Primary herpes simplex virus type 1 infection of the eye triggers similar immune responses in the cornea and the skin of the eyelids

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Herpetic stromal keratitis (HSK) and blepharoconjunctivitis in humans are thought partly to result from immunopathological responses to herpes simplex virus type 1 (HSV-1). The corneas of NIH mice were inoculated with HSV-1 (strain McKrae) and mice were examined for signs of disease and infection on days 1, 4, 7, 10, 14 and 21. The eyes and eyelids of infected and control mice were processed for immunohistochemistry and double stained for viral antigens and one of the following cell surface markers (Gr-1, F4/80, CD4, CD8, CD45R or MHC class II) or one of the following cytokines (IL-2, IL-4, IL-6, IL-10, IL-12 or IFN-γ). All infected mice developed signs of HSK by day 4 and blepharitis by day 7 and these both persisted until day 21, when signs of resolution were apparent. Virus was detected during the first week of infection and became undetectable by day 10. Large numbers of Gr-1+ cells (neutrophils) infiltrated infected corneas and eyelids in areas of viral antigen and CD4+ T cells increased significantly in number after virus clearance. In both sites, the predominant cytokines were IL-6, IL-10, IL-12 and IFN-γ, with few IL-2+ and IL-4+ cells. These observations suggest that the immune responses in the cornea are similar to those in the eyelids but, overall, the responses are not clearly characterized as either Th1 or Th2. In both sites, the neutrophil is the predominant infiltrating cell type and is a likely source of the cytokines observed and a major effector of the disease process.

Introduction

Herpes simplex virus type 1 (HSV-1) infections of the eye can trigger a potentially blinding disease known as herpetic stromal keratitis (HSK). The precise mechanisms of this disease are unclear but there is evidence for immunopathological processes that persist after the virus has apparently been cleared from the eye (Hendricks, 1999).

In addition, HSV-1 infection is frequently associated with blepharoconjunctivitis and eyelid ulcers in both humans and animal models (Egerer & Stary, 1980; Liesegang, 1989; Shimeld et al., 1990a). Evidence of HSV-1 infection has been demonstrated in the eyelids of mice as early as 40 h after corneal inoculation and persists there for at least 7 days thereafter (Shimeld et al., 2001). Also, viral antigens have been identified in the eyelids on day 5 after corneal inoculation but were no longer present on day 11 and beyond (Maggs et al., 1998).

Despite extensive research into the immune responses to HSV-1 in the cornea, particularly with respect to the development of HSK, much less is known about such responses in the eyelids.

T cell-deficient mice fail to develop HSK when infected with the virus (Metcalf et al., 1979); however, the ability to develop such keratitis was restored when exogenous, HSV-sensitized CD4+ T cells were transferred to these mice (Russell et al., 1984). Failure to develop HSK was also shown in a different model of T cell depletion. However, these mice developed severe periocular skin lesions (Hendricks & Tumpey, 1991). Both IL-2 and IFN-γ seem to be important in mediating T cell responses in the cornea and the skin (Hendricks et al., 1992; Inoue et al., 2001). In addition, both lymphocyte function-associated antigen-1 and intercellular adhesion molecule-1 play an important role in clearing HSV-1 infection in the eyelids but the functions of these adhesion molecules in HSK was less clear (Dennis et al., 1995).

From these observations, it seems that, in addition to the virus, the immune responses and CD4+ T cells in particular
Table 1. Scoring system for clinical signs of HSV-1 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 lid swelling and hair loss</td>
<td>Moderate lid swelling and hair loss</td>
<td>Severe lid swelling and hair loss</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Skin vesicles</td>
<td>Exudative crusts</td>
<td>Skin ulceration</td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>None</td>
<td>30% corneal ulceration</td>
<td>60% corneal ulceration</td>
<td>No corneal epithelium</td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>None</td>
<td>Mild infiltration</td>
<td>Moderate infiltration</td>
<td>No iris details visible</td>
<td></td>
</tr>
<tr>
<td>Uveitis</td>
<td>None</td>
<td>Mild iris hyperaemia</td>
<td>Moderate iris hyperaemia</td>
<td>Hypopyon and mydriasis</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Primary antibodies used for immunohistochemical staining of cell surface markers, cytokines and HSV-1 antigens

Some antibodies were not purified and the tissue culture supernatant (TCSN) was used instead. Control antibodies are also included.

