Immune cell infiltration in corneas of mice with recurrent herpes simplex virus disease

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Reactivation of latent herpes simplex virus type 1 (HSV-1) infection was induced by UV irradiation of the corneas of latently infected mice. On days 1–4 after stimulation, infectious virus was sought in nervous and ocular tissue. On days 4, 7 and 10, eyes with either recurrent epithelial or stromal disease and appropriate controls were stained to identify immune cells and HSV-1 antigens. The maximum incidence of infectious virus was on day 2 when 5/10 ophthalmic parts of the trigeminal ganglion yielded HSV. Thus in this mouse model, as in humans, reactivation of virus in the trigeminal ganglion is the likely source of virus producing recurrent disease and shedding in the tear film. On day 4, when virus antigens were still present, granulocytes were the predominant infiltrating cell in corneas with either type of disease. Small numbers of T cells, dendritic cells and cells expressing MHC class II were also present. In stromal disease, the granulocyte infiltrate persisted and T cells remained sparse. In contrast, in epithelial disease, granulocyte numbers rapidly declined and both CD4+ and CD8+ T cells (present at a ratio of 1:1) increased significantly. The secondary immune response to virus antigen is more rapid and vigorous than that during primary corneal infection. Granulocytes may play a role in the initial clearance of virus, however, the other types of cells present early on provide the potential for a local secondary immune response. The high proportion of CD8+ cells in epithelial disease compared with stromal disease suggests that they may be acting as suppressors.

Introduction

Studies using a variety of animal models have provided valuable information on the pathogenesis of herpes simplex virus type 1 (HSV-1) disease. The relevance of such information to human disease is likely to be dependent, at least in part, on how accurately the models mimic infection in humans. We have developed a mouse model of recurrent corneal disease which mimics the clinical disease seen in humans (Shimeld et al., 1989, 1990a), however, to investigate how closely other features of HSV pathogenesis in this model resemble that seen in humans further characterization was necessary. Firstly, the source of reactivated virus which produces this disease was investigated. The trigeminal ganglion (TG) is thought to be the most likely source of virus in humans but there is evidence from humans and animals that the cornea itself may be persistently or latently infected with HSV-1 (Kaye et al., 1991; Cleator et al., 1994; Easty et al., 1987; Cook et al., 1991; O'Brien & Taylor, 1989). Although such a source cannot be precluded in the mouse model, we show that, as in man, reactivation of virus in the TG is the most likely source of virus to produce recurrent disease and shedding of virus in the tear film. Secondly, the model uses passive immunization prior to primary infection; this is essential in the preparation of animals suitable for the induction of recurrent disease. The presence of antibodies to HSV-1 at the time of infection may be a disadvantage of the model since such passive immunity may interfere with the developing active immune response. In this study, we show that passive immunization does not affect the ability of mice to mount a delayed type hypersensitivity (DTH) response; an immune reaction thought to be of particular importance at sites of local recurrence of antigen.

During primary infection the host encounters HSV antigens for the first time and following reactivations of latent infection there are repeated exposures to antigens. These secondary immune responses differ from those that occur during primary infection in that they are usually more rapid and more vigorous. Such differences are associated with phenotypic changes in lymphocytes that have had previous contact with the antigen (Springer, 1990). In humans with recurrent herpetic corneal infection, the immunological response is thought

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to play a major role in the development of stromal disease. Although the primary immune response to virus in the cornea has been well studied (Doymaz & Rouse, 1992) investigations of a secondary response in this tissue have, until recently, been hindered by lack of a suitable animal model.

In the model we have developed, animals show either a mild epithelial infection which resolves, or a more severe stromal keratitis where corneas remain opaque and vascularized. The main aim of this study was to use a model for a detailed quantitative immunohistochemical study, using monoclonal antibodies to identify the immune cells which infiltrate the corneas, in both types of recurrent herpetic disease. Our immunohistochemical investigation was performed on fixed paraffin wax-embedded eyes using a newly developed method which allows the simultaneous preservation of antigen in combination with excellent tissue morphology (Whiteland et al., 1995).

