Natural Killer Cells Prevent Direct Anterior-to-Posterior Spread of Herpes Simplex Virus Type 1 in the Eye

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PURPOSE. Anterior chamber (AC) inoculation of the KOS strain of herpes simplex virus type 1 (HSV-1) results in morphologic sparing of the ipsilateral retina, whereas the retina of the uninoculated contralateral eye becomes infected and undergoes acute retinal necrosis. Natural killer (NK) cells are an important component of the primary immune response to most virus infections. The purpose of this study was to determine whether NK cells are involved in preventing early direct anterior-to-posterior spread of HSV-1 after AC inoculation.

METHODS. Normal BALB/c mice were inoculated with 4 × 10⁴ plaque-forming units (PFU) of the KOS strain of HSV-1 using the AC route. NK activity was measured in the spleen, the superficial cervical and submandibular lymph nodes, and the inoculated eye by lysis of chromium-labeled, NK-sensitive YAC-1 target cells. Histopathologic scoring and immunohistochemical staining for HSV-1 were performed in NK-depleted (injected intravenously with anti-asialo GM₁) or mock-depleted (injected intravenously with normal rabbit serum) mice.

RESULTS. In mock-depleted mice, NK activity in the spleens, superficial cervical and submandibular lymph nodes, and inoculated eyes peaked at postinoculation (pi) day 5 and declined by pi day 7. Treatment with anti-asialo GM₁ eliminated NK activity in the eye and at nonocular sites. The histopathologic scores at pi day 5 indicated more damage to the retinas of NK-depleted mice than to those of mock-depleted mice, and immunohistochemical staining for HSV-1 showed spread of the virus to the sensory retina only in NK-depleted mice.

CONCLUSIONS. NK cells were activated within 5 days after AC inoculation of the KOS strain of HSV-1. Activation of NK cells appears to play a role in preventing direct anterior-to-posterior spread of the virus in the inoculated eye which, in turn, protects the retina of this eye and helps to explain why the architecture of the retina of this eye is spared. (Invest Ophthalmol Vis Sci. 2000;41:132–137)

After unilocular anterior chamber (AC) inoculation of the KOS strain of herpes simplex virus (HSV-1) in euthymic BALB/c mice, extensive virus infection accompanied by a massive inflammatory response is observed in the anterior segment of the injected eye.¹ However, in spite of the virus infection and inflammation in the anterior segment, HSV-1 does not spread from the infected AC directly to the retina of the injected eye after unilocular AC inoculation.²⁻⁴ Although virus infection and retinitis are observed in the inoculated eye of T-cell–depleted mice, virus entering the retina of these mice spreads there through retrograde transport in the optic nerve of the injected eye on or about postinoculation (pi) day 9.⁵ Direct anterior-to-posterior spread of virus is not observed in T-cell–depleted mice at any time after inoculation. Thus, although T cells are involved in preventing spread of virus to the ipsilateral optic nerve,⁵ they do not appear to be responsible for preventing direct anterior-to-posterior spread of the KOS strain of HSV-1 after unilocular AC inoculation. Therefore, it is likely that a non–T-cell–dependent mechanism prevents spread of virus to the retina of the injected eye.

An early, but nonspecific, mechanism of protection operative in many types of infections is natural killer (NK) cells.⁶ NK cells, a subset of large, granular lymphocytes, are activated by the cytokines interferon (IFN)-α, IFN-β, and interleukin (IL)-12 and can secrete cytokines with antiviral activity such as IFN-γ and tumor necrosis factor (TNF)-α when activated.⁷⁻⁸ NK cells lyse virus-infected cells after conjugate recognition of those cells by a major histocompatibility complex–unrestricted mechanism. However, the eye constitutively expresses NK-suppressing factors, including TGF-β and macrophage migration inhibitory factor, which have been shown to diminish NK activity in vitro.⁹⁻¹³ NK cells have been shown to be important in resistance to a number of primarily intracellular pathogens, including HSV. Intradiscal injection of HSV increases NK cell activity, and NK cells have been shown to modulate replication of HSV-1 in the liver and brain.¹⁴⁻¹⁶ The purpose of the studies described in this article was to examine the role of NK cells in the injected eye after unilocular AC inoculation of HSV-1. The results of these studies suggest that NK cells prevent direct anterior-to-posterior virus spread of KOS strain of HSV-1 in the injected eye after unilocular AC inoculation.
TABLE 1. Histopathologic Evaluation of the Retina after Uniocular AC Inoculation of HSV-1

<table>
<thead>
<tr>
<th>Histopathologic Observation</th>
<th>Histopathologic Score</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Destruction of sensory retina</td>
<td>None</td>
</tr>
<tr>
<td>Inflammatory cell infiltration</td>
<td>None</td>
</tr>
<tr>
<td>Choroidal hypercellularity</td>
<td>None</td>
</tr>
<tr>
<td>Pyknotic debris</td>
<td>None</td>
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**METHODS**

**Animals**

Female BALB/c mice, 6 to 8 weeks old, were obtained from Taconic Farm (Germantown, NY). Animals were housed in accordance with National Institutes of Health guidelines. All animal experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were maintained on a 12-hour light-dark cycle and given unrestricted access to food and water.