<table>
<thead>
<tr>
<th>Antibody clone</th>
<th>Specificity</th>
<th>Species (isotype)</th>
<th>Concentration (dilution)</th>
<th>Source</th>
<th>Control tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB6-8C5</td>
<td>Gr-1 antigen (neutrophils, eosinophils vascular endothelium)</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>0.33 µg/ml (1:1500)</td>
<td>Pharmingen</td>
<td>Spleen</td>
</tr>
<tr>
<td>C1:A3-1</td>
<td>F4/80 antigen (macrophages, dendritic cells)</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>TCSN (1:20)</td>
<td>Serotec</td>
<td>Spleen</td>
</tr>
<tr>
<td>YE2/36</td>
<td>MHC class II (B cells, macrophages, dendritic cells, Langerhans cells)</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>TCSN (1:50)</td>
<td>Serotec</td>
<td>Spleen</td>
</tr>
<tr>
<td>RM4-5</td>
<td>CD44 antigen (T cells)</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>20 µg/ml (1:25)</td>
<td>Pharmingen</td>
<td>Spleen</td>
</tr>
<tr>
<td>KT15</td>
<td>CD8 antigen (T cells)</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>100 µg/ml (1:10)</td>
<td>Serotec</td>
<td>Spleen</td>
</tr>
<tr>
<td>RA3-6B2</td>
<td>CD45R/B220 antigen (B cells, NK cells, non MHC-restricted T cells)</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>0.66 µg/ml (1:300)</td>
<td>TCS/Caltag</td>
<td>Spleen</td>
</tr>
<tr>
<td>S4B6</td>
<td>IL-2</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>100 µg/ml (1:10)</td>
<td>Harlan Seralab</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td>BVD4-1D11</td>
<td>IL-4</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>33 µg/ml (1:15)</td>
<td>Pharmingen</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td>MP5-20F3</td>
<td>IL-6</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>66 µg/ml (1:15)</td>
<td>Cambridge Bioscience</td>
<td>HSV-infected eye</td>
</tr>
<tr>
<td>2A5</td>
<td>IL-10</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>3-3 µg/ml (1:300)</td>
<td>Harlan Seralab</td>
<td>Intestinal mucosa</td>
</tr>
<tr>
<td>C15.6</td>
<td>IL-12</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>100 µg/ml (1:10)</td>
<td>Biosource</td>
<td>HSV-infected eye</td>
</tr>
<tr>
<td>RP64</td>
<td>IFN-γ</td>
<td>Hamster IgG</td>
<td>20–66 µg/ml (1:30–100)</td>
<td>Harlan Seralab</td>
<td>HSV-infected eye</td>
</tr>
<tr>
<td>Polyclonal</td>
<td>HSV-1 antigens</td>
<td>Rabbit IgG</td>
<td>33·6 µg/ml (1:250)</td>
<td>Dako</td>
<td>HSV-infected TG</td>
</tr>
<tr>
<td>A110-1</td>
<td>IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>100 µg/ml (1:5)</td>
<td>Pharmingen</td>
<td>Multiple</td>
</tr>
<tr>
<td>B39.4</td>
<td>IgG&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>100 µg/ml (1:5)</td>
<td>Pharmingen</td>
<td>Multiple</td>
</tr>
<tr>
<td>A95-1</td>
<td>IgG&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Rat (IgG&lt;sub&gt;b&lt;/sub&gt;)</td>
<td>100 µg/ml (1:5)</td>
<td>Pharmingen</td>
<td>Multiple</td>
</tr>
<tr>
<td>Polyclonal</td>
<td>Hamster IgG</td>
<td>Hamster IgG</td>
<td>20–66 µg/ml (1:30–100)</td>
<td>Pharmingen</td>
<td>HSV-infected eye</td>
</tr>
</tbody>
</table>

play a significant role in the pathogenesis of stromal disease. However, while attenuation of these responses ameliorates corneal disease, such immune suppression exacerbates the severity of disease in the skin of the eyelids.

