Cytokine Production in a Murine Model of Recurrent Herpetic Stromal Keratitis

Thomas H. Stumpf,1 Carolyn Shimeld,1 David L. Easty,1 and Terry J. Hill2

PURPOSE. To determine the pattern of cytokine production in the cornea and its relationship with viral antigens, in our murine model of recurrent ocular herpes simplex virus (HSV)-1 infection.

METHODS. Six weeks after corneal inoculation with HSV-1, the eyes of latently infected and control mice were UV irradiated and examined for signs of disease and virus reactivation. The eyes of five mice with recurrent stromal disease and two controls were processed for immunohistochemistry on days 4, 7, 10, and 14 after irradiation. Sections were double stained for viral antigens and one of the following cytokines: interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12, and interferon (IFN)-γ.

RESULTS. Fifty percent of mice showed signs of recurrent stromal disease, the severity of which peaked on day 10 after UV irradiation. There was a large cellular infiltrate in the stroma of all the corneas with recurrent disease and the predominant cytokines were IL-1β, IL-6, IL-10, IL-12, and IFN-γ; all present in large numbers of cells on the days studied. There were very few cells producing IL-2 and IL-4. Control eyes had no significant cytokine-producing cells in the stroma.

CONCLUSIONS. These observations suggest that recurrent herpetic stromal keratitis (HSK) may not be characterized by a classic T-helper (Th)1 or Th2 response. However, the large number of IFN-γ+ and IL-12+ cells and the relative absence of IL-4 favors a Th1 response, and despite the numerous IL-10+ cells, the overall balance of cytokine production appears to be proinflammatory. (Invest Ophthalmol Vis Sci. 2001;42:372–378)

Herpetic stromal keratitis (HSK) is a potentially blinding disease, initiated by recurrent infection of the cornea with the herpes simplex virus (HSV)-1. It is thought to be an immunopathologic process that persists after the virus has been cleared from the eye.1

It has been shown that T lymphocytes play an essential role in the mouse model of primary infection, and that HSK does not develop in T-cell-deficient mice.2 However, the keratitis develops when such mice are given HSV-sensitized T cells.3

Recently, a more complex picture of HSK is emerging and several hypotheses have attempted to explain this disease.4 There is evidence to support a role for both a T-helper (Th)15,6 and a Th27,8 response. In addition, cytotoxic CD8+ T cells9 and an autoimmune response against corneal autoantigens10 have also been implicated.

Some of these contradicting hypotheses may have resulted from different mouse and virus strains used and nearly all the reports investigated the responses after primary infection. However, in humans, HSK is a feature of recurrent corneal infections and occurs in an individual with established viral immunity, a feature absent from the models of primary infection.

Our mouse model of recurrent HSV-1 infection mimics the corneal disease seen in humans11,12 and using this model, we have demonstrated a vigorous cellular infiltrate in the corneal stroma, which consists mainly of neutrophils, but also some CD4+ T lymphocytes and macrophages.13 Using this model, it has recently been shown that both interleukin (IL)-1 and tumor necrosis factor (TNF)-α are important factors in the pathogenesis of recurrent HSK, but little is known about their source or the involvement of other cytokines.14

We now report on a wide range of cytokines and the distribution of HSV-1 antigen in recurrent HSK, using a recently developed quantitative immunohistochemical method.15

METHODS

Animals

All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Specific pathogen-free, 8-week-old female NIH/OLA inbred mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg; Rompun; Bayer, Bury St. Edmunds, UK) and ketamine (100 mg/kg Vetalar; Pharmacia & Upjohn, Crawley, UK).

Latent Infection and Recurrent Disease

HSV-1 strain McKrae was grown and titrated on Vero cells. Latent infection and recurrence of HSV-1 infection was induced as described previously.11 In brief, mice were passively immunized with an intra-peritoneal injection of normal human serum (Chemicon, Temecula, CA) known to contain HSV-1 antibodies. The serum was diluted in phosphate-buffered saline (PBS) to give an effective dose (ED50) of 8000 plaque-forming units (pfu). After 24 hours, a 5 µl volume containing 102 PFU of HSV-1 was applied to the left cornea of anesthetized mice, which was subsequently scarified. Control mice were mock inoculated using the medium of uninfected Vero cells. Only mice with normal eyes after primary infection were used for reactivation.

At least 6 weeks after primary infection, mice were anesthetized and their corneas irradiated with UV B light for 90 seconds (UV lamp emitting a peak of 4.03 mJ/cm² per second at 320 nm; Hanovia, Slough, UK). Mice were examined by slit lamp for signs of recurrent ocular disease using the criteria in Table 1. Clinical assessments were made immediately before UV irradiation and on days 1 to 7, 10, and 14 after irradiation. Eye washings were taken after each examination for virus isolation.16

On each of the days 4, 7, 10, and 14, five mice with the most severe signs of recurrent HSV-1 eye disease and two control mice had their eyes processed for immunohistochemistry. Recurrent disease was defined as virus shedding in tears and/or stromal opacification for more than 2 consecutive days. The most diseased eyes were chosen because of their large cellular infiltrate, making the immunohistochemical detection of cytokines more likely.