**Virus**

The KOS strain of HSV-1 was used in this study. Virus stocks were titered on Vero cell (ATCC, Rockville, MD) monolayers, as described previously.5

**Ocular Injection**

Mice were anesthetized by intramuscular injection of a cocktail containing 0.02 ml Rompun (X-JECT 5.A; Uetus Animal Health, St Joseph, MO) and 0.03 ml ketamine per 25 g body mass. The AC of the right eye of each mouse was inoculated with 4.0 × 10⁴ plaque-forming units (PFU) of KOS in a volume of 2 μl. The eyes were washed three times in HBSS and resuspended in RPMI-5. To have enough effector cells from the eye for the assays, three or four eyes were collected at each time point.

**Chromium Release Assay**

One million YAC-1 target cells were labeled with 100 μCi chromium-51 (Du Pont–NEN, Wilmington, DE) for 1 hour in 500 μl RPMI 1640 with 5% fetal calf serum (RPMI-5) at 37°C. After labeling, the target cells were washed three times and seeded into 96-well plates at a concentration of 10⁴ cells/100 μl per well. Effector cells were obtained from the spleen, the superficial cervical and submandibular lymph nodes, or the inoculated eye. Splenic effector cells were prepared by grinding the spleens between frosted slide glasses, followed by aspiration through a 22-gauge needle to make a single-cell suspension. Spleen cells were washed three times in Hanks’ balanced salt solution (HBSS), and erythrocytes were lysed by ammonium chloride treatment; the remaining spleen cells were washed three times in HBSS and resuspended in RPMI-5. Cells from the superficial cervical and submandibular lymph nodes ipsilateral to the site of injection were isolated by teasing the tissue through a 200-μm nylon mesh. The cells were then washed three times and resuspended in RPMI-5. Eyes were enucleated after cardiac perfusion, and the conjunctiva and extraocular muscles were removed under the dissecting microscope. The eyes were teased through a 70-μm mesh, and the cells were washed three times in HBSS and resuspended in RPMI-5. To have enough effector cells from the eye for the assays, three or four eyes were collected at each time point.

**Histopathologic Evaluation of the Retina**

Eyes were fixed in buffered formalin, embedded in paraffin, and sectioned. The sections were then stained with hematoxylin and eosin. A modification of the semiquantitative histopathologic scoring system described by Azumi and Atherton was used to evaluate the extent of retinal destruction, inflammatory cell infiltration, hypercellularity of the choroid, and amount of pyknotic debris as shown in Table 1. The maximum possible histopathologic score for a single retina was 13.
Immunohistochemistry

Mice were killed and perfused with PBS. The eyes were removed, embedded in Tissue-TEK O.C.T. (optimum cutting temperature) compound (Miles, Elkhart, IN), and sectioned at 8 µm using a cryostat. All sections were air dried and stored at −70°C. Before staining, sections were fixed in acetone for 5 minutes, washed twice with PBS, and placed in 0.3% hydrogen peroxide in methanol for 15 minutes. Sections were then washed twice with PBS and incubated with buffered casein solution (Powerblock; Bio Genex, San Ramon, CA) for 15 minutes. Excess casein solution was blotted, and the sections were incubated with a 1:200 dilution of rabbit polyclonal anti-HSV-1 antibody (Dako, Carpinteria, CA) for 2 hours. The sections were washed twice with PBS, incubated with a 1:500 dilution of biotinylated goat anti-rabbit IgG (Vector) for 45 minutes, washed twice with PBS, and incubated with Vectastain ABC (Vector), according to the manufacturer’s directions. The sections then were washed three times with PBS and reacted with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma) supplemented with 0.3% nickel chloride and 0.01% hydrogen peroxide. Color development was monitored microscopically. All sections were counterstained with methyl green.

