Articles

Contribution of Virus and Immune Factors to Herpes Simplex Virus Type I-Induced Corneal Pathology

Robert L. Hendricks and Terrence M. Tumpey

In vivo T-lymphocyte subpopulation depletion techniques were used to identify the roles of L3T4+ (CD4) and Lyt-2+ (CD8) T-lymphocytes in the pathogenesis of corneal stromal disease induced by two different strains of Herpes simplex virus type 1 (HSV-1). Histologic examination of infected corneas revealed significant differences in the composition of the inflammatory corneal infiltrates induced by the RE and KOS strains of HSV-1. The RE strain induced a predominantly polymorphonuclear leukocyte (PMN) infiltrate, which began approximately 1 week after infection and progressed through day 21. Depletion of CD4 cells before corneal infection with RE HSV-1 greatly reduced the incidence and severity of corneal disease; depletion of CD8 cells had no effect. The strain KOS HSV-1 induced an early PMN infiltrate that became predominantly mononuclear by day 21. Depletion of CD4 cells did not change the incidence or severity of KOS HSV-1-induced corneal stromal disease. The corneal lesions of these mice contained numerous CD8 cells. Depletion of CD8 cells before KOS HSV-1 infection of the cornea moderately reduced the incidence of stromal disease. However, in CD8-depleted mice with the disease, PMNs were the most prevalent infiltrating cells, and the disease appeared identical to that seen in RE HSV-1 infected corneas. Simultaneous depletion of CD4 and CD8 cells before KOS HSV-1 infection eliminated stromal disease. However, when T-cell depletion was discontinued in these mice, stromal disease developed in concert with the appearance of T-cells in the lymphoid organs and corneas. Thus, T-lymphocytes are a necessary component of HSV-1 corneal stromal disease. These results further suggest that RE HSV-1 preferentially activates CD4 cells in the cornea, and KOS HSV-1 preferentially activates CD8 cells in the cornea. Invest Ophthalmol Vis Sci 31:1929–1939, 1990

Despite extensive research, it remains unclear why certain individuals who are infected with Herpes simplex virus type 1 (HSV-1) develop recurrent herpetic ocular disease while others infected with HSV-1 do not. Two basic hypotheses have been advanced and supported by experimental findings. The first hypothesis holds that the pattern of disease that develops after primary infection is determined by the genetics of the infecting virus. Genetic characteristics of certain HSV-1 viruses favor colonization of the trigeminal ganglia, followed by periodic reactivation, and establishment of recurrent corneal disease. According to the second hypothesis, the pattern of disease that develops after primary HSV-1 infection is determined by the genetics of the host. Individuals are thought to inherit a predisposition to HSV-1 disease that is presumably related to some aspect of their immune response and/or natural defense against the virus. A third and, in our view, more likely possibility is that both host and viral factors influence susceptibility to herpetic stromal disease.

In support of the concept that the virus determines corneal pathology, Centifanto-Fitzgerald and colleagues showed that certain strains of HSV-1 had a propensity for inducing stromal keratitis after topical infection of the cornea; other strains were found to produce only epithelial lesions. Stromal keratitis is the type of disease that is most frequently associated with the blinding aspects of herpetic ocular disease.

The hypothesis that host factors determine susceptibility to HSV-1 corneal disease was supported by observations that different inbred strains of mice vary
in their susceptibility to herpetic disease in general and to ocular disease in particular. In the latter study, susceptibility to HSV-1 corneal disease mapped outside of the major histocompatibility complex (MHC). Interestingly, there is growing evidence that HSV-1 stromal keratitis is due to an immunopathologic response to HSV-1 antigens in the corneal stroma. Metcalf et al reported that congenitally athymic nude mice did not have stromal lesions after corneal infection with HSV-1 but their euthymic litter mates did. Russell et al extended these findings by showing that nude mice could be made susceptible to stromal lesions after adoptive transfer of T-lymphocytes from HSV-1 immune euthymic mice. Ksander and Hendricks demonstrated that A/J mice, a strain that is normally highly susceptible to HSV-1 corneal lesions, could be rendered resistant by the induction of cell-mediated immune tolerance to HSV-1 antigens. Thus, the susceptibility of certain strains of mice to HSV-1 stromal keratitis may reflect hyperresponsiveness rather than hyporesponsiveness of T-lymphocytes to HSV-1 antigens in those strains.

