Histological and electron microscopic studies of experimental herpetic keratitis in the rabbit

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Histological and electron microscopic observations, together with virus cultures, were made in the eyes of 50 New Zealand white rabbits which received bilateral intrastromal inoculation with the RE strain of herpes simplex virus. Virus cultures of whole corneas were positive for the first 2 weeks following inoculation, but were consistently negative thereafter. An inflammatory response to HSV infection, consisting of polymorphonuclear leukocytes and lymphocytes, was seen in the limbus within 7 hours after inoculation of the cornea. A massive accumulation of lymphocytes and plasma cells appeared in the limbus, suggesting that the limbus may behave as a lymphoid tissue, where differentiation and maturation of lymphoid cells occur as they acquire immunocompetence. Neovascularization of the cornea was associated with a heavy infiltration of the surrounding stroma with polymorphonuclear leukocytes, as well as numerous plasma cells and a few lymphocytes and macrophages. Numerous abnormal, pleomorphic keratocytes were found in the stroma. Lymphocytes were frequently found closely adhering to these abnormal keratocytes, suggesting a T-cell attack on a target cell. A model which describes the mechanism by which herpes virus infection leads to corneal scarring is suggested.

Key words: herpetic keratitis, rabbit cornea, herpesvirus hominis, electron microscopy, histology, immunopathogenesis

Recent studies in this laboratory have shown that intrastromal injection of the RE strain of herpes simplex virus (HSV) induces a high incidence of disciform edema and necrotizing keratitis in rabbit corneas without prior sensitization of the host. Histological examination of these corneas indicated that a complex inflammatory reaction was associated with the induction of herpetic keratitis in the rabbit, and immunofluorescent antibody techniques showed that viral antigens were present in the keratocytes of the inflamed corneas in animals with early necrotizing keratitis. Immunoferritin labeling procedures with antihuman IgG-anti-horse ferritin hybrid antibodies have also been used to demonstrate the presence of HSV antigens in infected corneal cells in vitro, in epithelial cells of the cornea, and on the surface membranes of stromal keratocytes in the corneas of rabbits with early necrotizing keratitis. The presence of numerous lymphocytes, many of which were found in intimate contact...
Fig. 1. Limbus 7 hr after inoculation of cornea with HSV. A marked infiltration of both PMN and lymphocytes is evident. Note the inflammatory cells (PMN) in the capillaries (arrows). Some plasma cells are also found (circle). (H&E; ×300.)

Fig. 2. Limbus 2 days after inoculation of cornea with HSV. A heavy infiltration of PMN and lymphocytes is seen in the limbus, and many PMN are found in the epithelium (circles). (H&E; ×600.)
Fig. 3. Three days after infection. Numerous PMN (circles) are found in the stroma beneath the epithelial lesion (H&E; ×800.)

with degenerating keratocytes of the stroma, strongly suggested that cell-mediated immunopathogenesis involving a T-cell attack on a target keratocyte may be a significant factor in the pathology of this disease.

In the present study we have used histological and ultrastructural techniques, together with virus cultures, to further describe the complex inflammatory response to herpes simplex in the rabbit cornea.

Materials and methods

Animal model. Fifty New Zealand white rabbits, weighing 2 to 3 kg each, were given bilateral intrastromal injections of 0.02 ml of the RE strain of HSV as described previously, except that the titer of the virus suspension was 10,000 plaque-forming units per milliliter. Biomicroscopic observations were made with a Mentor slit lamp prior to the death of the animals from an overdose of Nembutal. Corneas were obtained at 1 and 7 hr after injections, and at 2 to 3 day intervals for 35 days thereafter.

Control animals received intrastromal injections of a lysate of human embryonic kidney cells in Eagle's medium with 1% fetal bovine serum, prepared as described below except that virus was omitted. Biomicroscopic observations were made of these eyes, and corneas were obtained after 1 day and at weekly intervals for histological study.

Table I. Virus cultures of rabbit corneas after intrastromal inoculation with herpes simplex virus

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<th>Days after injection</th>
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* Each point represents triplicate cultures from a single cornea in tube cultures of human embryonic kidney cells.

