Patterns of Herpes Simplex Keratitis in Inbred Mice

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The authors have investigated the course of herpes simplex type 1 (HSV) keratitis in three different inbred strains of mice infected with four different HSV isolates. Severity of ocular disease and mortality is dependent upon both the virus isolate and the host strain. In particular, the likelihood of progression from self-limited dendritic keratitis to severe necrotizing stromal keratitis varies markedly among the virus–host strain combinations tested. When mice from strains resistant to stromal disease are crossed with mice from strains susceptible to stromal disease, the F₁ offspring are resistant, suggesting that the gene(s) controlling resistance is dominant. Corneal stromal keratocytes and embryo fibroblasts from inbred mice differ significantly in their ability to support the replication of HSV in vitro. HSV replicates more efficiently in vitro in keratocytes from mice susceptible to stromal keratitis than it does in keratocytes from mice resistant to stromal keratitis. These findings provide evidence in an animal model for both virus- and host-related mechanisms that determine susceptibility to stromal keratitis. Invest Ophthalmol Vis Sci 26:1360-1367, 1985

In man, the clinical spectrum of ocular herpes simplex virus type 1 (HSV) infection includes conjunctivitis, epithelial keratitis, stromal keratitis, disciform corneal edema, uveitis, and retinitis. Corneal epithelial HSV infections often resolve without significant residual visual impairment, while recurrent disease involving the deep stroma often causes corneal scarring and visual loss.

In stromal keratitis, the host immune response plays a major role in tissue destruction.¹⁻⁷ It has also been shown that the course of experimentally produced keratitis in rabbits is genetically determined by the infecting virus strain.⁸

Such investigations of virus and host factors that determine the course of HSV keratitis are best performed in an inbred, genetically defined host so that individual host variation is minimized. To provide such a model, we have studied the course of HSV keratitis in inbred mice.

Materials and Methods

Virus

Four isolates of herpes simplex virus type 1 (HSV) were obtained from patients with active herpetic keratitis. These viruses were grown in secondary rabbit kidney cell cultures using Eagle’s minimum essential medium with Hank’s salts containing 2% fetal calf serum (MEM-FCS). When cytopathologic effect was 3–4⁺, the cells were frozen, thawed, and sonicated. Cell debris was removed by centrifugation and the virus-containing supernatant was stored in aliquots at −70°C. Virus titers were 4.2⁻⁵.⁸⁻⁶ plaque-forming units (PFU)/ml, measured by plaque assay on Vero cells.⁹

Animal Inoculation

Four- to 8-week-old BALB/c, A/J, C57BL/6, and (C57BL/6 × BALB/c)F₁ (CB6F₁) male mice were obtained from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimatize to their new environment for at least 1 wk. Animals were anesthetized with methoxyflurane and examined with a slit lamp. Mice with preexisting ocular abnormalities were excluded from experiments.

Under general anesthesia, the corneal epithelium of the left eye of each animal was gently scratched with a 25-gauge needle, using six crisscross strokes. A sterile cotton-tipped applicator was then dipped into the virus suspension (5.0 × 10⁵ PFU/ml) and rubbed on the cornea for 15 sec. Control mice were inoculated with MEM-FCS alone. Animals were marked with methoxyflurane and examined with a slit lamp. Mice with preexisting ocular abnormalities were excluded from experiments.

Under general anesthesia, the corneal epithelium of the left eye of each animal was gently scratched with a 25-gauge needle, using six crisscross strokes. A sterile cotton-tipped applicator was then dipped into the virus suspension (5.0 × 10⁵ PFU/ml) and rubbed on the cornea for 15 sec. Control mice were inoculated with MEM-FCS alone. Animals were marked with methoxyflurane and examined with a slit lamp. Mice with preexisting ocular abnormalities were excluded from experiments.

Three times each week the mice were examined with a modified slit lamp using white and cobalt blue-filtered light after instillation of fluorescein (0.1 mg/ml in saline). The number, size, and location of dendrites and epithelial defects were recorded for each eye. The eyes were graded on a scale of 0–4⁺ for stromal infiltrate, uveitis, and vascularization. Both inoculated and un-
inoculated contralateral eyes were observed. Mice were examined daily for evidence of systemic disease and deaths were recorded.