Methods

Production of latently infected mice. Specific pathogen-free, 8-week-old female NIH/ OLA inbred mice were obtained from Harlan/Olac (Bicester, Oxford, UK) and were maintained as a breeding colony in the Department of Pathology and Microbiology. Anaesthetized mice (Shimeld et al., 1995) were inoculated by scarification of the left cornea with a 26G needle (Tullo et al., 1983) through a 5 µl drop of medium containing 1 x 10^6 p.f.u. of HSV-1 strain McKrae (Williams et al., 1965). Control mice were inoculated in the same way with a preparation of uninfected Vero cells made in the same manner to the virus inoculum (mock inoculum). Twenty-four hours before inoculation with virus, animals were inoculated intraperitoneally (i.p.) with 0.5 ml of rabbit serum containing antibodies to HSV-1 (Shimeld et al., 1995). The serum was diluted with PBS to give a dose of 8000 ED~0. On occasions, mice were mock immunized by an i.p. injection of normal rabbit serum diluted 1:100 in PBS (all sera were from Dakopatts, High Wycombe, UK) and were maintained as a breeding colony in the Department of Pathology and Microbiology. Anaesthetized mice (Shimeld et al., 1995) were inoculated by scarification of the left cornea with a 26G needle (Tullo et al., 1983) through a 5 µl drop of medium containing 1 x 10^6 p.f.u. of HSV-1 strain McKrae (Williams et al., 1965). Control mice were inoculated in the same way with a preparation of uninfected Vero cells made in the same manner to the virus inoculum (mock inoculum). Twenty-four hours before inoculation with virus, animals were inoculated intraperitoneally (i.p.) with 0.5 ml of rabbit serum containing antibodies to HSV-1 (Shimeld et al., 1995). The serum was diluted with PBS to give a dose of 8000 ED~0. On occasions, mice were mock immunized by an i.p. injection of normal rabbit serum diluted in a similar manner. Only mice that survived primary infection were used for reactivation of latent infection.

Reactivation of latent infection. At least 30 days after corneal inoculation, mice were anaesthetized and the left cornea and lids treated with UV irradiation as described previously (Shimeld et al., 1995). Chloramphenicol eye ointment (Daniels Pharmaceutical, Derby, UK) was applied immediately after irradiation and daily thereafter for 10 days.

Dissection of tissues. For immunohistochemical studies mice were killed and their left eyes removed. Spleens were removed from some animals. For studies on the isolation of infectious virus reactivated in vivo, mice were killed and a 2 mm diameter disc was trephined from the left cornea. By microdissection the remainder of the eye was divided into two parts, the anterior part contained the corneal scleral rim, iris and ciliary body, the posterior contained retina, choroid and sclera. The following tissues were then removed from the left side: the upper and lower eyelids, the superior cervical ganglion (SCG) and the three parts of the TG: ophthalmic (TG1), maxillary (TG2) and mandibular (TG3) (Tullo et al., 1982).

Isolation of infectious virus from tissues and eyewashings. Tissues were each ground in 0.5 ml of medium and then frozen and thawed three times to disrupt the cells. The resulting cell-free suspensions were put onto a monolayer of Vero cells in 25 cm² flasks and incubated at 37 ºC in 5% CO₂ until cytopathic effect was seen, or for 7 days. After slit-lamp examination, 20 µl of medium was irrigated and aspirated ten times on the left eye and immediately put onto Vero cells for the isolation of virus (Tullo et al., 1982).

Clinical examination and classification of recurrent disease. Mice were anaesthetized and the cornea, iris and lids examined for signs of disease using a slit-lamp microscope. In accordance with the results of preliminary studies, recurrent corneal disease in mice was classified into two types: (i) epithelial disease with discrete focal epithelial ulceration (which could be punctate or dendritic) underlaid by slight corneal opacification through which the iris was visible; or (ii) stromal disease with larger areas of ulceration and opacification often involving the entire cornea with a density sufficient to obscure part or all of the iris. Iris hyperaemia occurred with both types of disease.

Measurement of DTH responses. DTH responsiveness was determined using the ear swelling assay. Antigen was prepared in PBS, from a UV-inactivated pool of HSV-1 strain McKrae produced in Hep2 cells. Mock antigen was prepared from uninfected Hep2 cells in a similar manner. Mice were challenged by an intradermal inoculation of the dorsal surface of the right pinna of 10 µl containing 3 x 10^4 - 10^5 p.f.u. Each left pinna was inoculated in a similar manner with mock antigen. Ear thickness was measured before and 24 hours after challenge using a Mitutoyo engineer's micrometer. Results are expressed as ear swelling of right (antigen) ear minus ear swelling of left (mock antigen) ear in units of 10^-3 mm.