Tissue Fixation and Immunohistochemistry

Tissues were fixed and processed as described previously.15 In brief, animals were killed and perfused with periodate-lysine-parafomalde-
Table 1. Scoring System for Clinical Signs of Ocular Disease

<table>
<thead>
<tr>
<th>Disease</th>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lid</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>Uveitis</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Details of Primary Antibodies Used for Immunohistochemical Staining of Cytokines, HSV-1 Antigens, and Respective Control Antibodies

<table>
<thead>
<tr>
<th>Antigen/Antibody</th>
<th>Species</th>
<th>Clone</th>
<th>Isotype</th>
<th>Concentration (Dilution)*</th>
<th>Positive Control Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>0.13 (1:750)</td>
<td>Spleen</td>
</tr>
<tr>
<td>IL-2</td>
<td>Rat</td>
<td>S4B6</td>
<td>IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>100 (1:10)</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td>IL-4</td>
<td>Rat</td>
<td>BUD4-1D11</td>
<td>IgG&lt;sub&gt;1a&lt;/sub&gt;</td>
<td>33 (1:15)</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td>IL-6</td>
<td>Rat</td>
<td>MP5-20F3</td>
<td>IgG&lt;sub&gt;1b&lt;/sub&gt;</td>
<td>66 (1:15)</td>
<td>HSV infected eye</td>
</tr>
<tr>
<td>IL-10</td>
<td>Rat</td>
<td>2A5</td>
<td>IgG&lt;sub&gt;1a&lt;/sub&gt;</td>
<td>3.3 (1:300)</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td>IL-12</td>
<td>Rat</td>
<td>C15.6</td>
<td>IgG&lt;sub&gt;1a&lt;/sub&gt;</td>
<td>100 (1:10)</td>
<td>HSV infected eye</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Hamster</td>
<td>RP64</td>
<td>IgG</td>
<td>20–66 (1:30–100)</td>
<td>HSV infected eye</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>33.6 (1:250)</td>
<td>HSV infected TG</td>
</tr>
<tr>
<td>IgG, isotype</td>
<td>Rat</td>
<td>A110-1</td>
<td>IgG&lt;sub&gt;1a&lt;/sub&gt;</td>
<td>100 (1:5)</td>
<td>—</td>
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<tr>
<td>IgG2a, isotype</td>
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<td>B99-4</td>
<td>IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>100 (1:5)</td>
<td>—</td>
</tr>
<tr>
<td>IgG, isotype</td>
<td>Rat</td>
<td>A95-1</td>
<td>IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>100 (1:5)</td>
<td>—</td>
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<tr>
<td>IgG</td>
<td>Hamster</td>
<td>Polyclonal</td>
<td>—</td>
<td>20–66 (1:30–100)</td>
<td>—</td>
</tr>
<tr>
<td>Normal serum</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>—</td>
<td>0.13 (1:750)</td>
<td>—</td>
</tr>
</tbody>
</table>

* Expressed as micrograms per milliliter.
reactivation (Fig. 2A), and the uveitis was generally worse in these mice compared with controls (Fig. 2C). Lid disease was first detected on day 4 in three mice and by day 10, eight mice were affected. The severity of these signs decreased by the end of the second week (Fig. 2D). Photographs of two eyes with recurrent disease after UV irradiation are shown in Figures 3B and 3C.

The remaining 29 latently infected mice had signs of UV irradiation injury, characterized by corneal epithelial ulceration and uveitis, but they did not show any signs of recurrent HSV-1 infection.

Virus Isolation and Antigen

Of the 74 latently infected mice, 30 shed virus in tears and 22 of the shedders subsequently showed development of stromal disease (Fig. 1). Fifteen mice had clinical signs of recurrent disease, but no virus was isolated from their tears. Viral shedding occurred between days 2 and 7 after UV irradiation, and the incidence peaked on days 3 and 4 when 16 and 17 mice, respectively, were shedding virus. There was no correlation between the amount or duration of viral shedding and the severity of the disease.

All 20 eyes used for immunohistochemistry had stromal disease and 16 had virus detected in the tears at some stage of the disease. Four eyes had signs of recurrent disease in the absence of virus in the tears. Of those 20 eyes, only three showed evidence of HSV-1 antigens—one on day 4 and two on day 7. These eyes included the only two that were shedding virus on the day that the eyes were processed. There was no correlation between the amount or duration of viral shedding and the severity of the disease.