The percentage of animals with active dendritic and stromal keratitis was calculated as the number of animals with disease divided by the number of animals alive at the time of observation. Because the percentage of animals with active stromal keratitis may be misleading if mortality is high or duration of keratitis short, the cumulative percentage of animals developing stromal keratitis was also calculated (cumulative number of animals with stromal keratitis/number of animals inoculated). The chi-square test with Yates's correction was used to test for significant differences among groups. The investigations described in this manuscript conform to the ARVO Resolution on the Use of Animals in Research.

Preparation of Keratocyte Cultures

Newborn mice were killed with anesthetic overdose at 2–5 days of age. The eyes were enucleated and rinsed in phosphate-buffered saline (PBS), and corneal epithelium was removed with a #11 scalpel blade. The cornea was excised with curved Vannas scissors and transferred to fresh PBS. Endothelium was removed with the tip of the scissors or with a sterile cotton-tipped applicator. Corneas were rinsed for 10 sec in antibiotic solution (polymyxin B, neomycin sulfate, and gramicidin—Neosporin®, Burroughs Wellcome; Research Triangle Park, NC), and then for 1 min in sterile PBS. The corneal stroma was then minced in a petri dish containing 1 ml of MEM with 10% fetal calf serum (FCS) and 100 ng fibroblast growth factor (Collaborative Research; Lexington, MA). A suspension of tissue fragments from both corneas of five mice was transferred to a 25 cm² tissue culture flask. The flask was left undisturbed for 3–4 days to allow the tissue fragments to attach to the bottom of the flask. Outgrowth of cells from explants was seen in 4–5 days. Fresh medium (1 ml) was added twice weekly until the flasks contained 5 ml, after which time one-half of the medium was replaced with fresh medium twice weekly. Cells were passed after treatment with trypsin-EDTA (Grand Island Biological Co; Grand Island, NY). In some cases, cultured keratocytes were frozen, stored in liquid nitrogen, and later thawed for use in experiments. Care was taken to assure that cultured keratocytes from each strain used in any given experiment were placed in culture at the same time and that they had been maintained in vitro for the same length of time with the same number of passages.

Preparation of Mouse Embryo Fibroblasts

Pregnant mice were killed with anesthetic overdose. Embryos were removed under sterile conditions and placed in a petri dish containing saline. The head, tail, limbs, and entrails were removed. The outer body was transferred to another petri dish containing MEM with 10% FCS. The tissue was minced and the suspension was transferred to 75 cm² flasks. Outgrowth of fibroblasts occurred in 2–4 days.

Virus Replication Assay

Stromal keratocytes from each strain of inbred mice were plated in six 35-mm petri dishes and grown to confluence. Three plates selected at random were treated with trypsin, and the number of cells in each plate was determined by counting with a hemocytometer. The remaining three keratocyte monolayers were then infected with HSV in adsorption buffer (PBS containing 2% FCS and 0.2% glucose) at a multiplicity of infection (MOI) of one or five PFU per cell. After 1 hr at 25°C, 0.75 ml of MEM containing 2% FCS was added to each monolayer and incubation was continued for 24 hr at 37°C in 5% CO₂. The plates were frozen and thawed twice at −70°C for 1 hr and then frozen overnight. On the following day, plates were thawed. The contents were carefully removed by pipette and transferred to tubes for plaque assay on Vero cells.

Results

Following inoculation with herpes simplex type 1 (HSV), inbred mice developed dendritic keratitis (Fig. 1), which persisted for 2 to 7 days. In some animals, the dendrites were accompanied by a mild, localized stromal infiltrate. Microscopic examination of sectioned corneas at this stage revealed localized epithelial
Fig. 2. Electron micrograph of the cornea of a BALB/c mouse with dendritic keratitis four days after inoculation with HSV. Virus particles (arrows) are seen in degenerating epithelial cells in the area of the dendrite (inset).

Cell disruption and typical HSV particles in the areas where dendrites were seen (Fig. 2).

During the second week after inoculation, dense stromal infiltrates were seen, often associated with persistent epithelial defects and corneal vascularization (Fig. 3). In some cases, necrotizing stromal keratitis was severe and the eyes perforated. Uveitis occurred 4–16 days postinoculation, with a peak prevalence on day 9. Some infected mice died from presumed systemic HSV infection, usually 7–11 days after inoculation.