Fixation and processing of tissue for immunohistochemistry. Tissues were fixed and processed as described previously (Whiteland et al., 1995). In brief, using a 30 gauge needle, two holes were made in the back of the eye and through one of these periodate-lysine-paraformaldehyde (PLP) (Whiteland et al., 1995) was gently injected until the fixative flowed out of the other hole. Spleens were cut into 2 mm slices. Tissues were then fixed overnight in PLP at 4 ºC, rapidly dehydrated and infiltrated under vacuum with low temperature wax. Serial 6 µm sections were cut and transferred to glass microscope slides precoated with poly-L-lysine (three sections per slide).

Immunohistochemistry

(i) Staining for HSV-1 antigens. Sections were stained by the peroxidase-anti-peroxidase method in sequence: 3% hydrogen peroxide, 20% normal swine serum, rabbit anti-HSV-1 serum diluted 1:500 in PBS containing 0.1% BSA, swine anti-rabbit immunoglobulin diluted 1:100 in PBS, rabbit peroxidase-anti-peroxidase complex diluted 1:100 in PBS (all sera were from Dakopatts, High Wycombe, UK) and diaminobenzidine (DAB) (Sigma). Slides were incubated with the primary antibody at 4 ºC overnight and with the other antibodies at room temperature for 30 min. Sections were washed twice in PBS between steps. Negative control slides were incubated with diluent instead of primary antibody and were included in each staining run. Sections were lightly counterstained with haematoxylin.

(ii) Staining of leukocytes and MHC class II. Slides were stained as described previously (Whiteland et al., 1995). In brief, endogenous peroxidase was blocked by 0.3% hydrogen peroxide and some sections were digested with trypsin, depending on the antibodies (Table 1). Non-specific binding sites were blocked by 1:5% normal rabbit serum and sections incubated with primary antibody overnight at 4 ºC. The clones, specificity, source and dilutions of the monoclonal antibodies used are listed in Table 1. The slides were then incubated with biotinylated rabbit anti-rat IgG, followed by avidin-biotin peroxidase complex (ABC) and DAB. The primary and secondary antibodies were diluted in PBS containing 0.1% BSA and the ABC complex in PBS. Sections were washed twice in PBS between steps; except after...
Table 1. Monoclonal antibodies used in the study

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specificity</th>
<th>Form*</th>
<th>Dilution</th>
<th>TT†</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>G7</td>
<td>Thy 1 (T cells, NK cells, neurofilaments)</td>
<td>pur</td>
<td>1:250</td>
<td>0</td>
<td>PharMingen, San Diego, USA</td>
</tr>
<tr>
<td>YCD3-1</td>
<td>CD3</td>
<td>pur</td>
<td>1:100</td>
<td>5</td>
<td>Gibco BRL, Perth, UK</td>
</tr>
<tr>
<td>RM4-5</td>
<td>CD4 (on T cells)</td>
<td>pur</td>
<td>1:20</td>
<td>5</td>
<td>PharMingen</td>
</tr>
<tr>
<td>KT15</td>
<td>CD8 (on T cells)</td>
<td>sn</td>
<td>neat</td>
<td>10</td>
<td>Serotec, Oxford, UK</td>
</tr>
<tr>
<td>3C7</td>
<td>CD25 (activated T and B cells)</td>
<td>pur</td>
<td>1:15</td>
<td>5</td>
<td>PharMingen</td>
</tr>
<tr>
<td>RA3-6B2</td>
<td>CD45R (B220 antigen on B cells, NK cells and non-MHC-restricted T cells)</td>
<td>pur</td>
<td>1:200</td>
<td>0</td>
<td>Bradsure Biologicals Ltd, UK</td>
</tr>
<tr>
<td>YE2/36</td>
<td>Class II MHC</td>
<td>sn</td>
<td>1:50</td>
<td>5</td>
<td>Serotec</td>
</tr>
<tr>
<td>C1:A3-1</td>
<td>F4/80 antigen (macrophages and dendritic cells)</td>
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<td>1:20</td>
<td>5</td>
<td>Serotec</td>
</tr>
<tr>
<td>M1/70</td>
<td>CD11b (macrophages and granulocytes)</td>
<td>sn</td>
<td>1:10</td>
<td>5</td>
<td>Serotec</td>
</tr>
<tr>
<td>RB6-8C5</td>
<td>Gr-1 antigen (granulocytes, vascular endothelium and erythrocytes)</td>
<td>pur</td>
<td>1:1500</td>
<td>5</td>
<td>PharMingen</td>
</tr>
</tbody>
</table>

