Role of T-Lymphocytes in the Pathogenesis of Herpetic Stromal Keratitis

Robert G. Russell, Mark P. Nasisse, Hal S. Larsen, and Barry T. Rouse

Our study was designed to investigate the mechanism of the stromal reaction in experimental ocular infection of murine eyes with herpes simplex virus (HSV). Severe stromal keratitis with scarring occurred in BALB/c mice after infection of the scarified cornea but similar reactions did not occur in athymic mice. However, if athymic mice were given adoptive transfers of lymphoid cells, a severe necrotizing and ulcerative keratitis accompanied by scarring resulted. The lesion progressed more quickly in recipients of lymphoid cells specifically immune to HSV and containing cytotoxic T-lymphocyte activity. In such mice, necrosis and ulceration were marked on the sixth day after transfer compared with 9–12 days for those given nonimmune cells. Removal of T-lymphocytes from the immune lymphoid population by treatment with specific antiserum and complement abrogated the adoptive transfer of the stromal reaction. Our results further demonstrate that stromal keratitis represents a host immunopathologic response to HSV infection in which T-lymphocytes are essential participants. Multiple mechanisms of T-cell immunopathology appear to be operating, including a reaction mediated by cytotoxic T-lymphocytes. Invest Ophthalmol Vis Sci 25:938–944, 1984

In the United States, herpes simplex virus (HSV) is an important cause of severe ocular disease and a common cause of corneal opacities. Whereas epithelial keratitis seems to result directly from HSV replication in epithelial cells, the stromal keratitis, characteristic of repeated attacks, is assumed to result from a host response to viral antigens present in the stroma.1-6 However, other factors also are involved such as the properties of the infecting strain.7,8 In stromal keratitis, viral antigens but not infectious virus can be demonstrated in keratocytes,9 and lymphocytes have been observed in close association with virus containing cells.2,9,10 This suggests that a host reaction is mediating the disease. However, an immunopathologic sequence of events for stromal keratitis needs to be established firmly as does the actual mechanism of the host response. Metcalf and colleagues6 provided an important clue that T-lymphocytes were involved since the stromal reaction failed to occur in mice genetically unable to mount T-cell responses. In this communication, we confirm Metcalf’s findings and present further evidence to support an immunopathologic role of the host response in stromal keratitis. Our results strongly imply that T-lymphocytes are involved centrally.

Materials and Methods

Mice

Athymic nude (nu/nu) male mice of the BALB/c background were obtained from the Memorial Research Center, University of Tennessee (Knoxville, TN), and BALB/c male mice (H-2b) were obtained from Cumberland View Farms (Clinton, TN). The mice were received at 5 weeks of age and ocular infection experiments were conducted in mice at 6–8 weeks of age.

Virus

The RE strain of herpes simplex virus type 1 (HSV-1) was passaged in HEP/2 cells, obtained from Flow Laboratories (McLean, VA). Cells were grown in and maintained in McCoy’s 5A medium with 5% calf serum. The titrations of infectious virus were determined by plaque counts on VERO rabbit kidney cells, as described elsewhere.11

Corneal Inoculation

Mice were anesthetized by intraperitoneal injection of 0.65–0.9 mg pentobarbital. The cornea was scratched heavily with a 25-gauge needle. A small cotton swab saturated with 100-μl virus preparation containing 2 × 106 plaque forming units (PFU)/ml was rubbed on the scratched cornea.
**Mouse Immunization and Preparation of Spleen Cells for Adoptive Transfer**

A detailed account of the procedures for obtaining cultures of immune spleen cells containing cytotoxic T-lymphocytes (CTL) was described in a previous communication. Briefly, BALB/c mice were infected intraperitoneally with 10^6 PFU (0.2 ml inoculum) of KOS strain of HSV-1. Spleen cell cultures were prepared 4 weeks or more post-infection from HSV-1 immunized mice. For in vitro cultures, the spleen cell suspensions (in RPMI 1640 supplemented with a solution of 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 5 X 10^-5 M 2-mercaptoethanol) were cultured in plastic petri dishes at densities of 2 X 10^6 cells per ml and 1 X 10^6 cells per cm^2 of surface area. These cultures were reinoculated with ultraviolet- (UV) inactivated HSV-1 (KOS strain, 10^7.0 plaque forming units per ml prior to inactivation) and cultured for a period of 5 days in a humidified CO2 (5%) incubator to obtain secondary stimulated HSV-1 immune cells.

Spleen cells from nonimmune BALB/c mice were cultured under similar conditions. The cultures were not infected with HSV-1.