Much controversy still exists as to which cytokines play an important role in HSK. Evidence from one model of HSK using BALB/c mice and primary infection with HSV-1 strain RE supports a role for Th1-type CD4<sup>+</sup> T cells, with the expression of IL-2, IL-12 and IFN-γ (Babu et al., 1995; Kanangat et al., 1996). However, another group using the same strain of mice but the McKrae strain of virus found expression of IL-4 and IL-6, as well as IFN-γ, but no IL-2 or TNF-α, suggesting a Th2 polarization of the immune response (Ghiasi et al., 1995). In addition, Th2-type T cells have been shown to exacerbate stromal keratitis following primary infection with HSV-1 strain KOS in several strains of mice (Jayaraman et al., 1993).

Despite the importance of T cells in HSK, the neutrophil is the predominant infiltrating cell and is probably responsible for most of the tissue damage (Chen et al., 1996; Thomas et al., 1997). Polymorphonuclear cells have also been found in large
Immune responses to HSV-1 in corneas and lids

Methods

Animals. Specific-pathogen-free, 8-week-old female NIH/OLA inbred mice were anaesthetized by intraperitoneal injection of xylazine (10 mg/kg Rompun, Bayer) and ketamine (100 mg/kg Vetalar, Pharmacia & Upjohn). All animals were cared for in accordance with UK Home Office regulations for the use of animals in research.

Inoculation of mice with HSV-1. HSV-1 (strain McKrae) was grown and titrated on Vero cells. Before inoculation, mice were examined to exclude those with ocular pathology. The corneas of anaesthetized mice were inoculated by scarification with 5 µl of 10^4 p.f.u. HSV-1 and control mice were inoculated using mock samples of virus. Mice were examined by slit lamp for signs of ocular disease using the criteria outlined in Table 1. Clinical assessments were made immediately before inoculation and on days 1, 4, 7, 10, 14 and 21 thereafter. Eye washings were taken after each examination for virus isolation (Tullo et al., 1983).

On each of the above days, five mice with signs of disease and two control mice were sacrificed and had their eyes processed for immunohistochemistry. At the same times, the eyelids of some of these mice (12 infected and 6 control) were processed in a similar manner.

Since 47 mice developed viral encephalitis and were thus excluded from histological analysis, a further two eyes and eight eyelids were obtained from other similarly infected mice, from which no detailed clinical and virological observations were available.

Tissue fixation and immunohistochemistry. Tissues were fixed and processed as described previously (Whiteland et al., 1995, 1997). In brief, animals were perfused with periodate-lysine-parafomaldehyde buffer (PLP). Eyes were taken and perfused with PLP and the eyelids dissected before fixing the tissues overnight at 4 °C. The eyelids were then rapidly dehydrated in ethanol and Histoclear (National Diagnostics) and infiltrated under vacuum with low temperature wax. Serial 6 µm sections were cut and three sections were transferred to poly(lysine)-pre-coated glass microscope slides. A total of 150 serial sections were cut from each of the 30 diseased and 12 control eyes. The same number of sections was also cut from the eyelids of the 20 diseased and 6 control mice. Both tissue samples and sections were stored at -20 °C.

Immunohistochemical staining. For each eye, slides were stained in pairs and the tissue sections on the two slides were from two separate areas of the eye, approximately 450 µm apart. Pairs of slides from each eye were double stained for HSV-1 antigens and one of the following cell surface markers: Gr-1, F4/80, CD4, CD8, CD45R/B220 or MHC class II. Separate pairs of slides were also double stained for HSV-1 antigens and one of the following cytokines: IL-2, IL-4, IL-6, IL-10, IL-12 and IFN-γ. The clone, source and dilutions of the primary antibodies are listed in Table 2.

For the eyelids, three sections from each of the upper and lower eyelids were double stained for HSV-1 antigens and the cell surface markers as above. Eyelid sections were also stained for the cytokines listed above.

Sections were stained for cell surface markers and cytokines as described previously (Whiteland et al., 1995, 1997). In brief, after overnight incubation with the primary antibody, the following biotinylated secondary antibodies were used: goat anti-hamster IgG for IFN-γ and rabbit anti-rat IgG for the other cytokines and all the cell surface markers. Avidin-biotin peroxidase complex and DAB (brown stain) reagent were sequentially added (Vector). Between steps, sections were washed in PBS for the cell surface markers and 0.1% PBS-saporin for the cytokines. Positive and negative tissue controls as well as isotype antibody controls were included in each run.

numbers in HSV-1-induced blepharitis (Smith et al., 2000). In addition, it has recently been suggested that neutrophils may be the source of some of the cytokine production observed in HSK (Daheshia et al., 1998; Stumpf et al., 2001; Whiteland et al., 1997).