Cytokine Staining

A total of 896 sections were examined under the microscope, and positively stained cells were counted (Fig. 4). In general, cytokine staining was confined to infiltrating cells, and the relative absence of staining seen in UV-irradiated control animals was related to the scarcity of infiltrating cells.

After UV irradiation, mice with recurrent stromal disease had an obviously larger cellular infiltrate compared with UV-irradiated control mice (Figs. 3E, 3D, 4A). A small number of cells in these control corneas stained positively for IL-6, IL-10, IL-12, and IFN-γ at some time points after UV irradiation (Figs. 3F, 4).

In contrast, corneas with recurrent stromal disease had large numbers of infiltrating cells, particularly in areas underlying epithelial ulcers and near the limbus (Fig. 3E). They were most numerous in the anterior stromal area but were also seen in the epithelium, particularly in HSV-1 antigen–positive areas and near the edges of ulcers. Although eyes with recurrent disease had increased cellularity of the iris, only one eye had HSV-1 antigen in the iris, and this was associated with a distinct focus of cells. Increased cellularity was also noted in the anterior chamber and between the endothelium and Descemet’s membrane.

In the corneas with recurrent stromal disease, the number of cells staining positively for IL-1β, IL-6, IL-10, IL-12, and IFN-γ was significantly greater than in control corneas (P < 0.05). Examples of such staining can be seen in Figures 3G and 3I through 3L, and the mean cell counts for these cytokines are shown in Figures 4B and 4E through 4H. With the exception of IL-1β, where there was significantly less staining on day 14 compared with earlier days (P < 0.05; Fig. 4B), the mean cell counts for the other cytokines investigated did not change significantly between different time points. IL-1β staining was also seen in central corneal epithelial cells, in both the infected and the control group, until day 10 but not on day 14 (data not shown).
There were very few IL-2+ (Fig. 3H) and IL-4+ cells in recurrent stromal disease, and numbers did not vary much with time and were not significantly different from control corneas ($P > 0.05$; Figs. 4C, 4D). In the corneas with recurrent stromal disease, there was no difference in the pattern of cytokine staining between those 16 mice that shed virus and the 4 mice that did not.

**DISCUSSION**

**Clinical Disease and Recurrent HSV-1**

UV irradiation was clearly responsible for the transient damage to the ocular tissues, which was at its worst between days 2 and 3 in the control animals. It seems likely that such trauma was important in inducing reactivation of latent virus in the trigeminal ganglion (TG) and the subsequent recurrent ocular infection.

The severe ocular signs seen clinically in those mice that had recurrent disease and the large cellular infiltrate that ensued after UV irradiation was not seen in control eyes. This response was likely to be the result of recurrent infection with HSV-1 and not the result of irradiation alone. This observation was supported by the fact that most of the eyes with recurrent disease had shed virus in tears.

However, in eight of the latently infected mice that shed virus, stromal disease did not develop. This may have been due to subclinical disease or an insufficient dose of virus infecting the cornea. Alternatively, recurrent infection of the conjunctiva or eyelid structures may have led to the shedding of small amounts of virus in the tears without the induction of stromal keratitis.

In contrast, the characteristic stromal disease of HSK developed in 15 of the latently infected mice in the absence of detectable virus in their tears. Viral shedding may have occurred between eye washings, or, alternatively, the virus may have infected the deeper layers of the epithelium without detectable surface shedding, as demonstrated in Figure 3L and by others.17

Compared with primary HSV-1 infection, transient and relatively low levels of virus and viral antigen were detected in recurrent disease. Together with the focal nature of the ocular lesions, these observations are consistent with our previous report that such ocular infection originates from reactivation of latency in very few neurons in the TG.18 However, even such a limited presence of virus in the cornea and/or iris appears sufficient to induce HSK, because much of the disease may result from rapid inflammatory responses in an animal with primed antiviral immunity.

**Cytokines in Recurrent Infection**

It has been shown that UV irradiation of mock-inoculated mice is associated with an increase in the number of F4/80+ and CD11b+ cells up to day 7.13 Furthermore, using the same model, it has been shown that there is an initial decrease in the number of Langerhans’ cells followed by an increase between 7 and 21 days after UV irradiation.17 In this study, we demonstrated that such mice had a small number of IL-6+, IL-10+, IL-12+, and IFN-γ+ cells in the stroma on days 4 and 14 after UV irradiation, as well as IL-1β staining of the epithelium up to day 10. The production of IL-1β by the epithelium and the other cytokines by perhaps macrophages and dendritic cells in the stroma, may contribute to the recruitment and activation of antigen-presenting cells, leading to a rapid innate immune response after recurrent infection of the cornea.