The course of HSV keratitis varies markedly, depending upon the virus isolate used (Fig. 4). For example, isolates 1 and 4 produce stromal disease more often than isolates 2 and 3. This difference is not simply a result of differences in the ability to establish the initial epithelial infection, since those virus isolates least efficient in producing dendrites (1 and 4) most often caused stromal keratitis. The relative severity of disease produced by the four HSV isolates was the same in each of the three individual inbred mouse strains. Figure 5 illustrates the result in BALB/c mice.

Figure 6 shows that the course of HSV keratitis also depends on the host mouse strain. Seventy-five percent to 85% of the animals of each strain developed dendritic keratitis, indicating a similar rate of epithelial infection, but the likelihood of progression to stromal disease was far greater in BALB/c than in C57BL/6 mice. A/J mice
Fig. 4. Virus isolate-determined variation in the course of HSV keratitis. Ten each of C57BL/6, BALB/c, and A/J mice were inoculated in the left eye with HSV isolate 1, 2, 3, or 4 (5.0 × 10^5 PFU/ml) and observed for 30 days. Each panel represents 30 mice. Because stromal infiltrates may resolve and because mice with stromal keratitis may die from presumed systemic or central nervous system HSV infection, the percentage of surviving animals with stromal disease (Stromal Keratitis-Active) may decrease with time. The cumulative percentage of inoculated animals developing stromal keratitis (Stromal Keratitis-Cumulative) is therefore shown in addition on this and succeeding figures. The number of animals that developed stromal disease differs significantly in the following cases (chi-square test): isolate 1 vs isolate 2 (P < 0.05), isolate 2 vs isolate 4 (P < 0.005).

developed stromal keratitis with an incidence similar to that of BALB/c mice, but mortality was higher than in the BALB/c strain. For each of the four HSV isolates, the pattern of host susceptibility to stromal disease was the same: A/J ≈ BALB/c > C57BL/6. Figure 7 illustrates the result with isolate 3.

Fig. 5. Virus isolate-determined variation in the course of HSV keratitis in BALB/c mice. Ten BALB/c mice were inoculated in the left eye with HSV isolate 1, 2, 3, or 4 (5.0 × 10^5 PFU/ml) and observed for 30 days. Each panel represents 10 mice. Similar results were obtained in each of the other two host mouse strains.
HSV Isolates 1-4

C57BL/6
- Dendritic Keratitis
- Stromal Keratitis
- Active
- Stromal Keratitis
- Cumulative
- Mortality

BALB/c

A/J

Days after Inoculation

Animals with Disease (%)

Fig. 6. Host strain-determined variation in the course of HSV keratitis. Groups of ten C57BL/6, BALB/c, or A/J mice were inoculated topically in the left eye with HSV isolates 1, 2, 3, or 4 (5.0 × 10^5 PFU/ml) and observed for 30 days. Each panel represents 40 mice. The number of animals that developed stromal disease differs significantly in the following cases (chi-square test): BALB/c vs C57BL/6 (P < 1.0 × 10^-7), A/J vs C57BL/6 (P < 2.0 × 10^-6).

The course of HSV keratitis in (C57BL/6 × BALB/c)F₁ (CB6F₁) mice did not differ significantly from that of disease in C57BL/6 mice, suggesting that the gene(s) controlling resistance is dominantly inherited (Fig. 8).

The number of dendrites present on each infected cornea, the size, and the morphology of dendrites produced by each of the four HSV isolates tested on days 2, 4, and 7 postinfection were compared. No virus or host strain–related differences were observed (data not shown).

In ten (8%) of 120 animals, uveitis developed in the uninoculated right eye. This would seem to represent endogenous spread of virus, rather than exogenous infection of the uninoculated eye, since it always occurred within 2 wk of inoculation and since the right cornea never showed evidence of dendritic or stromal keratitis. Six of these animals were A/J, three were BALB/c, and one was C57BL/6, suggesting that this phenomenon may be under genetic control of the host.