Recently, attempts were made to identify the T-lymphocyte population that is responsible for the stromal damage associated with HSV-1 corneal disease. Two different experimental approaches were used with apparently contradictory results. Hendricks et al. used mutant strains of HSV-1 with altered expression of HSV-1 cell surface glycoproteins to differentially suppress the delayed-type hypersensitivity (DTH) and cytotoxic T-lymphocyte (CTL) responses to HSV-1. This study showed that HSV-1 stromal disease was virtually eliminated in mice whose HSV-1-specific CTL response was suppressed, but it developed normally in mice whose HSV-1-specific DTH response was suppressed. These findings suggest that HSV-1-specific CTL may contribute to the destruction of corneal tissue associated with stromal keratitis. More recently, Newell et al tested the effect of depleting specific T-lymphocyte subpopulations in vivo on susceptibility to the development of HSV-1-induced stromal keratitis. In this study, depletion of L3T4+ (CD4) cells greatly reduced the susceptibility of Balb/c mice to stromal keratitis after HSV-1 corneal infection. In contrast, depletion of Lyt-2+ (CD8) cells had no effect on the development of stromal disease. The CD4 depletion was shown to abrogate the DTH and antibody response to HSV-1, but it did not affect the CTL response. These studies suggest that the DTH response to HSV-1 may be responsible for destruction of the corneal stroma after HSV-1 corneal infection.

The solution to this apparent paradox may lie in the strain of HSV-1 used in these two studies. Hendricks and colleagues used the KOS strain of HSV-1, while Newell and associates used the RE strain. We therefore compared the KOS and RE strains of HSV-1 to identify the roles of CD4 and CD8 T-lymphocytes in the pathogenesis of corneal stromal disease. Our findings showed that the inflammatory cellular infiltrates induced by these two strains of virus were markedly different. Furthermore, the effect of depleting a particular subpopulation of T-lymphocytes on the development of stromal disease varied with the strain of virus used to infect the cornea. It appears, therefore, that susceptibility to HSV-1 stromal disease involves a complex interaction between HSV-1 and the immune system, which can be influenced by the genetic composition of the virus and the host.

Materials and Methods

Mice and Infecting Virus

All animal experiments complied with the ARVO Resolution on the Use of Animals in Research. Female A/J mice (The Jackson Laboratories, Bar Harbor, ME), 8–12 wk old, were acclimated for 1 wk before initiating experiments. Mice were anesthetized with 2 mg of ketamine hydrochloride (Vetalar, Parke-Davis, Morris Plains, NJ) and 0.04 mg of acepromazine maleate (Aveco, Fort Dodge, IA) in 0.1 ml of RPMI-1640 medium injected intramuscularly in the left hind leg. Topical corneal infection was induced by applying 3 μl of a virus suspension (5 × 10⁴ plaque-forming units [PFU]) topically to a scarified cornea and rubbing it in with the eyelid. The KOS and RE strains of HSV-1 were identically prepared for use in these studies. The virus was grown in VERO or HEp-2 cells, and intact virions were purified on Percoll (Pharmacia, Piscataway, NJ) gradients as previously described. The virus used to infect mice was prepared from HSV-1-infected HEp-2 cells grown in RPMI-1640 plus 10% normal rabbit serum. The virus used for in vivo restimulation and DTH challenge was prepared from HSV-1-infected VERO cells grown in RPMI-1640 plus 10% fetal calf serum (FCS). The latter virus suspensions were inactivated by exposure to a germicidal lamp for 10 min at a distance of 5 cm (UV HSV-1).

Clinical Observation of HSV-1-Infected Corneas

The severity of corneal disease was monitored through slit-lamp examination of mouse eyes by an observer who was unaware of the treatment group to which the animal belonged. The degree of stromal opacity was scored on a scale of 0–3+, where 0 indicated no opacity; 1+, slight haze; 2+, moderate opac-
ity; and 3+, severe opacity obliterating the view of the iris. The area of opacity was also noted. However, since the pattern of results and the significance of group differences were the same when analyzed as to the degree or area of opacity, for the sake of simplicity, only the data for the degree of opacity are shown.

In Vivo Depletion of Functional Subpopulations of T-Lymphocytes

Groups of mice were depleted of their CD4 and/or CD8 T-lymphocyte subpopulation by in vivo treatment with rat monoclonal antibodies (MoAbs) specific for the L3T4 (clone GK 1.5; American Type Culture Collection, Rockville, MD, 1 mg per injection) and Lyt-2.2 (clone 2.43; American Type Culture Collection, 0.2 mg per injection) differentiation markers. The mice received intraperitoneal (IP) injections of MoAbs the day before monocular topical corneal infection with HSV-1 (day -1) and 2 and 6 days after infection (days +2 and +6). The mice then received weekly IP injections of the MoAb to maintain depletion during the observation period. Controls received IP injections of phosphate-buffered saline (PBS). The antibody solutions were coded, and all studies were done in a randomized double-blind fashion.