* pur = purified, sn = supernatant.
† TT, Optimal digestion time (min) for sections with 0.0025% trypsin in 0.1% CaCl₂.

digestion in trypsin when they were washed three times. Positive control slides were sections of spleen stained in a similar manner. Negative control slides were incubated with diluent instead of primary antibody. Sections were lightly counterstained with haematoxylin. All sera and ABC reagents were from Vector, Peterborough, UK.

Quantification of cells stained with monoclonal antibodies. Cells were counted in the area of maximum staining in the stroma of the cornea using a × 40 objective lens in two grid areas of 0.04 mm² on each of two sections. On occasions, Gr-1⁺ and CD11b⁺ cells were too numerous to count and were assigned a score of 200 cells/grid area (cga) (200 was the minimum number judged to be present), transformed cell count (tcc) 14-1.

Statistics. Normal probability plots showed that the data did not conform to a normal distribution, and the variances of different groups differed widely. Square root transformation resolved both of these features and allowed comparisons by analysis of variance. Multiple unplanned comparisons were made by the method of Tukey (Snedecor & Cochran, 1967); the level of significance was set at 5%. Fig. 1 is plotted with the transformed data. Student's t-test was used to assess the significance in the DTH experiment.

Results

DTH response

Seventeen mice were passively immunized and 30 were given normal rabbit serum diluted in a similar manner to the serum containing HSV-1 antibodies (mock immunized). Twenty-four hours later, animals were inoculated on the cornea with 10⁴ p.f.u. of HSV-1 strain McKrae. Eye disease was seen in 28/30 mock-immunized mice and 4/17 passively immunized animals. The latter four animals were excluded from the experiment since such disease makes them unsuitable for reactivation experiments (Shimeld et al., 1990a). Two months after inoculation of the cornea, mice were challenged with antigen and mock antigen. There was no significant difference in the DTH response between passively immunized and mock-immunized mice (P < 0.297); ear swelling (10⁻² mm ± SEM) was 9.7 ± 4.5 in passively immunized and 12 ± 3.9 in mock-immunized animals.

Isolation of infectious virus from tissues following reactivation of latent infection

Forty-seven days after primary infection, 39 latently infected mice were treated with UV irradiation; eyewashings were taken for the isolation of virus before irradiation and afterwards daily for 4 days. Groups of 9 or 10 mice were killed on days 1, 2, 3 and 4 and their tissues removed for the isolation of virus. Virus was first isolated from TG2 and the anterior part of the eye of 1/10 mice on day 1 (Table 2). The maximum incidence of isolation was on day 2 when 5/10 TG1, 1/10 corneas, 2/10 anterior parts, 2/10 posterior parts and 1/10 lids yielded virus. Virus was not isolated from TG3 or SCG at any time. Two of 29 eyewashings on day 2 and 3/19 on day 3 yielded virus. Virus was not isolated from eyewashings taken on day 1 or day 4 or before UV irradiation.

Investigation of mice with recurrent eye disease

One hundred and nineteen latently infected mice and 19 mock-inoculated animals were treated with UV irradiation; eyes were examined immediately before treatment and eyewashings were taken at this time and daily for 10 days. Twenty latently infected mice showed recurrent epithelial disease, 9 had stromal disease and 17
developed recurrent lid disease (as previously described; Shimeld et al., 1990a). On the following days after reactivation mice with recurrent disease were killed and their tissues removed for immunohistochemical staining: day 4 (4 with epithelial disease and 2 with stromal disease); day 7 (3 with epithelial disease and 3 with stromal disease); day 10 (3 with epithelial disease and 4 with stromal disease). Three mock-inoculated animals were killed on each of these days. All mice with recurrent disease had shed virus in their eyewashings. Serial sections were cut and every 11th slide was stained by haematoxylin and eosin and every adjacent slide for HSV-1 antigens. Blocks of slides covering the area within the cornea with the maximum number of infiltrating cells were selected and stained for immune cells. The eyes from three latently infected mice untreated with UV irradiation were processed in a similar way. Blocks of serial sections from the central corneas of three eyes from uninfected mice were stained to identify resident immune cells.

