
Herpes Simplex Virus Stromal Keratitis Is Not Titer-Dependent and Does Not Correlate with Neurovirulence

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We developed a murine model of ocular herpes simplex virus (HSV) disease which is particularly suited for testing stromal keratitis because most animals show some evidence of infection. Using this model, we characterized the ocular disease patterns caused by ten recent low-passage clinical isolates of HSV-1, as well as those caused by the established laboratory strains HSV-1 KOS and HSV-2 333. Viral strains were evaluated for their ability to cause stromal keratitis, blepharitis, vascularization of the cornea, and mortality. The model was not useful for scoring epithelial keratitis. The ocular disease caused by the recent isolates ranged from very mild disease to severe stromal keratitis. Some of the recent isolates caused disease as severe as the two laboratory strains. A comparison of the virulence characteristics expressed by various HSV strains indicated that the ability to cause stromal disease was correlated with vascularization of the cornea (correlation coefficient = 0.797, \( P < 0.001 \)) and was not correlated with the neurovirulence of the strains (correlation coefficient 0.045, \( P > 0.05 \)). The severity of stromal keratitis was not dependent on the amount of inoculum over the range tested and a strain causing severe stromal keratitis caused severe ocular disease even when mixed with a nonstromal strain at ratios of 10:1, 100:1, and 1000:1. Invest Ophthalmol Vis Sci 30:2474–2480, 1989

Ocular herpes simplex virus (HSV) infections are a major cause of blindness in industrialized nations.\(^1\) The spectrum of infection ranges from blepharitis and epithelial disease to severe necrotizing stromal keratitis.\(^2\) Severe infections can lead to scarring and clouding of the cornea, which result in significant visual impairment or blindness.

Both host and viral factors affect the severity of ocular HSV infections. Innate host resistance clearly affects the severity of infections in inbred strains of mice. Metcalf and Michaelis\(^3\) tested different inbred strains of mice and found that the same virus strain produced differing severities of ocular disease. DBA/2 mice were most susceptible, and were followed in susceptibility by BALB/c, C3H, and C57/BL6. Stulting et al\(^4\) confirmed these findings and showed that virus replicated more efficiently in corneal fibroblast cultures from DBA/2 mice than from C57/BL6 mice. Recently, Opremcak et al\(^5\) showed that host resistance correlated with the Igh-1 phenotype in the mouse.

The immune response also has been shown to play an important role in severe ocular herpes infections. Earlier studies have shown that lymphoid cells infiltrate the cornea during severe infections and antigen–antibody complexes were present in diseased corneas.\(^6,7\) Animals depleted of polymorphonuclear neutrophils (PMNs) did not develop severe stromal disease.\(^8\) Animals previously infected with HSV or immunized against the virus developed more severe eye disease and developed disease more quickly than did control animals.\(^9,10\) Athymic mice (nu/nu) failed to develop stromal disease, while heterozygous (nu/+ ) mice developed severe eye disease.\(^11\) Athymic mice given lymphoid cells in adoptive transfer studies regained susceptibility to severe eye disease.\(^12\) These results indicate that the severe stromal keratitis which develops in ocular infections is at least partially an immunopathologic phenomenon.

Viral strain differences also influence the outcome of an ocular infection; little, however, is known about the specific viral genes or virulence factors involved.
Certain strains of virus caused severe eye infections involving the stroma, while others caused only mild epithelial infections, when tested in animal models.\textsuperscript{10,13,14}  

Centifanto-Fitzgerald et al\textsuperscript{15} studied the ocular disease produced by recombinant laboratory strains of HSV in rabbits and suggested that a gene or genes mapping from 0.70 to 0.83 map units (mu) are involved in eye disease. However, the gene or genes involved in ocular disease which map in this region have not been identified and other regions of the HSV genome have not been studied. Genes contributing to virulence in the mouse or tree shrew map between 0.7 and 0.8 mu and have been identified,\textsuperscript{3,16-21} but it is not known whether these genes are important in ocular infections. The HSV DNA polymerase gene may play an important role in the ability of a viral strain to spread from the cornea to the central nervous system.\textsuperscript{22}  

Previous studies of ocular HSV infections have relied primarily on laboratory strains of HSV and intertypic recombinants and have scored disease as the percentage of animals showing disease.\textsuperscript{14,15,18,20,21} Selection during laboratory passage could result in altered pathogenicity. The use of intertypic recombinants could also affect the disease pattern.  