Lymphocyte Restimulation in Bulk and Limiting-Dilution Cultures

The regional (preauricular) lymph nodes (RLN) were excised on the designated day after HSV-1 infection, and single-cell suspensions were prepared. Preliminary experiments revealed that optimum stimulation in bulk culture occurred at a ratio of 0.001 PFU (before inactivation) of UV HSV-1 per RLN cell. Bulk cultures were prepared in assay medium (RPMI-1640 plus 5% FCS, 10 mM HEPES buffer, and antibiotics). Some of the bulk cultures were supplemented with recombinant murine interleukin 2 (rIL-2; Genzyme, Boston, MA) or recombinant murine interferon-γ (rIFN-γ; Interferon Sciences, New Brunswick, NJ). The limiting-dilution (LD) cultures were prepared as previously described.6 Stimulator cells for LD cultures were spleen cells from normal A/J mice that were incubated for 2 hr with UV HSV-1 (5 PFU per cell) in siliconized glass tubes. The cells were treated for 30 min with mitomycin C (50 μg/ml; Sigma, St. Louis, MO) and then washed, counted, and adjusted to 2 × 10^5/ml in LD medium (RPMI-1640 supplemented with 10% FCS, 15% supernatant from concanavalin A-stimulated Lewis rat spleen cells, 5 × 10^-5 M 2-mercaptoethanol, and antibiotics buffered to pH 7.2 with HEPES buffer). The LD cultures consisted of graded numbers of RLN responder cells that were plated in the wells of 96-well round-bottom microtiter plates in 0.1 ml of LD medium. Twenty-four control cultures did not receive responder cells. All cultures received 0.1 ml of stimulator cells (2 × 10^5 cells) and were incubated for 7 days at 37°C in a humid 5% CO_2-95% air atmosphere.

Cytotoxicity Assay

The HSV-1-infected L929 (L929-HSV) cells (clone CCL 1; American Type Culture Collection) were used as targets. The L929 cells (compatible with A/J mice at the H-2K locus) were infected with HSV at a multiplicity of infection of 5.0 for 2 hr and labeled with ^51Cr (200 μCi/2 × 10^6 cells) for 1 hr. At least 80% of the L929-HSV targets expressed HSV antigen as assessed by lysis with anti-HSV-1 antibody and complement. A 4-hr ^51Cr release assay was done as previously described.6 Bulk cultures were tested at three effector:target ratios. Each LD culture was tested for cytotoxic activity against 1.0 × 10^5 targets in a 4-hr ^51Cr release assay.

DTH Assay

The DTH was elicited by injecting 5 × 10^5 PFU of UV HSV-1 in a volume of 10 μl into the dorsal side of the mouse ear pinna. Ear swelling was measured 24 hr later using an engineer's micrometer (Mitutoyo, Tokyo, Japan). The amount of ear swelling (ie, postchallenge minus prechallenge ear thickness) in HSV-1-infected mice was compared with that of similarly challenged but unimmunized mice.

Histologic and Immunohistochemical Examination of Infected Corneas

These studies were done as previously described.10 To summarize briefly, the infected eyes were enucleated and immediately fixed in 10% neutral buffered formalin, and 5-μm paraffin sections were prepared. The sections were stained with hematoxylin-eosin, mounted with Permount (Fisher Scientific, Fairlawn, NJ), and cover-slipped for microscopic examination. For immunohistochemical staining, the eyes were imbedded in O.C.T. (Tissue Tek; Miles, Naperville, IL), snap-frozen in an isopentane-dry ice bath, and 6-μm sections were cut at -20°C. The sections were fixed in cold acetone for 10 min and then exposed to the primary antibodies overnight at 4°C. The following rat MoAbs were used as primary antibodies (both from American Type Culture Collection): anti-Lyt-2 (clone 53-6.72) and anti-L3T4 (GK 1.5). The sections were then stained using the streptavidin-biotin com-
plex immunoperoxidase staining procedure (Zymed Laboratories, South San Francisco, CA).

Statistical Analyses

The significance of group differences in the DTH response and cytotoxic activity in bulk culture assays was assessed using Dunnett's multiple comparison analysis. The significance of group differences in the degree of stromal opacity on each observation day, and in the time from infection to the onset of stromal disease, was assessed by an analysis of variance. The significance of group differences in the incidence of stromal disease was assessed by the Pearson chi-square test.

The data from LD assays were analyzed with a computer program which was provided by Dr. Peter Krammer (Heidelberg, FRG). The frequency (f) of cytotoxic cell precursors was determined by both the maximum likelihood and the minimum chi-square methods, and the 95% confidence limits of f were calculated. The chi-square value for the goodness of fit to the Poisson model and the P value corresponding to the chi-square value were determined. All reported LD data conform to the single-hit kinetics of the Poisson distribution. The significance of differences in f values was assessed by the global test for homogeneity of independent slopes.

Results

Efficacy of In Vivo T-Lymphocyte Subpopulation Depletion

Mice were included in each study group to monitor the efficacy of in vivo T-lymphocyte subpopulation depletion. At 1-wk intervals after corneal infection, mice were killed, and draining RLN cells and spleen cells were stained for CD4, CD8, and Thy-1.2 markers. These were then analyzed on an Epics V FACS (Coulter, Hialeah, FL). Representative histograms of lymph node cells obtained 10 days after HSV-1 infection from depleted and control animals are shown in Figure 1. In vivo treatment with anti-L3T4 MoAb resulted in a 90%-95% reduction of CD4 cells (Fig. 1C), with no reduction of the CD8 population (Fig. 1D). Similarly, in vivo treatment with anti-Lyt-2 resulted in a 90%-95% depletion of CD8 cells (Fig. 1F), with no reduction in the CD4 population (Fig. 1E). In all experiments, the frequency of Thy-1.2+ cells closely approximated that of the nondepleted population (not shown), suggesting that in vivo MoAb treatment resulted in depletion of the selected population rather than masking of the cell surface marker. Similar profiles were obtained for RLN and spleen cells throughout the 21-day observation period.