Clinical signs and shedding of virus in the tear film

The eyes from the uninfected mice and from the latently infected animals untreated with UV irradiation appeared normal. The eyes from mock-inoculated mice treated with UV irradiation developed temporary mild epithelial ulceration, corneal oedema and iris hyperaemia as described previously (Shimeld et al., 1989). By day 10, all eyes had very slight corneal vascularization and one had a large area of corneal haze. In contrast, animals with recurrent corneal disease developed well-defined epithelial ulcers. These were accompanied by infiltration (varying from slight and focal to heavy and widespread) and marked iris hyperaemia.

Virus was not isolated from eyewashings taken before UV irradiation. 58/119 (49 %) of latently infected mice shed virus in eyewashings on at least one occasion. Analysis of the eyewashings from the eyes used in the immunohistochemical study showed that the day when virus was first shed after UV irradiation varied; 7/19 on day 2, 5/19 on day 3 and 7/19 on day 4. The duration of shedding also varied from 1–5 days and the amount from 2 to > 100 p.f.u. There was no correlation between the amount of virus, the duration of shedding or the timing of appearance of virus in eyewashings with the severity of eye disease.

Immunohistochemical staining

(i) HSV-1 antigens

Virus antigens were found in eyes (6/6) taken on day 4: 1/6 in the conjunctival epithelium and the others (5/6) in the corneal epithelium. Three of the five eyes had more than one focus of infected corneal epithelial cells and areas of staining varied in size from 1–2 cells to 10–20 cells and were often distant from each other. Two eyes of the five also had plaques of virus antigens in the iris stroma. No virus antigen was seen in the corneal stroma. There did not appear to be any difference between eyes with epithelial or stromal disease in the number or size of foci of virus antigen. No virus antigens were seen in eyes with disease taken on days 7 and 10 or in eyes from mock-inoculated animals.

(ii) Immune cells

In uninfected animals, the only population of immune cells in the cornea (2 cga) were process bearing, F4/80+ (macrophages and dendritic cells) scattered throughout the stroma with a slightly denser distribution in the anterior part of this tissue. An occasional stained cell was seen in the corneal epithelium near the limbus. A focus of immune cells was present at the limbus; these cells were

Table 2. Isolation of virus from tissues and eyewashings following UV irradiation of latently infected mice

<table>
<thead>
<tr>
<th>Day after UV irradiation</th>
<th>TGI*</th>
<th>TG2</th>
<th>TG3</th>
<th>SCG</th>
<th>C</th>
<th>A</th>
<th>P</th>
<th>L</th>
<th>Eyewashings</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0/10</td>
<td>1/10</td>
<td>0/10</td>
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<td>0/10</td>
<td>0/10</td>
<td>0/9</td>
<td>0/39</td>
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<tr>
<td>2</td>
<td>5/10</td>
<td>1/10</td>
<td>0/10</td>
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<td>1/9</td>
<td>2/9</td>
<td>0/9</td>
</tr>
</tbody>
</table>

* Only small amounts of virus were isolated from nervous tissue, an average of 21 p.f.u. and a maximum of 52 p.f.u. per sample. On occasions, much larger amounts of virus (> 100 p.f.u.) were isolated from all the types of peripheral tissue tested.
mostly F4/80+ with lesser numbers of Gr-1+ cells (granulocytes), CD11b+ cells (macrophages and granulocytes), CD3+ or Thy-1+ cells (henceforth referred to as 'T cells') and an occasional cell expressing MHC class II. Apart from a rare T cell, F4/80+ cells were the only immune cells seen in the iris.

In corneas from latently infected non-UV-irradiator mice taken at least 3 months after corneal inoculation, two to three small foci of infiltrating cells were detected. These foci were in the anterior stroma and contained: T cells (26 cga) of which the great majority were CD4+ cells, CD11b+ cells (macrophages and granulocytes) (12 cga), F4/80+ cells (11 cga) MHC class II-expressing cells (14 cga) and Gr-1+ cells (2 cga). Apart from these foci, the number and distribution of stained cells in corneas from latently infected mice were similar to that in uninfected animals.