We now report a detailed morphological comparison of the nature of the immune response in the cornea and the skin of the eyelids following corneal infection with HSV-1. The characteristics of the infiltrating cells and the pattern of cytokine production in both these tissues are described. The quantitative immunohistochemical technique we used preserves the cell surface markers, cytokine and HSV-1 antigens in paraffin wax-embedded tissues and thereby provides good morphology in the tissue sections (Whiteland et al., 1995, 1997).
Fig. 2. For legend see facing page.
Following staining for cell surface markers (eyes and eyelids) and cytokines (eyes), slides were stained for HSV-1 antigens by sequentially incubating them with a rabbit anti-HSV-1 antiserum, swine anti-rabbit immunoglobulin and rabbit peroxidase-anti-peroxidase complex (Dako) before incubation with VIP reagent (purple stain) (Vector). Positive and negative control slides were always included. Double stained sections were counterstained with methyl green and the cytokine eyelid sections were counterstained with haematoxylin.

Microscopy and quantification. Stained cells were counted in the area of greatest infiltrate in the cornalian stroma or the subcutaneous tissues of the eyelids. The cells from two sections of each slide were taken in a grid area of 0.04 mm² at a magnification of 400x using the Quantimet image analysis system. Cell number was expressed as cells per area (cpa) and a maximum of 255 cells were counted for each area. Data are expressed as mean number of cells ± SEM. The Mann–Whitney test was used for statistical analysis and P < 0.05 was considered significant.

Results

Clinical disease

A total of 100 (88 infected and 12 control) mice were assessed clinically and eye washings were taken for virus isolation following inoculation with either HSV-1 or mock samples of virus. None of the control mice developed signs of disease following mock inoculation (Fig. 1).

On day 1 after inoculation with HSV-1, mice developed a mild uveitis and an epithelial ulcer, which affected approximately 30% of the corneal surface (mean clinical scores of 0.43 and 1.08, respectively) (Fig. 1a, c). By day 4, a stromal infiltrate was observed (mean clinical score of 1.24), which increased in severity up to day 14 (mean clinical score of 2.75) (Fig. 1b). Although a mild form of blepharitis was visible on day 4 in a small number of mice, significant lid disease was first noted on day 5, reached a peak on day 7 (mean clinical score of 2.45) and persisted to the third week (Fig. 1d). The severity of the lid and stromal disease after day 7 made subsequent clinical assessments of disease more difficult. By day 21, all clinical signs appeared to have resolved.

Virus isolation

Following inoculation with HSV-1, all 88 mice had virus isolated from their tears on days 1 and 4. By day 7, only 53.5% of mice were shedding virus and none shed virus thereafter. There was no correlation between the amount of virus shed and the severity of clinical disease or the chance of developing viral encephalitis. No virus was isolated from control mice.

Viral antigen

(i) In the cornea. Viral antigen was detected immunohistochemically in the epithelium of all corneas on day 1 (Fig. 2a, f). By day 4, four of five corneas had histological evidence of HSV-1 antigen (Fig. 2d), of which three also had viral antigen in the iris and/or ciliary body. On day 7, four of five eyes had HSV-1 antigen staining of the iris and ciliary body, with only two showing staining of the corneal and conjunctival epithelium. No viral antigen was detected in the cornea, iris or ciliary body after day 7.

(ii) In the eyelids. No viral antigen was visible in the eyelid tissues on day 1. However, antigen was seen in small areas of the conjunctival fornices in some eyes/eyelids at this time. On day 4, viral antigen was detected in all lids and was distributed at the epithelial surface, the base of the hair follicles and in the sebaceous glands (Fig. 2g). On day 7, the antigen was still visible in the deeper structures of the epidermis but the shedding of large areas of the epidermis markedly reduced the amount of viral antigen visible. No viral antigen was detected in the eyelids after day 7 or in mock-inoculated control eyes or eyelids at anytime.