Effect of In Vivo T-Lymphocyte Subpopulation Depletion on the Development of the DTH and CTL Response to HSV-1

A comparable level of DTH reactivity was induced by corneal infection with the KOS and RE strains of HSV-1 (Table 1). In vivo treatment with anti-L3T4 MoAb at days −1, +2, and +6 relative to the time of corneal HSV-1 infection, resulted in an 86%-100% reduction of the DTH response. In contrast, in vivo treatment with anti-Lyt-2 MoAb had no effect on the DTH response induced by RE HSV-1, although it consistently caused a moderate reduction in the DTH response induced by KOS HSV-1. Thus, the DTH response induced by RE HSV-1 is mediated by CD4 cells, and that induced by KOS HSV-1 is mediated largely by CD4 cells with a minor contribution of CD8 cells.

The two strains of HSV-1 also induced comparable levels of CTL activity in the lymph nodes. In this model, in vivo depletion of CD4 cells reproducibly resulted in an approximately 50% reduction of
Effect of in vivo T-lymphocyte subpopulation depletion on the CTL Response to KOS HSV-1

Table 1. Effect of T-lymphocyte subpopulation depletion on the DTH response to HSV-1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>In vivo antibody treatment*</th>
<th>Ear swelling (×10^4 in) ± SEM</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>20.54 ± 1.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KOS</td>
<td>69.35 ± 2.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KOS Anti-L3T4</td>
<td>27.01 ± 2.56 P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KOS Anti-Lyt-2.2</td>
<td>44.39 ± 3.59 P = 0.003</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>27.68 ± 1.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>53.92 ± 6.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE Anti-L3T4</td>
<td>27.07 ± 0.67 P = 0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE Anti-Lyt-2.2</td>
<td>56.53 ± 5.31 P = 0.763</td>
<td></td>
</tr>
</tbody>
</table>

* Groups of four mice received topical corneal infections with 5 × 10^6 PFU of the indicated strain of HSV-1.
† On days -1, +2, and +6 relative to the time of infection, each mouse received intraperitoneal injections of the indicated rat MoAb.
‡ Seven days after infection, each of the HSV-1-infected mice and four uninfected control mice were challenged in the ear pinna with UV HSV-1.

Ear thickness (in inches) was measured before and 24 hr after challenge, and the difference in these readings is recorded as ear swelling.

§ Comparison of each antibody-treated group with the mock antibody-treated group by Dunnett's multiple comparison analysis.

HSV-1 specific CTL activity of RLN cells as measured in a bulk culture cytotoxicity assay compared with that of RLN cells from mock-depleted controls (Table 2). However, optimal CTL activity was obtained by supplementing these cultures with rIL-2 (50-100 units/ml) or IFN-7 (2500-5000 units/ml). The cytotoxic activity in lymphokine-supplemented cultures was reduced 90%-95% by treatment of the restimulated RLN cells with anti-Lyt-2 antibody plus complement immediately before the 51Cr release assay (data not shown). Thus, most of the lytic activity against HSV-1-infected targets in these cultures was mediated by CTLs.

In contrast, RLN cells from the same CD4-depleted mice had an increased frequency of HSV-1-specific CTL precursors (CTLp), probably due to the increased frequency of CD8 cells in the lymph nodes of these mice. These findings suggest that the expansion of CTLp in vivo may not require an accessory function of CD4 T-lymphocytes, whereas, in our hands, optimal differentiation of the CTLp into active cytotoxic cells in vitro does appear to require CD4 help.

The Influence of the Infecting Strain of HSV-1 on the Nature of the Corneal Inflammatory Infiltrate

We previously described the corneal inflammatory infiltrate that develops after topical corneal infection with KOS HSV-1.10 There is an early polymorphonuclear leukocyte (PMN) infiltrate on day 7, followed by a mixture of PMN and mononuclear leukocytes by day 14 (not shown). By day 21 after infection with KOS, the corneal infiltrate consisted predominantly of mononuclear leukocytes (Fig. 2A). The RE strain of HSV-1 induced a markedly different corneal inflammatory infiltrate, consisting predominantly of PMN (Fig. 2B). The RE HSV-1-infected corneas were also much more edematous than KOS HSV-1-infected corneas. The KOS HSV-1-infected corneas contained significantly (P = 0.039) more CD8 cells (an average of 37.5 cells per high power field, Fig. 3A) than CD4 cells (an average of 14.3 cells per high power field, Fig. 3B). The RE HSV-1-infected corneas contained significantly (P = 0.024) more CD4 cells (average of 39 cells per high power field, Fig. 3C) than CD8 cells (average of 20 cells per high power field, Fig. 3D).