As a first step in the analysis of virulence factors in ocular HSV infections, we tested the disease patterns produced by ten low-passage, recent clinical isolates of HSV-1, as well as the disease patterns produced by the commonly used laboratory strains HSV-1 KOS and HSV-2 333 in BALB/c mice. In our model system, a high percentage of the animals showed some evidence of infection and thereby allowed us to assess the strains on the basis of the percentage of animals infected and of the severity of disease. These 12 viral strains showed a wide variation in their ability to cause severe stromal disease. We found that 1) the stromal disease trait of a given strain did not correlate with mortality due to encephalitis; 2) the severity of stromal keratitis strongly correlated with vascularization of the cornea; 3) the severity of stromal disease did not change with the amount of viral inoculum over the range we tested; and 4) strains which caused severe stromal keratitis caused severe ocular disease even when mixed with avirulent strains at ratios of 1:10, 1:100, and 1:1000.  

Materials and Methods

Cell Cultures

Human foreskin fibroblasts (HFF) and African Green Monkey (VERO) cells were grown in Dulbeccos Modified Eagles medium (DME) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. Cells infected with virus were grown in DME containing 2.0% FBS with antibiotics as described above.  

Virus

HSV-1 KOS was provided by Dr. Sullivan Read (Loyola University, Maywood, IL) and HSV-2 333 was provided by Dr. Denise Galloway (Fred Hutchinson Cancer Center, Seattle, WA). Recent, low-passage isolates from patients with ocular HSV infections were provided by Dr. John W. Chandler (University of Wisconsin-Madison, Madison, WI). All viral strains were plaque-purified twice on VERO or HFF cells prior to testing. High-titer stocks of each isolate were prepared by infecting VERO cells at low multiplicity of infection (0.01) and harvesting infected monolayers when the cytopathic effect was 90 to 100%. The infected cells were subjected to three freeze-thaw cycles using a dry ice-ethanol bath, centrifuged at 1000 g to remove debris, and stored at -80°C. All experiments, with the exception of the titer dependence study, were carried out using the same high-titer stock preparations for each isolate. Viral titers were determined by plaque assay on VERO cells using DME with 2% FBS, antibiotics, and 2% methocell. All recent clinical isolates were identified as HSV-1 by kinetic neutralization tests and were passed less than four times prior to plaque purification.\textsuperscript{23} Clinical data on the disease caused by the isolates in patients were not available.  

Animal Inoculation

Three- to four-week-old BALB/c mice (Harlan-Sprague Dawley, Madison, WI) were allowed to acclimate to their new environment for 1 week prior to inoculation. Animals were examined microscopically for corneal defects prior to inoculation, and mice having corneal defects were excluded from the studies. Following anesthetization with 2.5% halothane, the right eye of each animal was scratched with a sterile 30-gauge needle with three vertical and three horizontal strokes. A 5-μl drop containing the desired amount of virus in DME with 2% FBS was then placed on the cornea and left there for 30 sec. Excess inoculum was removed with a cotton swab. Mice were then examined daily for the first 3 days and every other day thereafter for 15 to 18 days. Animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research and with NIH guidelines.
Disease Scoring

Mice were examined microscopically for evidence of disease. Stromal disease was scored: 1+, cloudiness, mild iris detail visible; 2+, iris detail obscured; 3+, cornea totally opaque; and 4+, perforation of the cornea. Vascularization was scored: 1+, <25% of the cornea involved; 2+, 25–50% involved; and 3+, >50% of the cornea involved. Blepharitis was scored: 1+, noticeably puffy eyelids; 2+, puffy eyelids with moderate crusting; 3+, eye 50% swollen shut and severe crusting; and 4+, eye totally crusted shut. Data are reported as the mean disease score for each group of mice on each day of observation (Fig. 1), or as the mean peak disease score (MPDS, Figs. 2, 3, 5). The MPDS was calculated by averaging the most severe disease score for each animal in a group regardless of the day when the peak score was observed. Epithelial disease could not be reproducibly distinguished from damage used to initiate infection and will not be discussed further.