Immunohistochemical staining for cell surface markers and cytokines

There was little variation in the number of stained cells (both cell surface marker- and cytokine-positive) at the different time-points in the mock-inoculated control eyes and eyelids. Consequently, an overall mean for each was used in the graphs for the control group.

Cell surface markers

(i) In the cornea. A small number of Gr-1⁺ cells (hereafter referred to as neutrophils) were seen in the corneas of mock-inoculated control mice 24 h after scarification (55 cpa) and the number dropped to between 0.5 and 1.41 cpa in subsequent days. Some F4/80⁺ cells were seen in the control corneas but the number of such cells did not vary significantly with time. Few other stained cells were seen in the control corneas at any time.

In contrast, HSV-1-inoculated corneas had a large and rapid infiltration of neutrophils and these were by far the most abundant cell-type present. They infiltrated the corneal stroma and were particularly associated with HSV-1 antigen-positive areas in the epithelium, which they also infiltrated (Fig. 2a).
Within 24 h, there were 112·4 cpa and this was followed by two peaks of infiltration, one on day 4 (200·9 cpa) and the other on day 10 (241·9 cpa) (Fig. 3a).

In contrast, F4/80+ cells did not start to increase significantly in the cornea compared to controls until day 7 (84·6 cpa) and reached a peak on day 14, (116·5 cpa) (Figs 2b and 3b).
Immune responses to HSV-1 in corneas and lids

Fig. 4. Mean number of cells ± SEM stained for various cytokines in the cornea (■) and eyelids (□) after HSV-1 inoculation of the cornea. (a) IL-2; (b) IL-4; (c) IL-6; (d) IL-10; (e) IL-12; (f) IFN-γ.

The number of MHC class II-positive cells also appeared to have two peaks of infiltration, which coincided with those of the neutrophils; a first peak on day 4 (27.5 cpa) and a second on day 10 (76.2 cpa) (Fig. 3c). Compared with the number of neutrophils, there were significantly less MHC class II+ cells. Interestingly, MHC class II expression was also seen on the
bulbar conjunctival epithelium and on the palpebral conjunctiva of the eyelids on day 7. This staining became less obvious on day 10 and was not seen again thereafter.

The number of CD4+ T cells was significantly greater than the controls at all time-points (P < 0.05). However, they were few in number until day 10, when they more than tripled from 23.4 CPA on day 7 to 74.5 CPA (Figs 2c and 3d). This rise in CD4+ cells coincided with the second peak of neutrophils and MHC class II+ cell infiltration.

The number of infiltrating CD8+ and CD45R+ cells remained relatively low throughout the experiment and most of the CD45R+ cells appeared to accumulate in the limbal area of the cornea.

(ii) In the eyelids. In contrast to the cornea, the control eyelid tissue had many resident F4/80+ and MHC class II+ cells but, as in the cornea, there were few neutrophils. There was no significant variation in the number of such cells over the study period.

Again, in contrast to the cornea, there was no change in the resident cell population in the eyelids within the first 24 h after corneal inoculation with virus. However, there was a massive rise in the number of neutrophils between days 1 and 4, when the cell numbers increased from 2.6 to 255 CPA (Figs 2g and 3a). This large cellular infiltrate preceded the clinical signs of eyelid disease. Large numbers of neutrophils were also seen on day 7. However, they became concentrated in areas of the epidermis which were being sloughed off (Fig. 2k). This probably led to the apparent reduction in the number neutrophils infiltrating the eyelid epidermis and dermis after day 7.

There was a small but significant rise in the number of F4/80+ cells between days 4 and 7 (P < 0.05), with a peak on day 10, (166–9 CPA) (Figs 2h and 3b). There was no significant change in the number of MHC class II+ cells in the dermis and epidermis following HSV-1 infection.

There was a gradual increase in the number of CD4+ T cells from 32-3 CPA on day 1 to 112-2 CPA on day 21 (Figs 2i and 3d). There was also an increase in the number of CD8+ T cells but this reached only 25.6 CPA on day 21 (Fig. 3e).

With the exception of day 7 (29.3 CPA), the number of CD45R+ B cells was not significantly different to the controls (P > 0.05).

Cytokine staining

(i) In the cornea. There were few cytokine-positive cells in control mock-inoculated corneas and the mean cell number ranged from 0.5 CPA stained positive for IL-2 on day 4 to 24.5 CPA stained positive for IFN-γ on day 1 after scarification.