The Effect of T-Lymphocyte Subpopulation Depletion on the Incidence and Nature of HSV-1-Induced Corneal Disease

We used in vivo T-lymphocyte subpopulation depletion to determine if different lymphocyte subpopulations were involved in the pathogenesis of corneal disease.

Table 2. Effect of in vivo T-lymphocyte subpopulation depletion on the CTL Response to KOS HSV-1

<table>
<thead>
<tr>
<th>In vivo antibody treatment</th>
<th>In vitro treatment</th>
<th>80:1</th>
<th>40:1</th>
<th>20:1</th>
<th>% 51Cr release E/T ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>53</td>
<td>42</td>
<td>24</td>
<td>11,739 (8615-18,418)</td>
</tr>
<tr>
<td>L3T4</td>
<td>None</td>
<td>28</td>
<td>23</td>
<td>17</td>
<td>7473 (5426-12,000)</td>
</tr>
<tr>
<td>L3T4</td>
<td>rIL-2 (100 U/ml)</td>
<td>54</td>
<td>41</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>L3T4</td>
<td>rIL-2 (10 U/ml)</td>
<td>37</td>
<td>14</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>L3T4</td>
<td>rIFN-γ (5000 U/ml)</td>
<td>51</td>
<td>50</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>L3T4</td>
<td>rIFN-γ (1000 U/ml)</td>
<td>40</td>
<td>33</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>L3T4</td>
<td>None</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>135,365 (99,059-213,685)</td>
</tr>
</tbody>
</table>

* Groups of mice were depleted of CD4 or CD8 T-lymphocytes by intraperitoneal injection of monoclonal anti-L3T4 or anti-Lyt-2 antibody, respectively, on days -1, +2, and +6 relative to the time of corneal infection with KOS HSV-1. Eight days after corneal infection, RLN cells were prepared and restimulated in vitro with HSV-1 antigens in bulk or limiting dilution cultures. Some of the bulk cultures were supplemented with recombinant mouse IL-2 (rIL-2) or recombinant murine interferon-γ (rIFN-γ). The CTL activity was measured against HSV-1-infected L929 cells in a 4-hr 51Cr-release assay. Data from bulk culture assays are expressed as the percent of 51Cr release at various effector/target (E/T) ratios. The data from limiting dilution assays are recorded as the inverse of the CTLp frequency and 95% confidence limits (95% CL).
disease after infection with the KOS and RE strains of HSV-1. Groups of mice were depleted of their CD4 or CD8 T-lymphocytes by in vivo treatment with anti-L3T4 or anti-Lyt-2 MoAb, respectively, 1 day before corneal infection with HSV-1. Depletion was maintained by repeated injections of MoAb. Control mice received IP injections of PBS. In a double-blind study, the mice were observed by slit-lamp biomicroscopy for the severity of corneal disease. Representative mice from each cage were killed, the infected eye was enucleated, and paraffin-embedded or frozen sections were prepared for histologic and immunohistochemical analysis.

Depletion of CD4 cells had no apparent effect on the incidence (Table 3) or severity (Fig. 4A) of stromal disease in corneas infected with the KOS strain of HSV-1. The corneas were devoid of CD4 cells (Fig. 5A), but numerous CD8 cells were present in the lesions (Fig. 5B). Depletion of CD8 cells before KOS HSV-1 infection of the cornea moderately reduced (not significantly, \( P < 0.05 \)) the incidence of stromal disease (Table 3) and delayed the mean time of onset (\( P = 0.006 \)) compared with mock-depleted controls. However, the mean disease severity of those CD8-depleted diseased mice was ultimately as great as that of the mock-depleted group (Fig. 4B). The CD8 depletion consistently changed the corneal infiltrate induced by KOS HSV-1 from a predominantly mononuclear infiltrate to one composed predominantly of PMN (Fig. 6), with some CD4 cells but no CD8 cells present (not shown). These corneas were also edematous and appeared similar to corneas infected with RE HSV-1 (Figs. 2B, 6).

As illustrated in Table 4, mice that were simultaneously depleted of CD4 and CD8 cells before corneal infection with KOS HSV-1 remained completely free of corneal stromal disease for 21 days after infection. One mouse that was depleted of T-cells for the full 42 days developed a mild stromal haze 30 days after infection, which persisted through the remainder of the observation period. These mice had severe periocular skin lesions but otherwise remained healthy. After 21 days of observation, treatment with the monoclonal anti-Lyt-2.2 and anti-L3T4 antibodies was discontinued in some of the mice, and the animals were observed for an additional 21 days. As the T-lymphocytes regenerated, corneal stromal disease began to develop, and the periocular skin lesions healed. By 42 days after infection (21 days after cessation of antibody treatment) 50% of the mice had stromal disease. At that time, the CD4 population had recovered to 43% of the normal level in the lymph nodes and 47% of normal in the spleen; the CD8 population had recovered to 33% of the normal level in the lymph nodes and 42% of the normal level in the spleen.