RESULTS

Activation of NK Cells in the Spleen, Lymph Nodes, and Eye after AC Inoculation of HSV-1

After AC inoculation of HSV-1 (KOS) into normal BALB/c mice, the ipsilateral retina does not become virus infected. Because there is no anatomic barrier to prevent virus spread within the eye and because T cells have been shown to protect the inoculated eye by preventing retrograde spread of virus from the brain,5 a non-T-cell mechanism, such as NK cells, appears to be responsible for preventing direct anterior-to-posterior spread of HSV-1 after unidirectional AC inoculation. Injection of HSV-1 into the AC usually results in induction of anterior chamber-associated immune deviation (ACAID), and both the aqueous and the vitreous of the eye contain multiple substances that depress NK activity.9–13 To determine whether AC inoculation of HSV-1 induces an NK response, the cytolytic activity of cells from the spleen, lymph nodes, and inoculated eye was measured by chromium release assays. On day 1, 3, 5, or 7 before the chromium-release assay, 4.0 × 10⁴ PFU of HSV-1 was injected into the AC of one eye. On the day of assay, mice were killed and cells from the spleen, lymph nodes, and inoculated eye were assayed for cytolytic activity against NK-sensitive YAC-1 target cells. As shown in Figure 1, the cytolytic activity of splenocytes (Fig. 1A), of cells from the superficial cervical and submandibular lymph nodes (Fig. 1B), or of cells from the eye (Fig. 1C) was first detected on pi day 3, peaked on pi day 5, and returned to baseline on pi day 7. Treatment with anti-asialo GM1 eliminated NK activity of ocular cells (Fig. 2) and splenocytes (not shown).

Destruction of the Ipsilateral Retina after Inoculation of KOS into the AC of NK-Depleted Mice

To determine whether the NK response after AC inoculation of KOS is involved in protecting the retina of the injected eye from viral infection and subsequent destruction, BALB/c mice were injected intravenously with anti-asialo GM1 serum or an equivalent volume and concentration of normal rabbit serum (control mice) on day −1; on day 0, 4.0 × 10⁴ PFU of the KOS
strain of HSV-1 was injected into the AC of one eye. Histopathologic scores of the inoculated eyes of NK-depleted mice were compared with those of the mock-depleted mice. At pi day 3, the retinas of mice in both groups had only slight atypical retinopathy, and there was no significant difference between the retinal scores of NK-depleted and mock-depleted control mice (not shown). However, by pi day 5, significantly more disease was observed in the retinas of NK-depleted mice than in the retinas of mock-depleted mice at pi day 5 ($p < 0.035$; Table 2). As shown in Figure 3, destruction of the sensory retina, inflammatory cell infiltration, and choroidal hypercellularity were observed in retinas of mice in the NK-depleted group (Fig. 3B), whereas in the mock-depleted mice, only mild retinal folding similar to that previously observed in the inoculated eyes of normal mice after AC injection of HSV-1 was seen (Fig. 3A).1,5

**Spread of HSV-1 to the Posterior Segment in NK-Depleted Mice**

To determine whether NK cells protect the inoculated eye from direct spread of HSV-1 to the posterior segment after AC injection, immunohistochemical staining for HSV-1 was performed on ocular sections from NK-depleted mice and mock-depleted mice. For these studies, mice were injected intravenously with anti-asialo GM$_1$ serum on day −1 and injected with $4.0 \times 10^4$ PFU of KOS through the AC route on day 0. To verify the specificity of the anti-HSV-1 antibody, adjacent sections were incubated with normal rabbit serum and processed identically with the sections treated with anti-HSV-1 antibody. These control slides showed no reactivity (not shown). At day 3 pi, HSV-1-positive cells were observed in the choroid in both NK-depleted mice and mock-depleted mice (not shown). HSV-1-positive cells were not observed in the sensory retina of mice in either group at this time. However, at day 5 pi, HSV-1-positive cells were observed in the sensory retina, but not in the RPE or choroid, of NK-depleted mice (Figs. 4B, 4D). In contrast, HSV-1-positive cells were not observed in the retina, choroid, or RPE of the mock-depleted mice at pi day 5 (Figs. 4A, 4C).