Following HSV-1 inoculation, there were large numbers of cells staining positive for IL-6, IL-10, IL-12 or IFN-γ.

Although some IL-6-positive cells were seen on day 1 after inoculation (32.8 CPA), this was not significantly different from the controls (P > 0.05). However, by day 4, the number of such cells had increased significantly to 131.2 CPA (P < 0.05) (Figs 2d and 4c). Thereafter, this number ranged between 104.3 CPA on day 7 and 157.6 CPA on day 21.

Similarly, IL-10+, IL-12+ and IFN-γ+ cells were all present on day 1, reached a peak on day 4 and, after a small fall in numbers on day 7, reached a second peak on day 10 (Figs 2e, f and 4d–f). This pattern of staining, with peaks on days 4 and 10, was similar to the number, location and distribution seen with neutrophil staining; however, the number of cells stained for these cytokines was lower than the number of Gr-1+ cells.

There was no significant change in the number of cells staining positive for either IL-2 or IL-4 following HSV-1 inoculation.

(ii) In the eyelids. There were few cytokine-positive cells in control eyelids and the mean cell number ranged from 0 CPA for IL-2 to 20.75 CPA for IFN-γ, both on day 21.

Following HSV-1 inoculation, there were large numbers of cells staining positive for IL-6, IL-10, IL-12 or IFN-γ in the eyelids (Figs 2j–l). The peak of staining for these cells was on day 4, when the number of stained cells ranged between 83.5 CPA for IL-6 to 217.6 CPA for IL-12 (Figs 3c–f). The number of stained cells on day 7 was very similar for each of the cytokines and dropped markedly on day 10.

Similar to the staining observed in the infected cornea, the number, location and distribution of staining for IL-6, IL-10, IL-12 and IFN-γ in the eyelids was similar to the staining seen for neutrophils. However, as in the cornea, the number of cells stained for these cytokines was lower.

There was no significant change in the number of cells staining positive in the eyelids for either IL-2 or IL-4 following HSV-1 infection.

Discussion

Although epithelial scarification was used as a method of inducing HSV-1 infection, the epithelium of uninfected control mice healed within 24 h of scarification and their eyes were then indistinguishable from normal mouse eyes. In contrast, where scarification was performed in the presence of HSV-1, the mice developed characteristic lesions of herpetic eye disease, similar to those described previously (Shimeld et al., 1990a, b; Miller et al., 1996). However, unlike many other mouse/virus strain combinations, HSK became apparent as early as day 4 after inoculation and increased in severity until day 14. Although there was a small reduction in the number of some of the infiltrating cell types in the cornea on day 7, this was not evident clinically.

The severity of the uveitis peaked on day 4, which correlated with the appearance of viral antigen in the iris and ciliary body. In addition, the eyelid disease started on day 5 after corneal inoculation and was preceded 24 h earlier by viral antigens and massive cellular infiltration into the dermis and epidermis. As proposed previously (Shimeld et al., 1990a), the
timing and the limitation of these lesions to the neurosensory dermatome of the ophthalmic and maxillary nerves would suggest that the appearance of viral antigens beyond the inoculation site, at intra-ocular and extra-ocular sites, is most likely to have occurred by zosteriform spread of virus in the nerves supplying these structures. For such a spread to occur, it has been suggested that some degree of virus replication is necessary in the trigeminal ganglion (TG) and possibly in the CNS. This suggestion is supported by the lack of virus activity in the TG and periocular tissue following corneal inoculation of mice with a virus (a thymidine kinase-null mutant) known to be defective in its replication in nervous tissue (Summers et al., 2001).

Virus clearance from the eye and eyelid tissues after day 7, as shown by the absence of viral antigen, did not result in a reduction in the inflammatory responses. Indeed, these increased, even in the absence of detectable virus, for another week before showing any signs of resolution. With respect to the eyelid disease of those mice that survived beyond day 10, where the epithelium had healed, it remained hairless, suggesting that the hair follicles were destroyed in the inflammatory response, possibly through the epidermal shedding process.