In contrast to the control animals, there were many more infiltrating cells in the corneas of mice with recurrent disease. The large numbers of IFN-γ+ and IL-12+ cells in those corneas and the scarcity of IL-4+ cells would suggest a Th1-type re-
sponse. In addition, the abundance of IFN-γ may exert a local antiviral effect, perhaps accounting for the low level of HSV-1 antigen. Moreover, this cytokine is recognized as a powerful potentiator of cell-mediated immune responses, particularly those of neutrophils and macrophages, which are known to be present in this model.15

However, there were very few IL-2+ cells, and IL-2 is a key cytokine in Th1 responses and in T-cell proliferation. Some studies have implicated IL-2 in HSK in the mouse primary infection model but none have demonstrated large quantities of this cytokine in the cornea.5,20–22 Therefore, in our study, despite the relative absence of IL-2 in the cornea, T-cell proliferation and IL-2 production could have been occurring in the regional lymph nodes or spleen at the height of recurrent HSK.

Large numbers of IL-1β+, IL-6+, and IL-10+ cells were also seen in the present study in mice with HSK. Both IL-1β and IL-6 are produced by many cell types, including lymphocytes and keratocytes, in response to infection, trauma, or immunologic challenge.19 They have both been regarded as proinflammatory cytokines with actions on both Th1- and Th2-type responses. However, the small number of B lymphocytes seen in recurrent HSK13 suggests that both IL-1β and IL-6 act as proinflammatory cytokines in a cell-mediated response rather than promote a humoral response. The reduction in IL-1β+ cells on day 14 appeared to correlate with resolution of the clinical signs and thus IL-1β may be a target for therapeutic intervention in the future.

IL-10, which was also seen in large numbers of cells, is considered to have anti-inflammatory properties and is characteristically associated with Th2 responses. This cytokine has been demonstrated during the resolution of HSK in the primary infection model,5 where it is thought to inhibit Th1 cytokines and produce a shift toward a Th2 type response. Moreover, IL-10 treatment reduces the severity of HSK in the primary model.23–25 The early appearance of IL-10 in true recurrent disease may reflect the fact that such disease involves a secondary, and therefore a more rapid, immune response than in primary infection. This immunomodulatory cytokine may contribute to the focal nature of the inflammation, possibly by inhibiting the actions of Th1 type T cells. However, there appears to be an overall proinflammatory cytokine balance.

Our observations suggest that recurrent HSK may not be characterized as a classic Th1 or Th2 response, a feature noted in several other viral infections.20 As with recurrent skin lesions, the relatively short duration, mild severity, and rapid

**FIGURE 3.** Clinical and histological photographs of UV irradiation in control and latently infected mice. (A) Control eye and (B) eye with recurrent HSK on day 4. Arrow: Area of stromal infiltration. (C) Recurrent HSK on day 7. Arrow: Area of lid disease. Anterior segment sections of the eyes stained with H&E on day 4 (D) control eye; (E) recurrent disease, showing a large stromal infiltrate. Corneal sections stained for cytokines; (F) IL-6 in control eye on day 7. Arrow: Single IL-6+ cell (brown); (G) IL-1β (brown) in recurrent HSK on day 4. (H) IL-2 in recurrent HSK on day 4. Arrow: Edge of ulcer staining positive for HSV-1 antigens (purple) and single IL-2+ cell (brown); (I) IL-6 (brown) in recurrent HSK on day 10. (J) IL-10 (brown) in recurrent HSK on day 10. (K) IL-12 (brown) in recurrent HSK on day 14. (L) IFN-γ (brown) in recurrent HSK on day 7. Arrow: HSV-1 antigen (purple) in the epithelium.
clearance of the virus in recurrent ocular disease is probably the result of a brisk secondary immune response. This may also explain the scarcity of viral antigen compared with primary infection. A broad-spectrum immune response, involving both Th1 and Th2 components, may be advantageous in producing both rapid clearance of the virus and restricted tissue damage. The latter would be particularly important in the cornea, where preservation of its transparency is paramount. The number, timing, and distribution of the cells staining positive for IL-1β, IL-6, IL-10, IL-12, or IFN-γ in this study suggests that these cytokines may be produced by the same cell type.
In a previous study we have shown that the neutrophil is the predominant and most rapidly infiltrating cell in recurrent HSK, and this was confirmed by H&E staining in the present study. Because the number, timing, and distribution of these infiltrating cells was very similar to the cytokine-positive cells, it seems possible that the neutrophils may be a source of IL-1β, IL-6, IL-10, IL-12, and IFN-γ, during recurrent HSK. Murine neutrophils have been reported to produce IL-10 and IL-12, and human neutrophils can produce IFN-γ. Our observations therefore support previous suggestions that neutrophils may be responsible for some of the cytokine production in HSK. We are now defining more precisely the role of such cells in cytokine production.

Acknowledgment

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References