Statistical Methods

Analysis of variance (ANOVA) and calculation of correlation coefficients was carried out on a Macintosh II computer using StatView 512+ programs. Statistically significant differences between the MPDSs of each strain were calculated at the 95% significance level using Fisher’s Protected Least Significant Difference (PLSD) test.

Results

Characterization of Disease

The ocular disease phenotypes of ten different recent clinical isolates and the laboratory strains HSV-1 KOS and HSV-2 333 were tested using BALB/c mice.
Table 1. Percentage of animals with disease after eye scratch infection

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of animals</th>
<th>Stromal keratitis</th>
<th>Vascularization</th>
<th>Blepharitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>11</td>
<td>81.8</td>
<td>90.9</td>
<td>90.9</td>
</tr>
<tr>
<td>333</td>
<td>10</td>
<td>77.7</td>
<td>44.4</td>
<td>90</td>
</tr>
<tr>
<td>970</td>
<td>11</td>
<td>100</td>
<td>90.9</td>
<td>81.8</td>
</tr>
<tr>
<td>401</td>
<td>11</td>
<td>90.9</td>
<td>90.9</td>
<td>100</td>
</tr>
<tr>
<td>347</td>
<td>12</td>
<td>40</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>311</td>
<td>10</td>
<td>20</td>
<td>66.7</td>
<td>90</td>
</tr>
<tr>
<td>360</td>
<td>10</td>
<td>63.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>365</td>
<td>9</td>
<td>60</td>
<td>55.6</td>
<td>66.7</td>
</tr>
<tr>
<td>412</td>
<td>11</td>
<td>18.1</td>
<td>45.5</td>
<td>100</td>
</tr>
<tr>
<td>994</td>
<td>11</td>
<td>0</td>
<td>54.5</td>
<td>81.8</td>
</tr>
<tr>
<td>OD4</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mock</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

and an inoculum of $1 \times 10^5$ plaque-forming units (pfu) per eye. Animals were scored for stromal disease, vascularization, and blepharitis.

Figure 1 shows the time course of the infections for all of the disease characteristics scored. Table 1 shows the percentage of animals that showed evidence of each disease scored. Stromal disease was generally not visible until 1 week post-infection and had not resolved when the studies were halted. With some strains (eg, KOS, 311, and 970), clouding of the cornea was visible as early as day 4 or 5 following infection.

Vascularization of the cornea was first visible between days 5 to 7 and continued to increase for the duration of the experiments. Comparison of the time courses of stromal disease and of vascularization suggested that these two characteristics may be related (see below). For example, comparison of strains 970, HSV-2 333, 394, 401, and HSV-1 KOS in Figure 1 shows that the time courses of stromal disease and of vascularization are parallel or overlapping.

Blepharitis was first visible between days 3 and 5, and peaked between days 7 and 9. The resolution (healing) of blepharitis varied widely, as seen by comparing strains 360 and 311.

To facilitate comparison of the strains, we calculated the MPDS for each of the strains. The results are shown in Figure 2. Figure 2A shows the MPDSs for stromal disease. Significant differences exist between the strains ($F = 9.004$, df = 134, $P = 0.0001$, one-way ANOVA). The strains can be divided into two groups based on the MPDS (by Fisher's PLSD test). The first group includes strains which cause severe stromal disease (HSV-1 KOS, HSV-2 333, 970, 401, and 394). The second group consists of strains which cause intermediate stromal disease (347, 311, 360, 365, and 412), or caused very little stromal disease (994 and OD4). Mock-infected corneas showed no evidence of stromal disease.

The strains also differ in their mean peak vascularization scores (Fig. 2B). Significant differences exist between strains with high vascularization scores (401, KOS, 394, and 970), those with intermediate scores (HSV-2 333, 347, 994, 311, 360, and 365), and those with low scores (OD4 [$F = 6.685$, df = 136, $P = 0.0001$, one-way ANOVA]).

The mean peak blepharitis scores are shown in Figure 2C. Statistical analysis indicates that the strains can be separated into three groups: strains 311, 360, 401, HSV-2 333, 347, 994, 311, 360, and 365, which had high blepharitis scores; strains 365, 994, and 412, which had intermediate blepharitis scores; and strain OD4, which caused very little blepharitis ($F = 10.499$, df = 136, $P = 0.0001$, one-way ANOVA).