Virus and virus cultures. The virus suspension used for inoculation of the rabbit eyes was prepared by seeding a culture flask (120 cm² surface
area) of human embryonic kidney cells (Microbiological Associates) with the RE strain of HSV. The infected culture was incubated at 37°C in 30 ml of Eagle's medium containing 5% fetal bovine serum. When at least 90% of the cells were infected, as shown by cytopathic effect, the culture was frozen and stored at −75°C overnight; then it was thawed and dispensed (1 ml aliquots) into glass vials. The virus suspension was stored at −75°C until used. The titer of the virus, determined by inoculating triplicate tube cultures of human embryonic kidney cells from serial dilutions of the stock suspension, was 1 × 10⁸ pfu/ml. This was diluted in Eagle's medium to obtain the suspension used for inoculating the corneas.

For assay of virus in infected eyes, corneal buttons were cut just inside the corneoscleral junction with iris scissors and placed in 1 ml of sterile tissue culture medium (Eagle's), then stored at −75°C in a freezer. The thawed corneas were minced with scissors and then sonicated for 1 min in an ice bath. The sonicate was used to seed three tube cultures of human embryonic kidney cells. The cultures were incubated at 37°C and examined at 1, 2, 3, and 6 days for evidence of cytopathic effect.

**Histology.** Corneas were fixed in either 4% formaldehyde or 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), then embedded in paraffin with an orientation of the tissue which permitted sections to be cut extending through the limbus and into the midstroma. These sections were stained with hematoxylin and eosin (H&E).

**Electron microscopy.** Corneas were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Pieces of tissue obtained from the limbus and stroma (processed separately) were washed in buffer, postfixed in 1% osmium, dehydrated in ethanol, and embedded in epon. Thin sections were cut with a diamond knife and stained for 15 min at room temperature with half-saturated uranyl acetate in 50% ethanol, then in Reynolds lead citrate for 2 min. The sections were examined and photographed with a Zeiss EM 9 S2 electron microscope.

**Results**

**Biomicroscopic observations.** The infected corneas passed through successive stages of inflammation, including epithelial keratitis, stromal edema, and necrotizing keratitis, as

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**Fig. 4.** Seven days after infection. Note the extensive damage to the endothelium, and invasion of this tissue with PMN (arrows). Infiltration of the endothelium by PMN is correlated with severe stromal edema in the rabbit. (H&E; ×900.)
Fig. 5. Eight days after infection. Neovascularization of the cornea is associated with the presence of large numbers of PMN in the stroma surrounding the capillaries. Plasma cells, macrophages, and lymphocytes are also numerous. A cluster of plasma cells (short arrows) is shown near a capillary (long arrow). (H&E; ×600.)

Fig. 6. Fifteen days after infection. A large mass of fibroblastic tissue (retrocorneal membrane) is found in the endothelium. Note the folds in Descemet's membrane (arrows). (H&E; ×150.)
Fig. 7. Limbus, 7 hr after infection. Two PMN and a small lymphocyte (note indented nucleus) are found in a capillary. The lymphocyte has made contact with the endothelial cell of the capillary. (Electron micrograph; calibration bar = 2 μm.)

Fig. 8. Lymphocytes in the interstitial space of the limbus, 24 hr after infection. (Electron micrograph; calibration bar = 2 μm.)
Fig. 9. HSV particles in the nucleus of a stromal keratocyte, 24 hr after infection. (Electron micrograph; calibration bar = 0.5 μm.)

Fig. 10. Cell debris and free virus particles (circles) released into the stroma as a result of cell lysis, 24 hr after infection. (Electron micrograph; calibration bar = 1 μm.)
Fig. 11. HSV particles in the nucleus of an infected epithelial cell, 24 hr after infection. (Electron micrograph; calibration bar = 1 μm.)

Fig. 12. Accumulation of immature plasma cells in the limbus. A PMN is seen in the lower center of the photograph. Seven days after infection. (Electron micrograph; calibration bar = 5 μm.)
Fig. 13. Lymphocyte (right) and a plasma cell closely adjacent to each other in the limbus, 7 days after infection. (Electron micrograph; calibration bar = 3 μm.)