**Cytotoxicity Assay**

The 5-day cultures of immune and nonimmune spleen cells were tested for CTL as previously described. Targets were A31 cells (H-2^d) infected with KOS strain of HSV-1 and uninfected A31 cells. Effector-to-target-cell ratios were 25:1 and the immune spleen cells showed 54% specific lysis against the syngeneic virus infected target after 4–5-hr incubation as measured by a ^51^chromium release assay. Nonimmune spleen cells cultured in vitro without antigen, lacked induction of cytotoxic lymphocytes.

**Adoptive Cell Transfer into Athymic Nude Mice**

Immune or nonimmune spleen cells (5 X 10^7 in 0.3 ml) were administered by intravenous inoculation 72 hr after ocular infection with the RE strain of HSV-1.

**Clinical and Pathologic Observations**

Four experimental groups of 25 mice each were used and were infected via the cornea with HSV-1: BALB/c mice; athymic mice; athymic mice given adoptive transfers of in vitro cultured immune spleen cells and finally athymic mice given adoptive transfers of in vitro cultured nonimmune spleen cells. To act as placebo controls, ten athymic mice and 10 BALB/c mice had their corneas scratched but received no virus infection.

Eyes were examined postinfection by slit-lamp biomicroscopy at two day intervals for a total of 18 days. Numerical scores of 0, 1+, 2+, 3+, and 4+ were used, these representing no change, mild, moderate, marked, and severe change, respectively. The corneas also were evaluated for opacity due to neovascularization, stromal edema, and scarring. A grade of mild was defined as the earliest detectable change. In the evaluation of edema and scarring, a grade of severe was reserved for changes involving the stroma of sufficient magnitude to result in complete corneal opacity. Periorcular dermatitis also was noted and was considered severe if at least one-half of the head was affected. Antibiotic (Neosporin ophthalmic solution, Burroughs Wellcome Co.; North Carolina) was applied topically two times daily.

Mice were killed by cervical dislocation at 3-day intervals to characterize the course of the keratitis histologically. Three BALB/c mice and three athymic mice not given adoptive transfer were obtained on day 3. Three mice from each group also were obtained on days 6, 9, 12, 15, and the remaining mice were killed at the termination of the experiment on day 18. The eyeball was removed and fixed in 10% formalin. For histologic study, approximately 80 representative sections were collected onto glass slides during complete serial sectioning of the eye. The sections were stained with haematoxylin and eosin.

**Cell Fractionation**

Spleen cells from HSV immune mice were stimulated with virus in vitro for 5 days then harvested, washed twice, and the viable cells isolated by centrifugation using Lympholyte-M (Accurate Chemical and Scientific Corp.; Westbury, NY) density separation medium. Antibody treatment was accomplished by reacting 5 X 10^7 spleen cells for 1 hr at 4°C with optimal concentration of either monoclonal antibody Thy 1.2 (a gift from Dr. Jon Sprent, Wistar Institute; Philadelphia, PA). The cells were then washed and 3.5 ml of a 1:8 dilution (optimal concentration) of Low-Tox-M rabbit complement was added and the mixture incubated for 1 hr at 37°C with occasional mixing. The treated cells were injected into seven recipient mice 48 hr after scarification and inoculation of the cornea with RE strain of HSV-1. To test that they were depleted of T-cells, an aliquot of anti Thy 1.2 treated cells were stimulated for 3 days with concanavalin A (10 μg/ml) and ^3^H thymidine incorporation measured. The cells failed to respond indicating an absence of T-lymphocytes. Seven control mice were injected with 5 X 10^7 cultured immune spleen cells. The clinical course was followed by slit-lamp biomicroscopy. The experiments were terminated on day 15.
and eyes were fixed in 10% buffered formalin for histological examination.

These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Results

Comparison of Ocular Infection with HSV-1 in BALB/c and Athymic Mice

Histopathological findings: In athymic and BALB/c mice examined histologically at day 3 postinfection, HSV-1 caused local necrosis of the corneal epithelium (Fig. 1). The epithelium regenerated rapidly causing a focal area of thickening (known as an epithelial facet) at day 6. A mild focal thickening remained at this site at day eighteen.

Following ocular infection with HSV-1 in athymic mice, the stroma of the eye was infiltrated diffusely with mild to moderate numbers of polymorphonuclear leukocytes (PMN) at day 6 and neovascularization was evident. These inflammatory changes subsided by day 9 to 12. None of these mice showed stromal necrosis or corneal ulceration (Table 1).

In BALB/c mice, coagulation necrosis of individual keratocytes was apparent at day 6 and this was accompanied by moderate lymphocytic infiltration of the stroma. These inflammatory lesions lasted from 3 to 6 days. Lesions were not as dense as those in athymic mice.