In agreement with the findings of Newell et al., depletion of CD4 cells significantly reduced the incidence (Table 3) and severity (Fig. 7A) of the stromal disease induced by RE HSV-1. Histologic examination of corneas from CD4-depleted and RE HSV-1-infected mice showed at most a mild inflammatory response. The CD8 cell depletion from RE HSV-1-infected mice did not affect the incidence (Table 3) or severity (Fig. 7B) of stromal disease, and the corneal infiltrate remained predominantly composed of PMN (not shown).

**Discussion**

Despite intensive research, HSV-1 corneal stromal disease remains the leading infectious cause of blindness in the United States and in other industrialized and third-world nations. Based on serologic findings,
most humans have been infected with HSV-1, but only a small percentage of infected individuals develop recurrent ocular disease. The factors influencing susceptibility to HSV-1 corneal disease appear to be quite complex. The genetics of the infecting virus may influence the pattern of corneal disease in that certain strains of HSV-1 have been shown to induce only superficial epithelial lesions; others produce the deep stromal lesions that frequently result in scarring and visual impairment. The genetics of the host also influence the pattern of corneal disease. Certain inbred strains of mice show greater susceptibility to HSV-1 corneal stromal disease than do other strains. Stromal lesions respond poorly to antiherpetic drugs and appear to be due largely or entirely to the inflammatory/immune response to HSV-1 antigens in the cornea.

Recent attempts to identify the particular component(s) of the immune response that contribute to the destruction of corneal tissue have produced conflicting results. Newell et al showed that depletion of CD4 cells rendered mice resistant to corneal stromal disease after infection with the RE strain of HSV-1; CD8 cell depletion had no effect. The strain RE HSV-1 appears to be more invasive in the cornea, with a greater propensity for inducing more severe stromal disease than other strains, including KOS HSV-1. We previously reported that the induction of

Table 3. Effect of in vivo T-lymphocyte subpopulation depleton on the incidence of stromal disease induced by HSV-1*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Infection</th>
<th>In vivo antibody treatment</th>
<th>Incidence of stromal disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KOS</td>
<td>Mock</td>
<td>8/20</td>
</tr>
<tr>
<td>2</td>
<td>KOS</td>
<td>Anti-L3T4</td>
<td>8/20</td>
</tr>
<tr>
<td>3</td>
<td>KOS</td>
<td>Anti-Lyt-2.2</td>
<td>15/30</td>
</tr>
<tr>
<td>4</td>
<td>RE</td>
<td>Mock</td>
<td>9/30</td>
</tr>
<tr>
<td>5</td>
<td>RE</td>
<td>Anti-L3T4</td>
<td>19/40</td>
</tr>
<tr>
<td>6</td>
<td>RE</td>
<td>Anti-Lyt-2.2</td>
<td>8/40†</td>
</tr>
<tr>
<td>7</td>
<td>RE</td>
<td>Mock</td>
<td>10/20</td>
</tr>
<tr>
<td>8</td>
<td>RE</td>
<td>Anti-Lyt-2.2</td>
<td>11/20</td>
</tr>
</tbody>
</table>

* Groups of mice received topical corneal infections with $5 \times 10^4$ PFU of the indicated strain of HSV-1. On days -1, +2, and +6 relative to the time of infection, and at weekly intervals thereafter, each mouse received an intraperitoneal injection of the indicated rat MoAb. Data are recorded as the ratio of eyes in which corneal stromal disease developed to total eyes after 21 days of observation.

† $P = 0.009$. 

Fig. 3. Immunohistochemical staining of corneas from mock-depleted mice obtained 21 days after corneal infection with KOS HSV-1 (A and B) or RE HSV-1 (C and D). Corneas were stained for CD8 cells (A and D) or CD4 cells (B and C). The KOS HSV-1-infected corneas show a preferential accumulation of CD8 cells, and the RE HSV-1-infected corneas exhibit a preponderance of CD4 cells. Arrows indicate positive reactions A and B, original magnification X82; C and D, original magnification, X41.
specific cellular immune tolerance to HSV-1 antigens reduces the susceptibility of A/J mice to corneal stromal disease after infection with KOS HSV-1. Resistance to stromal disease was associated with suppressed HSV-1-specific and MHC class I restricted CTL activity but not with DTH unresponsiveness. These studies suggested the involvement of CD8 cells, but not CD4 cells, in the pathogenesis of KOS HSV-1-induced corneal stromal disease.

We demonstrated that topical corneal HSV-1 infection results in infection and expression of the HSV-1 genome in corneal epithelial cells, stromal fibroblasts, and endothelial cells underlying the site of infection (submitted for publication). During the first 2–3 days after corneal infection, the virus replicates in and destroys corneal epithelial cells in small, defined areas, leading to the formation of dendritic or geographic epithelial lesions. The lesions are self-limiting and are no longer visible by day 5. In the absence of a cellular immune response to HSV-1, the cornea remains clear, and the corneal architecture is preserved. When the cellular immune response to HSV-1 is not compromised, corneal damage occurs.