Vascularization of the Cornea Correlates with the Severity of Stromal Disease

The development of stromal disease paralleled the appearance of vascularization of the cornea (Fig. 1). To determine if the degree of vascularization of the cornea correlated with the severity of stromal disease, we carried out a correlation analysis on the mean peak vascularization and stromal disease scores. There was a strong correlation between the two disease characteristics (correlation coefficient = 0.797, $P < 0.001$). Pairwise comparisons between the other
disease characteristics did not reveal any significant correlations.

**Mortality**

The viral strains were also scored for their ability to kill the mice (Fig. 3). Six strains (OD4, 365, 994, 401, 394, and 412) were not lethal, even when tested several times (412, 394, and OD4). Although in the experiment shown in Figure 3 one mouse infected with HSV-1 KOS died, in three other experiments none of the mice infected with this strain died. One mouse infected with strain 347 died on day 3. It was not clear whether this mouse died from HSV infection, since we did not attempt a virus isolation from the animal. Four strains (360, HSV-2 333, 311, and 970) were lethal. Strain 360 is particularly lethal, killing 100% of the mice by day 8.

When we compared the ability of the strains to kill mice with the ocular disease scores, we found that strains 360, 311, and 970 (all lethal) caused severe blepharitis. The time course of the blepharitis also appeared distinctive, with a very high disease peak for all three strains. Blepharitis scores did not, however, correlate with lethality (correlation coefficient 0.7, $P > 0.05$). The severity of stromal keratitis also did not correlate with neurovirulence (correlation coefficient 0.045, $P > 0.05$).

**Titer Dependence of Ocular Disease**

To determine if the amount of viral inoculum altered the stromal disease pattern, we scored ocular disease after infection with amounts of virus varying by 2 logs. We tested two strains which did not cause stromal disease (OD4 and 994) and two strains which caused severe stromal disease (401 and 394). The results are shown in Figure 4. We were unable to prepare viral stocks with titers high enough to test $1 \times 10^7$ pfu for strain OD4 and strain 994.

Strains OD4 and 994 did not cause stromal keratitis at any level of inoculum tested. Strains 401 and 394 caused severe stromal keratitis, with scores approximately 30-fold higher than the scores of mock-infected animals. We found that stromal disease scores did not vary significantly with strains 401 and 394, even though we used a 2-log difference in the amount of virus; this result suggests that stromal keratitis is not dependent on the amount of viral inoculum over the range we tested.

To further characterize the titer dependence, we carried out a mixing experiment. Strain OD4, which did not cause stromal keratitis, was mixed with strain 394, which caused severe stromal keratitis, at OD4:394 ratios of 10:1, 100:1, and 1000:1. The mixed inoculum was then applied to mouse corneas with the eye scratch method, and the mice were scored for stromal keratitis. As can be seen in Table 2,
at ratios of 10:1, 100:1, and 1000:1, strain 394 was able to cause severe stromal keratitis. These results showed 1) that a large excess of avirulent virus did not interfere with development of severe stromal keratitis; and 2) that stromal keratitis was not titer-dependent.

Discussion

Murine models are particularly suited for the study of ocular HSV infections for several reasons. Mice are less expensive than other animals; the variety of inbred strains available makes it possible to minimize genetic differences in host resistance; and the immune response in mice is extensively characterized. Our model seems well-suited for studying stromal keratitis, vascularization of the cornea, and blepharitis. The model is not suitable for studying epithelial disease. However, because stromal keratitis results in blindness, it is the most important disease characteristic. Our results confirm previous observations made with a variety of animal models. We found that strains vary considerably in the severity of ocular disease and that stromal keratitis does not appear until day 6 or 7.

Our results demonstrate that the severity of stromal disease in the murine model was not dependent on the size of the inoculum for the range of titers we tested. A 2-log difference in viral inoculum had no effect on the MPDS (Fig. 4). Wander et al. reported similar results in a rabbit model, in which there was little effect on the number of animals with stromal disease, even with a 4-log difference in inoculum.