Table 1. Histopathologic course of stromal keratitis caused by HSV-1 in immunocompetent BALB/c and restored athymic mice

<table>
<thead>
<tr>
<th>Group*</th>
<th>Days postinfection with HSV-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>BALB/c and HSV-1</td>
<td></td>
</tr>
<tr>
<td>Stromal necrosis</td>
<td>+</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>-</td>
</tr>
<tr>
<td>Scarring</td>
<td>-</td>
</tr>
<tr>
<td>Athymic and HSV-1</td>
<td></td>
</tr>
<tr>
<td>Stromal necrosis</td>
<td>+</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>-</td>
</tr>
<tr>
<td>Scarring</td>
<td>-</td>
</tr>
<tr>
<td>Athymic and HSV-1 immune spleen cells†</td>
<td></td>
</tr>
<tr>
<td>Stromal necrosis</td>
<td>+</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>-</td>
</tr>
<tr>
<td>Scarring</td>
<td>-</td>
</tr>
<tr>
<td>Athymic and HSV-1 nonimmune spleen cells</td>
<td></td>
</tr>
<tr>
<td>Stromal necrosis</td>
<td>+</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>-</td>
</tr>
<tr>
<td>Scarring</td>
<td>-</td>
</tr>
</tbody>
</table>

* Twenty mice were used in each group. All mice infected on day 0 by swabbing the scarified cornea with 100 μl of stock solution containing 2 x 10⁶ PFU/ml. During the period when stromal keratitis occurred in the normal BALB/c and adoptively reconstituted athymic mice, three mice were studied histologically on days 6, 9, 12, 15 and five mice on day 18. Numerical scores of -, 1+, 2+, 3+, 4+ represent no change, mild, moderate, marked, and severe change, respectively.

† Adoptive transfers of 5 x 10⁷ cells given 3 days after infection of the cornea with RE strain of HSV-1.
the corneal stroma (Fig. 2). Severe stromal necrosis of the central cornea, resulting in ulceration occurred slightly later, affecting 50% of mice at days 12 to 18 (Fig. 3, Table 1). This was accompanied by a marked PMN response and prominent scarring. Most of the remaining mice exhibited necrosis of individual keratocytes at days 12 to 18.

The course of stromal keratitis in athymic mice adoptively transferred with nonimmune spleen cells was similar to that in the BALB/c mice (Table 1). Necrosis of individual keratocytes occurred at day 9 (6 days after cell transfer). Severe central stromal necrosis and ulceration was evident at days 12 to 18 (9 to 15 days postadoptive transfer), and a marked scarring of the cornea occurred. Approximately 50% of mice in this group were affected.

In athymic mice adoptively transferred with immune spleen cells, the onset of necrosis was earlier, with severe damage at day 9 (6 days postadoptive transfer). Marked stromal necrosis of keratocytes and corneal ulceration of the central cornea occurred in animals at days 9 to 18 (Fig. 4, Table 1). Lymphocytic infiltration was present initially in the stroma, but the inflammatory response to the severe stromal necrosis and ulceration was predominantly PMN. Early fibroplasia at the periphery of the stromal necrosis began at days 9 to 12 and progressed to marked diffuse stromal scarring in affected mice.
Stromal keratitis in the BALB/c and athymic mice, restored with immune or nonimmune spleen cells, was most pronounced in the central cornea where HSV-1 infection occurred following scarification. At the limbus, there was mild to moderate infiltration by predominately PMN accompanied by lymphocytes. There was no endothelial involvement.

Clinical observations—slit-lamp biomicroscopy:
Superficial damage to the epithelium was observed by 3 days after infection in all mice. Marked corneal opacity, associated with stromal edema and neovascularization was evident in euthymic BALB/c mice and athymic mice adoptively transferred with nonimmune spleen cells. The opacity was observable at days 6 to 8, but corneas were clear by approximately day 14. Athymic mice given adoptive transfers of immune spleen cells showed marked corneal opacity earlier at days 4 to 6, but these reactions also resolved by day 14. In athymic mice infected with HSV only, the corneal opacity was more mild and had subsided fully by day 10 postinfection (Fig. 5).

Prominent scarring of the cornea occurred in BALB/c mice and in athymic mice adoptively transferred with immune or nonimmune cells, but such scarring failed to occur in athymic mice not given adoptive cell transfers. Scarring began at days 8–10 and was moderate to marked in about 50% of the mice from day 12 to 18. Corneal ulceration and necrosis was observed occasionally in some of the mice that developed corneal scarring. Our interpretation of the histologic lesions

Fig. 4. BALB/c, cornea infected with HSV-1. Higher magnification showing superficial necrosis of the cornea (large arrow), infiltrating PMN, neovascularization and early fibroplasia (small arrows) (hematoxylin and eosin, X450).