Fig. 14. Lymphocyte (above) in intimate contact with a stromal keratocyte. A broad simple zone of contact is seen between the lymphocyte and the keratocyte, although there appears to be fusion of the cell membranes at one point (arrow). Seven days after infection. (Electron micrograph; calibration bar = 0.5 μm.)
Fig. 15. Stromal keratocyte with a beaded nucleus, 10 days after infection. (Electron micrograph; calibration bar = 2 µm.)

Fig. 16. Stromal keratocyte with basal bodies. Note the indented nucleus. Fifteen days after infection. (Electron micrograph; calibration bar = 0.5 µm.)
Fig. 17. Lymphocyte with multiple indentations in the nucleus, 15 days after infection. (Electron micrograph; calibration bar = 1 µm.)

Fig. 18. Mitotic cell, presumably a lymphocyte, in the stroma, 15 days after infection. (Electron micrograph; calibration bar = 2 µm.)
Fig. 19. Cell (macrophage?) in the limbus in which viruslike particles are found in the cytoplasm (circle). The profile of a nerve axon (arrow) which contains similar particles, morphologically resembling HSV, is seen in the upper left. The inset shows a higher magnification view of the particle in the nerve fiber. Twenty-nine days after infection. (Electron micrograph; calibration bar = 1 μm.)

described in detail previously, except that the inflammatory response was more severe with the higher titer of the virus suspension used here. Several eyes were observed with a hyphema or hypopyon, and the corneas of one animal perforated as the result of severe necrotizing keratitis.

The eyes of all control animals remained clear throughout the observation period.

**Virus cultures.** Virus cultures were scored as positive for HSV if rounded cells were observed in the culture followed by the appearance of discrete foci of degenerating cells. As shown in Table I, there was a progressive increase in the incubation time required for appearance of cytopathic effect, suggesting a progressive decrease in virus titer as the inflammation progressed. Virus cultures were consistently negative after postinfection day 13. No evidence of recurrent infection was observed during the period of study.

**Histological observations** (Figs. 1 to 6). Animals were killed at various times after intrastromal inoculation of the cornea with HSV. The numbers in parentheses below represent the number of animals from which corneas were obtained for histological examination at each time.

**Control (6).** An occasional plasma cell or lymphocyte was found in the limbus of control eyes, but no evidence of a cellular infiltrate was observed in the stroma at any time.

At 1 hr (1). The cornea appeared normal except for lacunae in midstroma resulting from the injection of virus suspension.

At 7 hr (1). The cornea appeared normal, but there was a marked infiltration of polymorphonuclear leukocytes (PMN) and lymphocytes in the limbus. A few plasma cells were also seen in the limbus (Fig. 1).

At 1 day (2). A heavy infiltration of PMN and lymphocytes was seen in the limbus with...
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Fig. 20. Ciliated cell in the limbus at 29 days after infection. Viralike particles (circle) are also found in this cell. (Electron micrograph; calibration bar = 1 μm.)

From 2 to 3 days (4). Large numbers of PMN, plasma cells, and lymphocytes were found in the limbus. PMN had also infiltrated the epithelium over the limbus (Fig. 2) and were found in epithelial lesions of the adjacent stroma (Fig. 3).

From 7 to 8 days (4). A heavy infiltration of PMN, plasma cells, and lymphocytes was found in the limbus. Many PMN were found invading the endothelium, resulting in separation of endothelial cells and the underlying Descemet’s membrane (Fig. 4). Areas of neovascularization were found, with many PMN and some plasma cells, macrophages, and lymphocytes in the surrounding stroma (Fig. 5).

From 10 to 20 days (11). A heavy infiltration of plasma cells and lymphocytes persisted in the limbus. Neovascularization of the stroma with many PMN and a few macrophages, plasma cells, and lymphocytes in the surrounding tissue were seen. The endothelium was absent from many areas of the cornea. On day 15 a large retrocorneal mass of tissue was found adjacent to Descemet’s membrane in one cornea (Fig. 6). Retrocorneal membranes have also been described in the eyes of rabbits which received intracameral injections with the McKrae stain of HSV.5

From 22 to 29 days (8). A mild to moderate infiltration of the limbus, composed of plasma cells and lymphocytes, was observed. Some neovascularization of the stroma was seen, and a few infiltrating cells were found in the stroma.