As demonstrated by the nature of the infiltrating cells and cytokine production, the immune responses to HSV infection in the cornea and eyelids were similar. These responses were also similar to the responses seen in recurrent HSV-1 infection using the same virus and mouse strain combination, even though there was almost certainly a much smaller input of virus into the eye during recurrent disease (Shimeld et al., 1996; Stumpf et al., 2001). There was, however, a more rapid local immune response in recurrent disease, with significant stromal keratitis on day 4 after the reactivating stimulus (ultraviolet irradiation of the cornea), i.e. only 1–2 days after the likely arrival of the virus in the cornea. Such rapid response most likely results from the fact that recurrent disease occurs in an animal in which antiviral immunity is already primed. However, the number of cells in the cornea in recurrent infection was less than that in primary infection. This again may reflect the smaller antigenic challenge, which occurs in recurrent disease.

Following primary disease, there was a large and rapid infiltration of neutrophils in both the cornea and the eyelids. This observation in the cornea was different from other models, in which the first wave of neutrophil infiltration appears not to be clinically significant (Thomas et al., 1997). Such differences may relate to the particular virus and mouse strains used. The close association of neutrophil infiltration with HSV-1-positive areas suggests that these cells may be involved in the clearance of virus from the epithelium in the cornea and eyelid tissues. In the eyelids on day 7, large areas of infected epidermis were sloughed off together with their neutrophil infiltrates, leaving the underlying dermis exposed. This was followed by an infiltration of chronic inflammatory cells, such as macrophages and CD4+ T cells. Epithelial sloughing was also seen in the cornea but the tough collagenous structure of the stroma presumably helped to retain large numbers of cells within this tissue.

Our observations, like those of previous reports, suggest that the first peak of neutrophil infiltration into the cornea is driven by virus replication. In addition, as in previous studies, the second peak of neutrophils in the cornea on day 10 coincides with the increase in the number of CD4+ T cells (Chen et al., 1996; Thomas et al., 1997). Interestingly, the rise in such T cells was preceded by the expression of MHC class II antigens by the conjunctival epithelium, which may have contributed to T cell activation. In the eyelids, in contrast to the cornea, the number of CD4 T cells gradually increased from day 1 until the final day of observation, day 21.

Neutrophils do not normally express MHC class II molecules; however, the staining pattern for this marker closely resembled that of the neutrophils in the cornea, with peaks on days 4 and 10. It may be that the activated neutrophils in the cornea, unlike the eyelids, express MHC class II during HSK and therefore contribute to the persistence of inflammation. Such expression of MHC class II molecules on human neutrophils following in vitro culture (Radsak et al., 2000) and in other chronic inflammatory diseases, such as Wegener’s granulomatosis, has been reported previously (Hansch et al., 1999).

The large numbers of IFN-γ+ and IL-12+ cells in HSV-1-infected corneas and eyelids and the scarcity of IL-4+ cells would suggest a Th1-type response. IFN-γ is recognized as a powerful potentiator of cell-mediated immune responses, particularly those of neutrophils and macrophages (di Giovine et al., 1996) and the abundance of this cytokine may exert a local antiviral effect.

However, there were very few IL-2+ cells, a key cytokine in Th1 responses and in T cell proliferation. Some studies have implicated IL-2 in HSK but none have demonstrated large quantities of this cytokine in the cornea. For example, the amount of IL-2 mRNA detected in the cornea of HSV-infected mice was 10 times less than that for IFN-γ (Babu et al., 1995). In addition, relative to IFN-γ, the amount of IL-2 in the draining lymph node was much greater than in the cornea at the same stages of disease. Hence, in our study, despite the relative lack 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 disease. IL-2 was also detected in the supernatant of cells isolated from HSV-infected corneas but only after in vitro stimulation (Niemialkowski & Rouse, 1992). In a different model, passively administered anti-IL-2 antibodies reduced the severity of HSK (Hendricks et al., 1992). It was proposed that this effect was mediated by a reduction in the chemotactic gradient for neutrophils and by inducing their apoptosis (Tang et al., 1997). However, IL-2 production in the cornea was not measured directly.