Fig. 5. Athymic, cornea infected with HSV-1, adoptive transfer of immune spleen cells. The pathologic changes are similar to BALB/c infected with HSV-1, shown in Figures 3 and 4. There is severe superficial necrosis and ulceration of the central cornea (large arrow), prominent PMN infiltration (small arrows) and neovascularization (hematoxylin and eosin, X400).
and biomicroscopic observations taken together is that scarring occurred simultaneously with stromal necrosis. Corneal scarring, detected by slit-lamp biomicroscopy, therefore was a reliable indicator of severe stromal necrosis. Resolution and biomicroscopic observations taken together is that scarring occurred simultaneously with stromal necrosis, whereas corneal edema and neovascularization were responses to initial virus replication and epithelial damage.

**Role of T-Cells in Stromal Keratitis**

In an experiment designed to further assess the role of T-cells in eliciting the stromal reaction, the effect of HSV infection was compared in groups of seven athymic mice reconstituted with immune spleen cells and mice reconstituted with immune spleen cells from which the T-cells were removed by treatment with anti-thy 1.2 serum and complement. The experiment was terminated 15 days after infection (13 days after reconstitution) and the corneas examined histologically. All seven mice reconstituted with immune spleen cells developed severe stromal necrosis and scarring. However, this reaction occurred in only one of seven animals reconstituted with T-cell depleted spleen cells. Thus, our results indicate the essential role of T-cells in producing the stromal reaction.

**Discussion**

The results of the present investigation provide further evidence for the hypothesis that herpetic stromal keratitis represents an immunopathologic reaction in which T-lymphocytes play an essential role. Thus, our findings confirm the observations of Metcalf and colleagues and show that ocular infection of immune competent mice produced epithelial necrosis followed by a stromal necrotic reaction that results in scarring, in athymic mice stromal necrosis, and scarring fail to occur. Data were collected by both biomicroscopy and histology, but the latter technique proved more useful to detect corneal necrosis, ulceration, and scarring, which we considered the significant pathologic lesions in immunocompetent mice.

To gain further evidence that the stromal reaction was immunopathologic, the course of events was followed in HSV infected athymic mice subsequently given adoptive cell transfers. One cell population used for transfer was spleen cells from HSV immune mice that were reimmunized in vitro with HSV antigen. This cell population, which we have characterized extensively in previous publications, consists predominantly of T-cells, expresses high levels of HSV-specific H-2 restricted cytotoxicity, contains helper cell activity, and can mediate delayed type hypersensitivity (DTH) reactions. If T-cells were involved in the immunopathologic reaction, the HSV immune cell source was expected to elicit a prompt response in the athymic recipients providing some cells could find access to the site of infection. Indeed athymic mice adoptively given such cells developed a necrotizing stromal reaction and scarring similar to that observed in euthymic BALB/c mice. Moreover, peak responses were evident from 6 days after transfer compared with a similar response in BALB/c mice at 12 days after infection. The adoptive transfer of the stromal response was abrogated upon removal of the T-cells by negative selection with specific antisera so further implicating the role of T-cells in mediating the stromal response. Consequently, our results support and extend previous observations that herpetic stromal keratitis represents an immunopathologic reaction. However, further experiments are required to delineate the actual mechanism of the immunopathology in vivo. For instance, the tissue damage could be mediated directly by cytotoxic T-lymphocytes as we demonstrated against HSV infected cells in vitro. Secondly, HSV-specific T-cells could be mediating an inflammatory DTH response. A third possibility is that T-lymphocytes were acting as helper cells in reconstituting the ability of recipient mice to produce antibody, such antibody mediating pathology by an immune complex mechanism, antibody and complement lysis, or perhaps antibody-dependent cell cytotoxicity. Immunopathology mediated by antibody in combination with polymorphonuclear leukocytes was suggested to account for stromal keratitis in experimental infection of the rabbit eye.

The adoptive cell transfers we employed contained helper cells and did reconstitute antibody producing responsiveness (data not shown), and so it is not possible to discount fully antibody as playing a role in mediating the stromal reaction. Further resolution of the putative mechanism of immunopathology operative in vivo will require adoptive cell transfer studies with defined subsets of T-cells such as Lyt2+ cytotoxic that are incapable of providing helper function. Such studies are currently underway in our laboratory.

**Key words:** corneal herpetic disease, stromal keratitis, immunopathology

**Acknowledgment**

The RE stain of HSV-1 was supplied kindly by Dr. Reichert.

**References**