Four days after UV irradiation of mock-inoculated mice there was an increase in the number of immune cells at the limbus and in the cornea compared to uninfected animals. In the cornea, immune cells were evenly distributed and the majority were either F4/80+ cells (14 cga) (Fig. 1b) or CD11b+ cells (15 cga) (data not shown). A small number of Gr-1+ cells were seen in the cornea (Fig. 1a) and in the anterior chamber. Seven days after UV irradiation, the number of immune cells in the cornea had increased: F4/80+ cells (25 cga), CD11b+ cells (22 cga) and Gr-1+ cells (7 cga). At this time corneas contained a small number of T cells (tcc 1.079, equivalent to 2 cga) (Fig. 1c), Ten days after UV irradiation, there was a decrease in the numbers of infiltrating cells. Compared to day 7, the numbers of Gr-1+ cells had decreased significantly (P = 0.005) and there were no T cells or cells expressing MHC class II. The numbers of CD11b+ cells had fallen to 7 cga and F4/80+ cells to 17 cga.

In corneas with epithelial disease, the highest number of infiltrating cells were found in one or more discrete foci in the anterior part of the stroma. These foci varied in position throughout the cornea. Even stroma distant from foci appeared to have more infiltrating cells than were seen in the mock-inoculated controls. In eyes with stromal disease, large areas of the cornea were heavily infiltrated and stained cells were often present throughout the entire depth of the stroma.

With reference to specific cell types, on day 4, in eyes with both types of disease, large numbers of Gr-1+ cells had infiltrated into corneas (Fig. 1a, Fig. 2a). These were distributed throughout the entire tissue but were most dense in the anterior stroma under epithelial lesions where virus antigens were identified (137 cga in eyes with epithelial disease and 200 cga for stromal disease). Gr-1+ cells were often seen in the anterior chamber and on the endothelium, particularly under lesions. By day 7, in eyes with epithelial disease, there was a significant decrease (P = 0.0001) in the number of Gr-1+ cells in the stroma (3 cga) and this decrease was maintained on day 10. In contrast, in eyes with stromal disease, the numbers of granulocytes remained high on days 7 and 10 (116 cga). There was a significant difference between corneas with epithelial and those with stromal disease in the numbers of Gr-1+ cells (P = 0.002). Staining for CD11b+ cells showed similar numbers and distribution to that for Gr-1+ cells (data not shown) which suggests that most of the CD11b+ cells were granulocytes rather than macrophages.

At all time points investigated there were no statistically significant differences in the numbers of F4/80+ cells between controls and eyes with either type of disease (Fig. 1b). However, diseased corneas always had slightly higher numbers of these cells compared to controls.

There were significant differences, dependent on time, between mock-inoculated controls and corneas with epithelial disease but not between these controls and corneas with stromal disease in the numbers of T cells (P = 0.041) (data not shown), CD4+ cells (P = 0.01) (Fig. 1e) and CD8+ cells (P = 0.005) (Fig. 1f). On day 4, there were small numbers of T cells (tcc 1.77, equivalent to 3 cga) present in the areas of maximum infiltration in corneas with both epithelial and stromal disease. By day 10, in corneas with epithelial disease there was a significant (P = 0.023) increase in the total number of T cells (tcc 6.89, equivalent to 52 cga) and in CD4+ cells (tcc 4.96, equivalent to 28 cga) (P = 0.016) and CD8+ cells (tcc 5.37, equivalent to 31 cga) (P = 0.004) (Fig. 2b) compared to day 4. In contrast, in corneas with stromal disease, although the numbers of both types of T cells increased these rises were not significant. T cells were present in foci in the stromas of diseased corneas and were also frequently identified in the corneal epithelium above these foci.

