboring latent HSV-1 (X125). The magnification is lower than that in Figure 1 (125X versus 250X) to allow for a wider field of view. A–E show the peripheral and paracentral regions of the corneal epithelium on days 0, 4, 11, 14, and 35 post-UV-B irradiation, respectively. F–J show the central region of the corneal epithelium on days 0, 4, 11, 14, and 35 post-UV-B irradiation, respectively.

epithelium after viral reactivation. The virally induced migration of LC temporally precedes the development of herpetic stromal disease (Figs. 3, 6). These findings are consistent with those of previous studies of primary infection\(^\text{15,26}\) that demonstrated that an increase in the number of LC in the more central regions of the cornea during the time of primary HSV-1 infection can promote stromal disease.

The intensity of the UV-B used in our model may be expected to damage functionally the LC it encounters\(^\text{38}\) and also may underlie the initial drop in peripheral LC in both the uninfected and latently infected groups. However, the migratory LC that infiltrate the cornea in substantial numbers by day 7 post-UV-B-induced reactivation are unlikely to be the same cells that were exposed to the UV-B. Those LC we observe at this later time are, therefore, potentially capable of presenting HSV-1 antigens to incoming T-cells.\(^\text{8}\)

The central migration of LC demonstrated after HSV-1 reactivation shares some common features with the changes in corneal LC distribution observed after primary HSV-1 infection.\(^\text{15,17,18,20,26}\) However, in a recurrent infection, the migration of LC toward the central cornea is delayed in comparison with that observed during primary infection. The later time point for the peak in the number of LC observed in this study of reactivated virus (day 14) compared with that observed in earlier experiments of primary infection (days 7–11) may represent the lag time between the reactivation in the trigeminal ganglia and the appearance of the replicating virus at the ocular surface. Al-

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<th>Day Post-UV Reactivation</th>
<th>Peripheral</th>
<th>Paracentral</th>
<th>Central</th>
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<tbody>
<tr>
<td>0</td>
<td>33.6 ± 3.4</td>
<td>15.6 ± 4.1</td>
<td>5.7 ± 2.7</td>
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<tr>
<td>4</td>
<td>28.9 ± 7.0</td>
<td>7.8 ± 3.1</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>7</td>
<td>69.7 ± 8.0</td>
<td>17.7 ± 2.9</td>
<td>14.6 ± 5.5</td>
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<tr>
<td>11</td>
<td>137.6 ± 26.0</td>
<td>71.7 ± 20.0</td>
<td>47.2 ± 18.5</td>
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<tr>
<td>14</td>
<td>232.2 ± 59.1</td>
<td>179.5 ± 67.9</td>
<td>64.6 ± 30.0</td>
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<td>152.2 ± 41.8</td>
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<tr>
<td>35</td>
<td>208.7 ± 64.0</td>
<td>156.2 ± 69.6</td>
<td>110.5 ± 49.4</td>
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Data are expressed as LC per square millimeter ± SEM.
DEGREE OF
STROMAL
OPACIFICATION

0= CLEAR STROMA
1= MILD OPACIFICATION
2= MODERATE OPACIFICATION
3= DENSE OPACIFICATION WITH LOSS OF DEFINED IRIS DETAIL
4= TOTAL OPACITY WITH NO POSTERIOR VIEW

FIGURE 6. Corneal stromal opacification after UV-B irradiation of mice latently infected with HSV-1. Stromal opacification was rated on a scale of 0-4, in which 0 indicated a clear stroma; 1, a mild stromal opacification; 2, a moderate opacity with discernible iris features; 3, a dense opacity with loss of defined iris detail; and 4, total opacity with no posterior view. At days 21 and 35, the two groups differed significantly at the 99.9% confidence level ($P < 0.001$).

alternatively, the later peak in LC migration observed after reactivation (as opposed to primary infection) may reflect transient depletion of LC from the ocular surface in the period immediately after UV-B, requiring additional time to repopulate the conjunctiva and limbus before subsequent central migration can occur.

In preliminary experiments in this laboratory with HSV-1 reactivation in mice, we have shown the infiltration into the cornea of different classes of T-cells to peak at day 10 or later after viral reactivation (Fig. 7) and a delayed-type hypersensitivity response to HSV-1 antigens to rise sharply until day 10 and then to continue to increase more slowly (data not shown). These pilot studies have also demonstrated that a large number of macrophages are present in the stroma by day 3 postreactivation (Fig. 8), but few macrophages ever enter the epithelium. These findings, taken in conjunction with the data we have presented here, produce a sequence of observations in which neuronal reactivation of virus, retrograde transport, and subsequent corneal replication of HSV-1 is followed, first, by the migration of macrophages into the stroma (day 3) and, then, by the movement of LC toward the central regions of the corneal epithelium (beginning at day 7 and peaking at day 14, Fig. 3). These events are accompanied and followed, first, by infiltration of T-lymphocytes into the cornea (beginning at day 7 and peaking at day 10 or later) and, then, by a persistent corneal opacification (Fig. 6) and neovascularization (day 21 and beyond).

It is our working hypothesis that reactivated virus replicating in the cornea (Fig. 4 shows the detection of HSV antigens) induces macrophages and corneal epithelium to release cytokines (eg, interleukin-1 and tumor necrosis factor-$
\alpha$), which in turn, attract LC to the epithelium, either directly or through other downstream cytokines such as interleukin-8 or monocyte chemotactic activation factor. Antigen-presenting corneal LC and stromal macrophages, perhaps through the release of their own products or in conjunction with interleukin-8, induce T-cells (and, possibly, additional LC) to infiltrate the cornea. The infiltration of macrophages and T-cells and/or the release of their products results in corneal opacification. A repetition of this sequence as a result of multiple viral recurrences further increases stromal opacification.

FIGURE 7. Immunochemical staining of corneal T-cell infiltrates detected 10 days after UV-B irradiation of mice latently infected with HSV-1 (Thy 1.2 monoclonal antibody, X250).

FIGURE 8. Immunochemical staining of corneal macrophage infiltrates detected 3 days after UV-B irradiation of mice latently infected with HSV-1 (Mac-1 monoclonal antibody, X 250).
In addition, both interleukin-1 and -2 have been implicated in the production of corneal neovascularization, along with other components of the inflammatory response. Ongoing experiments designed to test the various components of this hypothesis may lead to therapeutic modalities to modulate the damaging ocular immune response to HSV-1 and to a better understanding of existing treatment regimens.

In summary, we found a correlation between the number of LC present in the cornea and the degree of persistent stromal opacification in our murine model of herpetic reactivation. To our knowledge, this is the first reported study of corneal LC dynamics after HSV-1 reactivation. Viral reactivation, as opposed to primary infection, has considerable clinical significance. In humans, it is, generally, reactivation of HSV-1, rather than primary infection, that eventually leads to visually significant corneal stromal disease. We have demonstrated that our murine reactivation model mimics this most significant component of the human ocular disease.

**Key Words**
Langerhans cells, herpes simplex virus type 1, viral reactivation, mouse model of reactivation, corneal epithelium

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**References**