At 35 days (4). A mild to moderate cellular infiltration persisted in the limbus. No inflammatory cells were found in the stroma.

Electron microscopic observations. (Figs. 7 to 20). Within 7 hr after inoculation of the rabbit cornea with HSV, inflammatory cells were found in the capillaries of the limbus. PMN predominated, but typical small lymphocytes were also frequently seen (Fig. 7).
Wandering lymphocyte

HSV inoculation of the cornea

Lysis of stromal cells releasing cell debris and viral antigens

Lymphokines (chemotactic factors for PMN and lymphocytes)

Accumulation of plasma cells and lymphocytes in the limbus; Migration of PMN to sites of active viral infection

Lymphocyte maturation in limbus

T-cell attack on target keratocytes in the stroma

Synthesis of abnormal collagen by affected keratocytes

Permanent stromal scarring

Fig. 21. Proposed mechanism for immunopathogenesis of herpetic stromal keratitis in experimental animals.

These inflammatory cells passed between the endothelial cells of the capillaries, and after 24 hr many cells of both types were found in the interstitial spaces of the limbus. Lymphoid cells were frequently found closely adjacent to each other (Figs. 8, 12, and 13).

Herpesvirus particles in nuclei of stromal keratocytes were seen 24 hr after intra-stromal inoculation (Fig. 9), and areas containing free virus particles and cell debris were also found (Fig. 10). The absence of a membrane coat surrounding the capsid of these extracellular virions suggests that they were released by cell lysis, rather than by shedding from intact cells. Large numbers of herpesvirus particles were found in degenerating nuclei of epithelial cells adjacent to lesions of this tissue (Fig. 11), suggesting that direct viral infection was responsible for the epithelial keratitis.

By postinfection day 7 large numbers of immature plasma cells (Fig. 12) were found in the limbus, and plasma cells in close contact with lymphocytes (Fig. 13) were frequently seen. In the stroma lymphocytes were also occasionally found in close physical contact with stromal keratocytes as early as day 7 (Fig. 14), presumably migrating from the limbus. Examples of lymphocytes in intimate contact with stromal keratocytes became more numerous as the inflammation progressed, as described previously.

A characteristic feature of the stroma on postinfection days 10 to 15 was the presence of numerous stromal keratocytes with abnormal features such as pleomorphic nuclei and basal bodies in the cytoplasm (Figs. 15 and 16). These morphological alterations suggest that physiological changes had occurred in the stromal keratocytes which were not destroyed by the initial viral infection. Lymphocytes with deeply indented nuclei (Fig. 17), indicating that the cell had been activated by contact with an antigen, and one cell which appeared to be a lymphocyte in mitosis (Fig. 18) were found.

Inflammatory cells (lymphocytes, PMN, and occasionally macrophages) were seen in the limbus on day 29. Viruslike particles, morphologically resembling HSV, were found in the cytoplasm of a cell which enveloped a corneal nerve also containing viruslike particles (Fig. 19). Viruslike particles were also seen in an unusual ciliated cell, presumably an abnormal keratocyte, found in the limbus (Fig. 20). There was no evidence of cell lysis or degeneration in these cells, and virus cultures were negative at this stage of the infection (Table I).

Discussion

Previous studies in this laboratory strongly suggest that cell-mediated immu-
nopathogenesis, similar to a host vs. graft response, is involved in the etiology of the necrotizing keratitis associated with degeneration of keratocytes and permanent scarring of the cornea.