There was a significant difference, dependent on time, between controls and corneas with epithelial disease but not between controls and corneas with stromal disease, in the numbers of cells expressing MHC class II (Fig. 1c). On day 4, very small numbers of cells expressing MHC class II, were seen in corneas from diseased and control mice (such cells were absent from this tissue in uninfected animals). By day 7, there were more of these cells in all tissues tested. On day 10, the number of cells expressing MHC class II had significantly increased in corneas with epithelial disease (P = 0.007) and significantly decreased in mock-inoculated corneas (P = 0.028) compared to day 4. In diseased and mock-inoculated corneas, at all time points tested, only very small numbers of CD45R+ cells (B cells, NK cells and non-MHC-restricted T cells) were seen (Fig. 1d).
Fig. 1. Infiltration of immune cells into corneas with recurrent HSV disease. Cells expressing Gr-1 (a), F4/80 (b), MHC class II (c), CD45R (d), CD4 (e) and CD8 (f) in the corneal stroma of mice with epithelial (black bars) or stromal (grey bars) recurrent herpetic disease or in mock-inoculated mice (white bars). Transformed cell count = square root of cell count (per mm² x 10³). Significant decreases in Gr-1⁺ cell counts were observed between day 4 and days 7 and 10 (P = 0.001) in mice with epithelial disease. Significant decreases in Gr-1⁺ cell counts were observed between day 10 and days 4 and 7 (P = 0.005) in mock-inoculated mice. Significant increases in cell counts for MHC class II expression were observed between day 4 and day 10 (P = 0.002) in mice with epithelial disease and a
Discussion

The timing of appearance and incidence of isolation of virus from the TG suggests that this ganglion is the most likely source of reactivated virus to produce recurrent ocular disease and shedding in the tear film. This confirms our previous studies in non-immunized animals (Shimeld et al., 1990a), a study in passively immunized mice (Laycock et al., 1991) and similar studies of induced reactivation in the cutaneous model of recurrent herpetic disease (Harbour et al., 1983). TG1, which contains the neurons which supply the cornea, yielded the highest incidence of virus. Isolation from TG2 was not surprising since this part of the ganglion also harbours latently infected neurons (Shimeld et al., 1990b). Reactivation in this part was to be expected since the lower eyelid, in addition to the cornea, was irradiated with UV and the eyelid is within the area innervated by TG2. Our failure to isolate virus from the SCG suggests that this tissue is not a significant source of recurrent virus in the eye.

The most probable route of spread of virus reactivated in trigeminal neurons is within the axons of ocular nerves; such nerves have stained for virus antigen after reactivation (Shimeld et al., 1989). Hence, this is the most likely source of virus isolated from posterior segments of eyes, although we cannot exclude reactivation of virus in the ciliary ganglion. Similarly infected nerves in anterior segments may be responsible for the isolates from this tissue. However, iris tissue is another likely source, since virus antigen was detected in this tissue, in this study and in previous work (Shimeld et al., 1989).

Areas within the corneas which contained the maximum number of infiltrating cells were chosen for detailed immunohistochemical study. This was particularly important for corneas with recurrent epithelial disease and those taken from animals prior to UV irradiation, since infiltrates were focal and often surrounded by almost normal tissue; a less scrupulous approach would have missed these foci.

In contrast to normal dermis of the ear (S. Manickasingham, personal communication) and normal rat corneal stroma (F. Figueirêdo, personal communication), MHC class II expression was not detected in normal mouse corneas, (although a few cells were seen at the limbus) suggesting that there may be a delay in antigen presentation at this site. The dose of irradiation used in this study produced temporary and slight damage to the cornea. However, such damage probably results in the release of mediators of inflammation since the number and types of immune cells were greater in corneas from irradiated uninfected animals in comparison to those resident in the normal mouse cornea.

Corneas from latently infected mice, taken before UV treatment, were similar to those from normal animals, with the exception of small foci containing a variety of immune cells. From their number and distribution it is likely that these foci are related to the small epithelial ulcers which were present during the acute infection; the predominant disease following inoculation of the cornea.
with virus in passively immunized mice (Shimeld et al., 1990b). This suggests long term persistence of immune cells in the cornea following recovery from the primary infection; a phenomenon also reported in the latently infected ganglion (Shimeld et al., 1995).