Significantly more virus was produced in cultured keratocytes by BALB/c mice than in those from C57BL/6 mice (Table 1). Similar results were obtained when embryo fibroblasts were tested (Table 2), suggesting that a genetically determined difference in the ability of cells from inbred mice to support virus replication might explain, at least in part, their difference in susceptibility to HSV stromal keratitis. It can also be seen in Table 1 that an occasional pair of keratocyte cultures showed better viral replication in C57BL/6 cells than in BALB/c cells. This finding was reproduc-

Fig. 7. Host strain-determined variation in keratitis caused by HSV isolate 3. Ten each of C57BL/6, BALB/c, and A/J mice were inoculated topically in the left eye with HSV isolate 3 (5.0 × 10^5 PFU/ml) and observed for 30 days. Each panel represents 10 mice.
uble when the same keratocyte line was retested on multiple occasions. In contrast, BALB/c embryo fibroblasts consistently allowed replication of HSV to a higher titer than C57BL/6 embryo fibroblasts in all experiments.

**Discussion**

Herpetic keratitis may appear in several clinical forms ranging from a relatively benign self-limited epithelial infection to severe necrotizing stromal keratitis with stromal degradation. The former process may not result in visual loss, while the latter often causes severe visual impairment.

Previous reports indicate that both viral- and host-determined factors play a role in determining the course of herpetic keratitis. Using the inbred mouse model, we confirm these findings and present further evidence that the progression from epithelial to stromal keratitis is controlled by both viral- and host-determined genetic factors. The host gene(s) conferring resistance to stromal keratitis appears to be dominant, since F₁ hybrids between resistant (C57BL/6) and susceptible (BALB/c) strains are resistant. The data also suggest that endogenous spread of virus from the inoculated eye to produce uveitis in the uninoculated opposite eye is under genetic control of the host.

Immunologic mechanisms are known to play a role in the pathogenesis of HSV stromal keratitis. Inbred athymic (nude) mice fail to develop necrotizing stromal keratitis which affects their heterozygous litter mates because they lack functional T-lymphocytes. Resistance to stromal keratitis may be controlled by genes coding for immunoglobulin or by genes closely linked to those loci.

Data regarding nonimmunologic host factors that might also be involved in controlling the spread of HSV infection is conflicting. Fibroblasts from inbred mice differing in susceptibility to HSV infection by the intraperitoneal route were found to support the replication of HSV equally well in vitro. Similarly, inbred mice differing in susceptibility to CNS infection following footpad injection reportedly showed no difference in the ability of ganglia to support the replication of HSV in vitro. In contrast, others have found that embryo fibroblasts from susceptible strains support HSV replication better than fibroblasts from resistant strains.

In the inbred mouse strains we studied, the ability of fibroblasts and keratocytes to support the replication of HSV in vitro correlates with their susceptibility to stromal keratitis. These data suggest that differences in host susceptibility to stromal keratitis are due, at least in part, to genetically determined nonimmunologic differences in the ability of host cells to restrict the replication of HSV.

Although the differences in virus yield from keratocytes from the inbred strains tested were statistically significant, one might question whether they are biologically significant, since only 1.4- to 5.4-fold differences were seen. Since the entire replication cycle for HSV lasts about 18 hr, our experimental conditions were such that only a single cycle of replication was examined. If 5.4-fold yield differences were maintained, for example, for six replication cycles (4 days), such as might occur during the initial phase of HSV infection in vivo, then these data would predict a $2.5 \times 10^4$-fold difference in virus production by cells of BALB/c and
Table 1. Replication of HSV in keratocytes from inbred mice