Mixing experiments with a nonstromal disease-producing strain and a stromal disease-producing strain further confirm that stromal disease did not depend on inoculum size. Stromal disease scores did not differ even though the inoculum varied by 2 logs (Table 2). We tested a total 4-log difference in inoculum with strain 394 and found no evidence for titer dependence. Results from the mixing experiments also indicate that the presence of an excess of relatively avirulent virus does not interfere with the development of stromal keratitis. It is not clear how the relatively small inoculum of a stromal strain causes severe stromal keratitis in the presence of excess mild virus. One possible explanation is that strain 394 replicates significantly faster or to higher titers than does OD4 in the cornea.

Our finding that the abilities to cause stromal disease and mortality did not correlate (correlation coefficient 0.045, \( P > 0.05 \)) suggests that they result from separate virulence factors. For example, strains KOS, 401, and 394 caused severe stromal keratitis, but were not lethal. Strains 360 and 311, which were effective in killing mice, caused intermediate to low stromal disease, while strain 970, which caused severe stromal disease, killed 50% of the mice by day 12 post-infection. Strains which were lethal (eg, 360 and 311), however, are difficult to assess because death occurred in some animals before stromal disease peaked. Although our results suggest the genes controlling neurovirulence and stromal keratitis are separate, we cannot rule out the existence of genes which may affect both traits.

Comparisons between the different disease characteristics suggests that the development of severe stromal disease correlates with the severity and onset of vascularization of the cornea. The association between vascularization and stromal keratitis is noteworthy since stromal disease is primarily an immunopathologic phenomenon; vascularization could provide easier access of the immune system to the infected cornea. Our results raise the possibility that drugs that inhibit blood vessel formation may be useful in treating herpetic keratitis.

HSV-1 KOS has traditionally been considered an avirulent strain because it is not lethal in mice. Our data confirm this observation (Fig. 3). However, we found that HSV-1 KOS caused severe stromal keratitis in mice, indicating that the classification of HSV-1 KOS as avirulent is relevant only for neurovirulence. Our HSV-1 KOS strain can be considered virulent for ocular disease. This observation confirms a recent report by Opriščak et al. who found that HSV-1 KOS caused stromal keratitis in mice. Two laboratories have now independently confirmed the ocular virulence of HSV-1 KOS. Wander et al. have recently shown that HSV-1 KOS does not cause stromal disease in a guinea pig model, suggesting the ocu-

<table>
<thead>
<tr>
<th>OD4 inoculum</th>
<th>Strain 394 inoculum</th>
<th>Ratio OD4:394</th>
<th>Number of animals</th>
<th>Stromal disease score (mean ± SD)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2×10⁶</td>
<td>2×10³</td>
<td>10:1</td>
<td>17</td>
<td>1.3 ± 0.8</td>
<td>100</td>
</tr>
<tr>
<td>2×10⁶</td>
<td>2×10⁴</td>
<td>100:1</td>
<td>14</td>
<td>1.8 ± 1.4</td>
<td>12</td>
</tr>
<tr>
<td>2×10⁶</td>
<td>2×10⁷</td>
<td>1000:1</td>
<td>14</td>
<td>2.0 ± 1.3</td>
<td>9</td>
</tr>
<tr>
<td>1×10⁶</td>
<td>—</td>
<td>—</td>
<td>15</td>
<td>0.13 ± 0.35</td>
<td>0</td>
</tr>
<tr>
<td>—</td>
<td>1×10⁵</td>
<td>—</td>
<td>9</td>
<td>2.8 ± 1.3</td>
<td>20</td>
</tr>
</tbody>
</table>

—Mock—

Table 2. Mixed infection with avirulent and virulent isolates
lar virulence of HSV-1 KOS may be species specific. Another possible explanation for the reported differences in the virulence of HSV-1 KOS is the different virulence characteristics of KOS variants used by different laboratories.

In summary, we have shown that recent low-passage clinical isolates of HSV have a wide range of pathogenic characteristics in mice and that some isolates cause disease as severe as that caused by established laboratory strains. We have shown that our model is useful for identifying differences in the ability to cause stromal disease; that stromal disease and vascularization are strongly correlated; and that mortality and stromal disease appear to result from different virulence factors. These strains could prove useful in future studies of the virulence factors involved in ocular herpetic infections.

Key words: herpes simplex virus, stromal keratitis, strain differences, titer dependence, neurovirulence

Acknowledgments

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References