**DISCUSSION**

After unilocular AC inoculation of the KOS strain of HSV-1 in BALB/c mice, acute retinal necrosis is observed in the un inoculated contralateral eye, whereas the retina of the ipsilateral eye is spared from virus infection and destruction.1-4 The mechanism that prevents virus from spreading to the retina of the injected eye has been puzzling. It is known that this mechanism cannot be T-cell-mediated, because in T-cell-depleted mice, virus does not spread directly from the infected anterior segment to the retina. Even though bilateral retinitis develops in T-cell-depleted mice, retinitis in the infected eye of T-cell-depleted mice results from late (on or after pi day 9) spread of virus from the suprachiasmatic nucleus contralateral to the side of inoculation to the optic nerve of the injected eye and not from direct anterior-to-posterior spread of the virus.2,4,5

In the normal eye, immune privilege is maintained and inflammatory damage is prevented by immunosuppressive cytokines, such as TGF-β and macrophage migration inhibitory factor, that are produced within the eye. However, these immunosuppressive ocular cytokines, which have also been shown to suppress NK activity,9-15 do not prevent induction of an NK response after unilocular AC inoculation of the KOS strain of HSV-1. The results from the studies presented in this article demonstrate that after unilocular AC inoculation of the KOS strain of HSV-1, there is both a local (eye and superficial cervical and submandibular lymph nodes) and a systemic (spleen) NK response, shown by cytolytic activity of mononuclear cells isolated from these sites against NK-sensitive YAC-1 targets. The reason ocular factors that normally suppress NK activity do not inhibit NK activation after AC inoculation of HSV-1 may be that the extensive virus replication and inflammation that occur within 1 to 2 days after AC inoculation1,17 outstrip the eye’s ability to produce these factors.

However, merely determining that AC inoculation of HSV-1 induces both a local and a systemic NK response does not provide information about the role of such a response during ocular infection or about how NK cells limit virus spread in the injected eye. Results from the immunohistochemistry and histopathologic studies suggest that depletion of NK cells allowed HSV-1 to spread to the ipsilateral retina early after infection and that subsequent virus replication in the retina led to increased retinal damage in NK-depleted mice.

**TABLE 2. NK Depletion Increases Retinal Damage after AC Inoculation of HSV-1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Retinal Score*</th>
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<tbody>
<tr>
<td>NK-depleted†</td>
<td>Anti-asialo GM$_1$</td>
<td>11, 7, 5, 3, 3, 3, 2, 0, 0</td>
</tr>
<tr>
<td>Mock-depleted</td>
<td>Normal rabbit serum</td>
<td>3, 2, 2, 2, 1, 1, 0, 0, 0</td>
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* Maximum posterior segment score for each eye.  
† Significantly different from mock-depleted group, $p < 0.035$; Mann-Whitney test; $n = 10$/group.
Interestingly, virus was observed at pi day 3 in the choroid, but not in the sensory retina, in both NK-depleted mice and mock-depleted mice. In both groups of mice, no virus-infected cells were detected in the choroid or RPE at pi day 5. However, at pi day 5, the retinas of NK-depleted mice had more histopathologic changes than the retinas of non-NK-depleted mice, and the retinal changes were observed coincident with virus infection of the retina. This observation suggests that after AC inoculation of HSV-1, the virus is able to spread to the choroid irrespective of the presence or

**FIGURE 3.** NK depletion results in destruction of the retina of the injected eye. Photomicrographs of retinal sections of (A) mock-depleted (histopathologic score: 1) or (B) NK-depleted (histopathologic score: 7) mice 5 days after AC inoculation with HSV-1. Hematoxylin and eosin; original magnification, $\times70$.

**FIGURE 4.** NK depletion allows virus infection of the ipsilateral retina after AC inoculation. Photomicrographs of retinal sections from infected, mock-depleted (A and C), or infected, NK-depleted (B and D) mice 5 days after inoculation of $4 \times 10^4$ PFU of the KOS strain of HSV-1 through the AC route. Immunohistochemistry was conducted using anti-HSV-1 serum. *Arrows:* virus-infected cells in the sensory retina. Original magnification, $\times70$ (A, C) and $\times280$ (B, D).
absence of NK cells. However, NK cells or their products eliminate HSV-1 from the choroid of normal mice by pi day 5, and in so doing, protect the retina of the injected eye from virus infection and destruction.

There is a paucity of information about how NK cells or their products limit virus spread and replication within the eye. NK cells have been reported in the anterior segment (iris and corneal limbus) 5 days after AC inoculation of HSV-1 in BALB/c mice. Recently, NK cells have been shown to modulate retinal destruction in a mouse model of cytomegalovirus retinitis. It is not clear whether these cells are normally present within the eye and are activated by virus infection within the ocular compartment, whether these cells are activated at an extraocular site and migrate to the eye, or whether there are both ocular and extraocular populations of such cells.

In summary, the results presented in this article support the idea that NK cells are activated after uniocular AC inoculation of the KOS strain of HSV-1 and that these cells are important in preventing spread of this virus from the infected AC to the retina of the inoculated eye. Additional studies will be required, however, to determine the mechanism by which such cells prevent virus spread and the origin and site of activation of these cells.

References