A model describing the mechanism of immunopathogenesis can be synthesized from the presently available data, as shown in Fig. 21. Following intrastromal inoculation of the cornea, virus replication occurs in stromal keratocytes, leading to cell lysis and release of free virus particles and cell debris containing viral antigens. Wandering lymphocytes, occasionally seen in normal cornea, contact these antigens and become sensitized. The sensitized lymphocytes may then produce lymphokines which include chemotactic factors for PMN and also lymphocytes. These inflammatory cells accumulate in the limbus, which appears to function as a lymphoid organ. PMN rapidly migrate from the limbus into the stroma, and accumulate in areas of active viral infection. The lymphocytes appear to undergo a period of maturation and differentiation in the limbus before they migrate into the cornea. Plasma cells also accumulate in the limbus.

Herpetic stromal keratitis in the rabbit is characterized by the presence of abnormal keratocytes in the stroma, suggesting that these cells have been altered as a result of the viral infection. Virus antigens have been demonstrated on the surface membranes of these stromal keratocytes, as well as on the surface membranes of rabbit corneal cells in vitro. Presumably, virus replication is terminated in the stromal cells by the production of interferon and other immune mechanisms, but synthesis of viral antigens may continue. The frequent occurrence of lymphocytes in intimate contact with stromal keratocytes in varying degrees of degeneration strongly suggests that a T-cell attack on target keratocytes may contribute to permanent scarring of the rabbit cornea, as well as blindness in human patients.

Evidence in support of this hypothesis has been provided by the experiments described by Polack et al. and Wind et al. on the host immune response and fate of corneal grafts between rabbits sensitized to HSV. Penetrating keratoplasties were done by exchanging corneal grafts between groups of systemically sensitized and locally sensitized animals. Of the grafts in which locally sensitized corneas were transplanted into systemically sensitized recipients, 100% developed complete opacity and peripheral corneal vascularization after initial clearing. Histological studies of the opaque corneas showed a pronounced infiltration of plasma cells and lymphocytes in the limbus. Degenerating keratocytes, some surrounded by several lymphocytes or plasma cells, were found in the stroma. Since the grafts were performed 4 to 6 weeks after the last inoculation with HSV and virus cultures of the opaque corneas were negative, it is unlikely that replicating virus was responsible for initiating the inflammatory response. Thus, the most likely explanation is that a host vs. graft reaction was initiated by the host immune system against the viral antigens present in the corneal transplant.

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The role of the limbus as a lymphoid tissue appears to have been recognized by Thompson and Olson in 1950. They found that injection of different antigens into the limbus of each eye was followed by the production of antibodies in the cornea specific for the particular antigen injected. Histopathological studies of the early stages of the corneal graft rejection also show that the immune reaction starts in the lymphoid tissues at the limbus and subconjunctival tissue in the grafted eye. Lymphoid cells are not normally found in the limbus, but appear to accumulate there in response to the presence of foreign antigen in the cornea.

Undoubtedly, the plasma cells which accumulate in the limbus following HSV inoculation of the cornea produce anti-HSV antibodies which diffuse into the cornea. It is conceivable that these antibodies may play a role in the immunopathogenesis of stromal herpetic keratitis. Sery et al. have shown that low doses (200 pfu) of HSV, strain H-4, induced corneal opacities in the right eye of 81% of the eyes injected. After observations for 6 to 7 weeks, a second dose of 20,000 pfu...
was injected into both right and left corneas. The strongest inflammatory response was now in the left eye, which had not received the initial injection, and the previously injected eyes remained clear and unaffected.

The authors concluded that the local antibody produced in the right eye during the initial infection played a protective rather than an antagonistic role, and prevented subsequent infection. In addition they suggested that immune complex formation does not induce corneal disease, since stromal reactions precede the appearance of neutralizing antibody in the cornea and serum.

However, Meyers and Pettit concluded, on the basis of immunohistopathologic studies of stromal keratitis, that the tissue injury which occurs in herpetic stromal keratitis results from the interaction of HSV antigens and anti-HSV antibody. This immune complex in turn activates the complement system to generate leukotactic factors for PMN, the major cellular infiltrate in their animals.

It appears that the corneal reaction to HSV infection may be determined by a combination of factors including dose and strain of virus, and the host response to these factors. Additional studies are required to determine the precise role of anti-HSV antibody, PMN and lymphoid cells in the immunopathogenesis of herpetic stromal keratitis.

REFERENCES