Large numbers of IL-6+ and IL-10+ cells were also seen in
the present study in mice with HSK and eyelid disease. IL-6 is produced by cells, such as lymphocytes and keratocytes, in response to infection, trauma or immunological challenge (di Giovine et al., 1996) and has been regarded as a pro-inflammatory cytokine with actions in both Th1- and Th2-type responses. However, in the present study, the small number of B lymphocytes seen in the cornea and eyelids suggests that IL-6 is acting as pro-inflammatory cytokine in cell-mediated immunity rather than promoting a humoral response.

IL-10 was also found in large amounts, with a peak of positive cells as early as day 4, but this cytokine is considered to have anti-inflammatory properties and is characteristically associated with Th2 responses. This cytokine has been demonstrated by others, particularly during the resolution of HSK (Babu et al., 1995), when it is thought to inhibit Th1 cytokines and produce a shift towards a Th2-type response. Moreover, IL-10 treatment reduces the severity of HSK in the primary model (Chun et al., 1998; Tumpey et al., 1994, 1998).

The cytokine responses seen in the present study and in particular the number, location and distribution of cellular infiltration were very similar to those observed in our studies on recurrent disease (Stumpf et al., 2001). Therefore, both primary and recurrent infection with HSV-1 (strain McKrae) in NIH mice may not be characterized as either a classical Th1 or a classical Th2 response, a feature noted with several other virus infections (Biron, 1998). Such a broad-spectrum response, involving both Th1 and Th2 components, may be advantageous in producing both a rapid clearance of virus and restricted tissue damage. The latter is particularly important in the cornea, where maintenance of its transparency is paramount for the preservation of vision.

In contrast to these responses in the eye and eyelids, the situation in the TG described in previous reports is very different. In this part of the peripheral nervous system, the response to HSV-1 infection involves macrophages/dendritic cells and both T and B lymphocytes with very few neutrophils (Shimeld et al., 1995). In addition, there is a very different cytokine profile with the predominance of TNF-α and IL-6, some IFN-γ, IL-2 and IL-4 but no IL-10 (Shimeld et al., 1997). This suggests that infection with HSV-1 causes different immune responses in different tissues and this may be dependent on the response of resident cells to virus infection as well as the early innate response.

It is possible that much of the tissue damage in HSK and in lid disease is caused by the neutrophils, since they are present in such large numbers and are known to release potent tissue damaging enzymes. Moreover, under the pathological conditions of HSK, these cells may acquire a prolonged life, possibly driven by the cytokine environment. As the number, timing and distribution of neutrophils were very similar to the cytokine-positive cells in both the cornea and the eyelids, it seems possible that they were a source of IL-6, IL-10, IL-12 and IFN-γ. In support of this, murine neutrophils have been shown to produce IL-10 and IL-12 (Romani et al., 1997) and human neutrophils have been shown to produce IFN-γ (Yeaman et al., 1998). Our observations therefore strengthen previous suggestions that neutrophils may be responsible for cytokine production in HSK (Daheshia et al., 1998; Stumpf et al., 2001; Whiteland et al., 1997).

Several theories have attempted to explain the immunopathology of HSK but the fundamental enigma is the reason for the persistence of the immune response after the virus has been cleared. Although CD4+ T cells appear to play an important role in this process, it is not clear which antigens are responsible for their activation. It has recently been shown that mice genetically modified to possess T cells that only recognize a peptide derived from hen egg ovalbumin fail to respond immunologically to HSV-1. Predictably, these mice are highly susceptible to infection but can develop stromal keratitis (Gangappa et al., 2000). This suggests that mechanisms other than viral antigen recognition are at play and are sufficient to cause HSK. Furthermore, this implies a significant role for bystander activation of non-virus-specific CD4+ T cells, perhaps activated by an inflammatory milieu of pro-inflammatory cytokines in the cornea. In addition, the present study has shown that the immune responses in the eyelids are essentially the same as in the cornea and the inflammation persisted in both tissues following primary infection. This would suggest that the underlying stimulus is not, as suggested by some investigators (Avery et al., 1995; Zhao et al., 1998), an autoimmunity to corneal autoantigens but the pro-inflammatory milieu of the HSV-1-infected tissues and possibly the deregulation of the factors involved in inflammatory resolution.

In conclusion, we have shown a similar broad spectrum of immune reactions to HSV-1 infection in the eye and the skin, including aspects of Th1 and Th2 responses. This will need to be taken into account in prospective immunological therapy, particularly in the complex disease of stromal keratitis.

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References


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