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Mouse strain*</th>
<th>Cell density (cells/plate × 10^4)</th>
<th>Multiplicity of infection</th>
<th>Virus production (PFU/cell)</th>
<th>P-value†</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>2.33</td>
<td>1</td>
<td>1.71 ± 0.30</td>
<td>0.006</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>1.38</td>
<td></td>
<td>0.32 ± 0.05</td>
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<tr>
<td>1</td>
<td>BALB/c</td>
<td>3.25</td>
<td>1</td>
<td>1.69 ± 0.06</td>
<td>0.0003</td>
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<td>C57BL/6</td>
<td>2.75</td>
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<td>0.63 ± 0.03</td>
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<tr>
<td>2</td>
<td>BALB/c</td>
<td>0.70</td>
<td>1</td>
<td>131.08 ± 8.01</td>
<td>0.007</td>
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<td></td>
<td>C57BL/6</td>
<td>1.19</td>
<td></td>
<td>96.15 ± 9.98</td>
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<tr>
<td>3</td>
<td>BALB/c</td>
<td>2.02</td>
<td>1</td>
<td>73.40 ± 5.56</td>
<td>0.001</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>2.20</td>
<td></td>
<td>22.91 ± 2.03</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BALB/c</td>
<td>1.45</td>
<td>1</td>
<td>37.78 ± 2.83</td>
<td>0.79</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>2.40</td>
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<td>45.28 ± 7.65</td>
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<tr>
<td>3</td>
<td>BALB/c</td>
<td>4.72</td>
<td>5</td>
<td>21.55 ± 1.67</td>
<td>0.03</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>4.31</td>
<td></td>
<td>9.78 ± 4.15</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BALB/c</td>
<td>6.51</td>
<td>5</td>
<td>19.63 ± 0.73</td>
<td>0.003</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>5.32</td>
<td></td>
<td>9.23 ± 1.57</td>
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</tr>
<tr>
<td>4</td>
<td>BALB/c</td>
<td>0.45</td>
<td>1</td>
<td>4.00 ± 0.42</td>
<td>0.0003</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>1.13</td>
<td></td>
<td>1.70 ± 0.04</td>
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</tbody>
</table>

* Keratocyte cultures from BALB/c or C57BL/6 mice obtained from pooled minced corneas (5 mice each strain) were infected with the virus isolates at a multiplicity of infection of one or five, as shown.
† One-tailed Student’s t-test.
‡ Mean ± standard error of the mean.

Table 2. Replication of HSV in embryo fibroblasts from inbred mice

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Mouse strain</th>
<th>Cell density (cells/plate × 10^4)</th>
<th>Virus production (PFU/cell)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>3.95</td>
<td>2.98 ± 0.19†</td>
<td>0.006</td>
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<td></td>
<td>C57BL/6</td>
<td>3.37</td>
<td>2.12 ± 0.05</td>
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<tr>
<td>1</td>
<td>BALB/c</td>
<td>5.88</td>
<td>0.93 ± 0.11</td>
<td>0.002</td>
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<td></td>
<td>C57BL/6</td>
<td>5.32</td>
<td>0.16 ± 0.01</td>
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<tr>
<td>2</td>
<td>BALB/c</td>
<td>2.92</td>
<td>34.42 ± 1.65</td>
<td>0.003</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>3.66</td>
<td>23.37 ± 0.93</td>
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</tr>
<tr>
<td>2</td>
<td>BALB/c</td>
<td>7.21</td>
<td>77.18 ± 8.72</td>
<td>0.001</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>9.79</td>
<td>8.53 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BALB/c</td>
<td>2.85</td>
<td>72.32 ± 3.93</td>
<td>0.0007</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>5.25</td>
<td>30.86 ± 1.04</td>
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</tr>
</tbody>
</table>

* One-tailed Student’s t-test.
† Mean ± standard error of the mean.

C57BL/6 mice at the end of that period. Such a difference would seem to be sufficient to explain a difference in the clinical course of HSV infection.

During the course of these experiments, keratocyte cultures were initiated from fresh embryo corneas on several occasions and later used for virus replication assays. Although BALB/c keratocytes routinely supported replication of HSV more efficiently than did C57BL/6 keratocytes, an occasional keratocyte culture yielded opposite results: more efficient replication in C57BL/6 than in BALB/c keratocytes. This finding was not simply the result of random variation in the assay, since repeated testing of the same keratocyte population (maintained frozen in liquid nitrogen) gave consistent results. In contrast, embryo fibroblasts from BALB/c mice always supported HSV replication better than fibroblasts from C57BL/6 mice. We hypothesize that there is limited heterogeneity in the ability of cells from each individual mouse to replicate HSV. Since the number of keratocytes in a mouse cornea is small, only a few cells become progenitors of the entire keratocyte culture tested later in the virus production assays. It seems possible that, on occasion, those progenitors might not be representative of the population as a whole in their ability to support HSV replication, explaining our finding of an occasional keratocyte culture that gave unexpected results.

Key words: herpes simplex virus, keratitis, inbred mice, HSV stromal keratitis, viral keratitis, immunogenetics, dendritic keratitis

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