Fig. 4. One day before unilocal corneal infection with KOS HSV-1, groups of 20 to 30 mice were depleted of CD4 (A) or CD8 (B) cells by intraperitoneal injection of rat anti-L3T4 or anti-Lyt-2 MoAbs, respectively. The infected eyes were then examined by slit lamp on the indicated days after infection and scored for the degree of stromal opacity. Disease severity in T-cell-depleted mice was compared with that of mock-depleted controls at each period by an analysis of variance. Estimated $P$ values are given.

Fig. 5. Immunohistochemical staining of corneas from CD4-depleted mice obtained 21 days after corneal infection with KOS HSV-1. The corneas were devoid of CD4 cells (A), but numerous CD8 cells (arrows) were present in the lesions (B). Original magnification, X41.
Our current findings suggest that the nature and mechanism of corneal damage depends on the virus that infects the cornea. Stromal disease induced by the RE strain of HSV-1 requires a response of CD4 T-lymphocytes and is characterized by an inflammatory infiltrate consisting predominantly of PMNs. The mechanism that favors PMN accumulation in RE HSV-1-infected corneas is not clear. This may be related to a greater release of HSV-1 antigens from cells infected with the more virulent RE strain of HSV-1, as previously described. Increased levels of free HSV-1 antigen in the corneal stroma could favor immune complex formation with antibody, resulting in an Arthus-type reaction. Alternatively, the released HSV-1 antigen may be pinocytosed and processed by an infiltrating antigen-presenting cell such as a Langhans cell or macrophage. There is evidence for a preferential expression of exogenously acquired soluble antigens in the context of class II MHC, which may favor the activation of CD4 cells in the corneas of RE HSV-1-infected mice. The resulting production of lymphokines such as tumor necrosis factor-α by the activated CD4 cells could account for the influx of PMNs into the cornea. Thus, the palliative effect of depleting CD4 cells before corneal infection with RE HSV-1 may be due to the resulting reduction in the antibody response (and Arthus reactivity) and/or to elimination of CD4 cell-derived lymphokines from the cornea.

In contrast, the KOS strain of HSV-1 appears to preferentially activate CD8 cells in the cornea as indicated by the following observations: (1) most T-lymphocytes infiltrating KOS HSV-1-infected corneas belonged to the CD8 subpopulation; (2) the incidence of KOS HSV-1-induced stromal disease was unaltered by CD4 cell depletion, and numerous CD8 cells were present in the corneal lesions of these mice and (3) suppression of the CD8-cell-mediated CTL response to HSV-1 antigens was associated with markedly reduced susceptibility to stromal disease after corneal infection with KOS HSV-1. When CD8 cells were depleted before KOS HSV-1 corneal infection, the disease pattern changed from one characterized by a mononuclear cell infiltrate with little edema to one characterized by a predominantly PMN infiltrate with substantial edema. In contrast, simultaneous depletion of both CD8 cells and CD4 cells before KOS HSV-1 infection virtually eliminated corneal disease. The latter two observations suggest that, in the absence of CD8 cells, KOS HSV-1 can activate CD4 cells in the cornea and induce disease that is similar to that induced by RE HSV-1.

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![Table 4. Concomitant regeneration of T-cell subpopulations and susceptibility to KOS HSV-1 corneal stromal disease*](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933152/)

<table>
<thead>
<tr>
<th>Period of CD4 and CD8 T-cell depletion (days PI)</th>
<th>% of lymph node cells</th>
<th>42 Days PI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy-1+ L3T4+ Lyt-2+</td>
<td>Stromal disease</td>
</tr>
<tr>
<td>None</td>
<td>9/20</td>
<td>60.30 41.62 15.86 11/20</td>
</tr>
<tr>
<td>−1−+42</td>
<td>0/10</td>
<td>8.11 0.64 0.04 1/10</td>
</tr>
<tr>
<td>−1−+21</td>
<td>0/20</td>
<td>26.22 19.47 5.45 10/20</td>
</tr>
</tbody>
</table>

*Groups of mice received corneal infections with KOS HSV-1. On the day before the days +2 and +6 postinfection (PI), and at weekly intervals thereafter, each mouse received an intraperitoneal injection of a mixture of rat MoAbs anti-L3T4 and anti-Lyt-2 to deplete the CD4 and CD8 T-lymphocyte populations, respectively. At 21 days PI, MoAb treatment was discontinued in some of the treated animals, permitting regeneration of the T-lymphocyte populations. All mice were examined for the appearance of corneal disease on alternate days through a 42-day observation period. Then the preauricular lymph nodes were removed, and the percentage of Thy-1+ (all T-cells), L3T4+ (CD4 T-cells) and Lyt-2+ (CD8 T-cells) were determined by immunofluorescence staining and flow cytometry. The incidence of stromal disease is presented as the ratio of the cumulative number of eyes exhibiting corneal stromal disease to total infected eyes by the indicated day PI.