In all corneas with recurrence of herpetic disease (epithelial or stromal) there was a similar rapid infiltration of granulocytes. Numbers far greater than in UV-treated controls had infiltrated by day 4, when virus antigen was still present in the eyes. Such infiltrates occur, together with antigen, following reactivation of virus after corneal transplantation in rats (Nicholls et al., 1996). Hence, granulocytes may play a role in the initial clearance of virus. However, the small numbers of T cells (which are probably memory cells (Mackay et al., 1990) and specific for the virus) together with MHC class II-expressing and F4/80+ cells (mostly dendritic) would provide the potential for a local secondary immune response. In a preliminary study in NIH mice, others have demonstrated large numbers of Mac 1+ macrophages in corneas 3 days after reactivation and the presence of Thy 1.2+ cells on day 10 (Miller et al., 1993).

In our study, the granulocyte response was earlier than that described in a variety of strains of immunocompetent mice with stromal keratitis induced by primary infection. During such infection, granulocytes were absent from the cornea on day 4 (Opremcak et al., 1990) and the maximum numbers were not detected until days 12–21 (Opremcak et al., 1990; Russel et al., 1984; Wang et al., 1988). The speed of the non-specific immune response in recurrent disease may appear to be unusual, particularly because evidence from eyewashings and immunohistochemistry suggest that there is considerably less virus antigen in these corneas compared to that found in primary infection. However, the early involvement of granulocytes may reflect the nature of the immune response in mice, since in these animals, in some circumstances, such cells play a vigorous role in DTH reactions (Crowle, 1975). Such responses are likely to be important at sites of recurrence of HSV antigen in the cornea and, as we have shown, the mice used in these studies, even though passively immunized, are able to mount an active response of this kind in the skin.

When antigen had been cleared, there were substantial differences in the immune cell infiltrate between corneas with the two types of disease. In epithelial disease the number of granulocytes had fallen dramatically to levels equivalent to those seen in controls and there was a significant rise in the number of T cells (CD4+ and CD8+) and cells expressing MHC class II. In contrast, large numbers of granulocytes remained in corneas with stromal disease and the levels of T cells and MHC class II expression did not increase significantly. Persistence of these granulocytes may inhibit the infiltration of other immune cells, for example, via the production of cytokines.

During primary disease of the cornea, only small numbers of T cells are first seen in BALB/c mice on days 7–8 (Opremcak et al., 1990; Wang et al., 1988) or day 11 in C.AL-20 mice (Heiligenhaus et al., 1994). In contrast, there was a rapid and marked T cell response in corneas with recurrent disease. Such a response is not surprising since the latently infected animals will be mounting a secondary immune response to viral antigens. A similar rapid infiltrate of CD4+ cells occurred in immune mice following secondary challenge by inoculation of virus onto the cornea (Ghiasi et al., 1995).

On day 10, corneas with recurrent epithelial disease had equal numbers of CD4+ and CD8+ cells. These latter cells may have suppressor activity, for example, via the secretion of cell surface proteins or cytokines (Kerhl et al., 1986). In contrast, corneas with stromal disease had lower numbers of T cells than corneas with epithelial disease and more CD4+ cells than CD8+ cells. A similar ratio of these cell types was demonstrated in diseased corneas during primary infection (Wang et al., 1988; Heiligenhaus et al., 1994) and in non-diseased corneas of immune mice following secondary ocular challenge (Ghiasi et al., 1995).

Reasons underlying the immune cell dynamics, in mice with epithelial as compared with stromal disease, need further investigation. These reasons could include differences in the immune response, e.g. of the TH1 or TH2 type, set up at the time of primary infection and/or differences in the amount of viral antigens reaching the eye during recurrence. We have found no evidence that severity of disease was related to amounts of viral antigen. Moreover, even in animals with stromal disease the basement membrane of the corneal epithelium appears to be an effective barrier to virus spread since antigen was restricted to epithelial cells. A similar distribution of virus antigen has been described by others in the mouse during recurrent disease (Laycock et al., 1991).

This mouse model has allowed, for the first time, the secondary immune response to recurrent HSV in the cornea to be investigated. The differences observed between this model of true recurrent disease and others involving primary infection, particularly as models of stromal disease, suggest the need for caution in the interpretation of such 'primary' models. Moreover, only in induced models of recurrent disease will effects on the local immune responses of the inducing agents (such as UV irradiation) themselves be evident. Such effects may occur, for example, through release of immunomodulating agents such as prostaglandins from local tissues (Harbour et al., 1983) and/or the release of immunomodulating factors, such as neuropeptides into the local
tissues from sensory nerve endings (Girolomoni & Tigelaar, 1990).

References