**Fig. 7.** One day before uniocular corneal infection with RE HSV-1, groups of 20 to 30 mice were depleted of CD4 (A) or CD8 (B) cells by intraperitoneal injection of rat anti-L3T4 or anti-Lyt-2 MoAbs, respectively. The infected eyes were then examined by slit lamp on the indicated days after infection and scored for the degree of stromal opacity. Disease severity in T-cell-depleted mice was compared with that of mock-depleted controls at each period by an analysis of variance. Estimated P values are given.
When simultaneous treatment of mice with anti-Lyt-2 and anti-L3T4 antibodies was discontinued 21 days after corneal infection with KOS HSV-1, the mice that were previously free of HSV-1 corneal lesions began to develop stromal disease in concert with T-lymphocyte recovery. This observation has interesting implications. It constitutes an important internal control for our experiment. It also suggests that, in the absence of T-lymphocytes, the infected corneas remain clear but may harbor HSV-1 antigens. When the T-lymphocyte populations are reconstituted, HSV-1-specific T-lymphocytes infiltrate the cornea and induce corneal disease. Alternatively, it is possible that the periorcular skin lesions provided a persistent source of virus, and HSV-1 antigens are not harbored in the cornea itself. However, if the cornea was continually infected by virus from the skin, one would expect to see persistent epithelial lesions caused by HSV-1 replication in corneal epithelial cells. This was not observed. In fact, the stromal disease developed in these animals with little or no epithelial involvement.

The preferential activation of CD8 cells in KOS HSV-1-infected corneas may reflect the reduced virulence of KOS HSV-1 relative to RE HSV-1. The KOS HSV-1-infected corneal cells may release less viral antigen into the extracellular matrix of the cornea, resulting in preferential expression of endogenously produced HSV-1 antigens in the context of class I MHC restriction elements and favoring the involvement of HSV-1-specific CD8 cells.

Depletion of CD8 cells only moderately reduced the incidence and did not affect the severity of the stromal disease induced by KOS HSV-1. The increased presence of PMN in the stromal lesions of the CD8-depleted and KOS HSV-1-infected mice that developed disease suggested that the reaction of CD8 cells to HSV-1 antigens in the cornea may help to reduce the chemotraction of PMNs. The mechanism by which CD8 cells reduce PMN infiltration into KOS HSV-1-infected corneas is not clear. Two obvious possibilities include: (1) elimination by HSV-1-specific CTL of KOS HSV-1-infected corneal cells before their release of a significant amount of virus antigen, or factors that are directly chemotactic for PMNs, or (2) suppression by CD8 cells of CD4 cell production of lymphokines such as tumor necrosis factor that may attract PMNs to the cornea.

The marked presence of CD8 cells and the paucity of PMN in the corneal lesions of CD4-depleted and KOS HSV-1-infected mice suggested that the CD8 cells themselves may have mediated significant tissue damage in the cornea, presumably due to their cytotoxic activity. In this regard, the effect of the CD4 cell depletion on the CTL activity of RLN cells was interesting. The expansion of the CTLp in vivo appears to be independent of CD4 help; a 90%-95% reduction of the CD4 subpopulation had no effect on the CTLp frequency. In contrast, the optimal proliferation and/or differentiation of the CTLp into active cytotoxic cells in vitro did appear to require an ancillary function of CD4 cells. The RLN cells from CD4-depleted mice consistently were approximately 50% less active than RLN cells from mock-depleted mice when reconstituted in bulk culture assays. The CTL activity of CD4-depleted mice could be restored by the addition of exogenous IL-2 or IFN-γ. It is possible that the CD8 cells produce sufficient IL-2 to support the proliferation of HSV-1-specific CTLp in the lymph nodes, but the amount of IL-2 produced by the CD8 cells may be insufficient to support optimal proliferation and/or differentiation into cytotoxic cells in vitro cultures. It should be noted that HSV-1-specific CTLp do not differentiate into cytotoxic cells in the lymphoid organs, suggesting that differentiation of CTLp may occur in the target tissue (viz, the infected cornea). Whether HSV-1-specific CTLp can differentiate into active cytotoxic cells in the infected cornea in the absence of CD4 cells is questionable. Based on our current findings, it is not clear if CTL activity is impaired in the corneas of CD4-depleted mice. The corneal lesions of these mice clearly contain numerous CD8 cells, but the function of these cells has not been determined.

Based on the results of this and previous studies, it appears that susceptibility to HSV-1-induced corneal stromal disease is determined by a complex interaction between the infecting virus and the immune system of the host. A better understanding of the viral and host factors contributing to tissue destruction may facilitate the development of immunology-based therapy for this blinding disease.

Key words: cytotoxicity, delayed-type hypersensitivity, Herpes simplex virus, keratitis, T-lymphocyte

References