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Mapping of Genes Involved in Murine Herpes Simplex Virus Keratitis: Identification of Genes and Their Modifiers

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Herpes simplex keratitis (HSK) is an inflammatory response to viral infection and self antigens in the cornea and is a major cause of blindness. Using two strains of mice which are susceptible (129/SVEV) and resistant (C57BL/6) to herpes simplex virus (HSV) strain KOS, (129/SVEV × C57BL/6)F_2 mice were generated and examined for their disease susceptibility in terms of clinical symptoms, ocular disease, and antibody production following corneal scarification with HSV (KOS). A genome-wide screen was carried out using microsatellite markers to determine the genetic loci involved in this response. Loci on chromosomes 4, 5, 12, 13, and 14 were shown to be involved in general susceptibility to clinical disease, whereas loci on chromosomes 10 and 17 were shown to be unique to ocular disease.

Herpes simplex virus (HSV) is a neurotropic DNA virus. When HSV is introduced into the eye, it infects corneal epithelial cells, keratocytes, and endothelial cells and induces herpes simplex keratitis (HSK), an inflammation of the cornea which is a major cause of corneal blindness. In addition to the direct cytopathic consequences of HSV infection, there is a considerable autoimmune component to this disease. There have been many studies showing the role of T cells in the disease (12, 13, 18, 31, 32, 34, 40, 41). That an autoimmune response is induced after HSV infection via corneal scarification has been shown in both the mouse and rat (2, 9, 17, 50), though evidence for this mechanism has not been found in humans (21, 46).

It is also clear that there are strain differences in the mouse for susceptibility to herpes keratitis (13, 32, 34, 41). Genes from the murine major histocompatibility locus (*H-2*) as well as non-*H-2* genes were found to be involved in immune responsiveness to this infection, which relates directly to susceptibility. Genetic studies using congenics have identified a keratitis-modifying locus on chromosome 12 of the mouse that is close to or identical with the immunoglobulin heavy chain (*Igh*) locus (13). This finding led to studies of the potential role of idiotypic *Igh* determinants in initiating and/or maintaining a state of self-tolerance (2). Such determinants have been shown to be cross-reactive with HSV determinants as well (50; K. Norose and E. Heber-Katz, unpublished data). However, the roles of other genes in resistance or susceptibility to HSK is unknown. Such genes might control the initial effectiveness of the immune response to the virus or the subsequent pathogenic autoreactivity unrelated to the *Igh* idiotype. These questions can be addressed by mapping genes that modify the severity and incidence of HSK in susceptible and resistant inbred mice and their progeny. This approach has been employed to understand the genetic basis underlying other autoimmune diseases (6, 16, 22, 28, 29, 49), but genes that control the autoimmune outcome of infectious agents are less well characterized (4, 8, 30, 37, 42).

In the present study, we carried out a genome-wide screen using the DNA-based mapping reagents or microsatellite markers (simple sequence length polymorphism [SSLP]) on chromosomes 1 through 19 of two parental mouse strains, 129/SVEV and C57BL/6, with the same major histocompatibility complex (MHC) haplotype (*H-2^b*). Using markers that exhibited polymorphism, we analyzed the F_2 progeny derived from matings of these two strains. Keratitis was measured by both clinical and histopathological parameters, and each was used independently as a quantitative trait phenotype for analysis with respect to the inheritance of microsatellite markers. As the first step in mapping the genes, we found multiple quantitative trait loci (QTL) that are involved in the clinical symptoms of keratitis susceptibility in addition to the previously identified locus on chromosome 12. We found multiple loci involved in ocular histopathology as well, which also included the QTL on chromosome 12. Like the QTL on chromosome 12, some of the loci were shared between these phenotypes, whereas some of the loci were unique to either clinical symptoms or histopathology. We also report the influence of sex on both the severity and incidence of HSK and the use of different genes in males and females for the particular phenotypes.

MATERIALS AND METHODS

Mice. The parental 129/SVEV mice were obtained from the Jackson Laboratory (Bar Harbor, Maine), and the C57BL/6 parental mice were acquired from the Taconic Laboratory (Germantown, N.Y.). Mice from both parental strains were bred and maintained under standard conditions at The Wistar Institute (Philadelphia, Pa.). F_1 and F_2 populations were generated to conduct the genetic studies. The female parent used for generating F_1 and intercross mice was 129/SVEV.

Infection. HSV type 1 (HSV-1) strain KOS (kindly provided by Nigel Fraser, University of Pennsylvania, Philadelphia) at a concentration of 2×10^8 PFU/ml

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was used to infect the mice. The cornea of the right eye was scarified, and each mouse received 5 μl or 10^6 PFU. The left eye was untreated.

Histology. For preparation of specimens for histology, mice were sacrificed on day 14 after infection and the eyes were enucleated, fixed in buffered 10% formalin, embedded in paraffin, cut in 5-μm-thick sections, and stained with hematoxylin and eosin.

Enzyme-linked immunosorbent assay (ELISA). HSV-1 (KOS) (10^4 PFU/50 μl/well) was added to 96-well PRO-BIND assay plates (Falcon 3915; BD and Co., Lincoln Park, N.J.) overnight at room temperature. The wells were washed three times, coated with 100 μl of a solution of 1% bovine serum albumin in phosphate-buffered saline for 2 h, washed three times, and then incubated with 50 μl of serum. The serum was used at final dilutions of 1:200, 1:1,000, and 1:5,000. Sera from uninfected C57BL/6 mice were used as a negative control, and the monoclonal HSV glycoprotein D-specific antibody B35 was used as a positive control. After 2 h, the plates were washed three times, alkaline phosphatase-coupled goat anti-mouse immunoglobulin (catalog no. A3562; Sigma) was added at a concentration of 1:10,000 for 1 h, and the plates were washed four times. The substrate *p*-nitrophenylphosphate was then added, and the plates were developed. The plates were then read at 650 and 405 nm using an SLT Rainbow SPECTRA 96-well plate reader.

Phenotyping. Scoring of susceptibility on day 6 for clinical disease and on day 15 for histological analysis after HSV infection was determined in several different ways.

(i) Clinical disease score. The clinical disease score was indicated as follows: 0, normal; 1+, light sensitivity; 2+, mild blepharitis; 3+, severe blepharitis; and 4+, ruffled fur and shaking.

(ii) Histological score. (a) Corneal pathology (group I). Corneal pathology was scored as follows: 0, clear cornea; 1+, slight cellular infiltration; 2+, moderate cellular infiltration; 3+, intense cellular infiltration; and 4+, severe necrotizing stromal keratitis.

(b) Anterior-chamber pathology (group II). Anterior-chamber pathology was scored as follows: 0, clear; 1+, slight cellular infiltration and/or fibrin; 2+, moderate cellular infiltration and/or fibrin; 3+, intense cellular infiltration and/or fibrin; and 4+, anterior synechia.

(c) Stromal keratitis score (group III). Stromal keratitis was scored as follows: 0, clear; 1+, corneal opacity and neovascularization in less than 25% of the cornea; 2+, less than 50% corneal opacity and neovascularization; 3+, less than 75% corneal opacity and neovascularization; and 4+, 75 to 100% corneal opacity and neovascularization.

Mice were assigned to one of three arbitrary severity classes based on their low, intermediate, or severe keratitis (HSK 0 to 1, 2, or 3 to 4). This trait was used to develop information on the linkage of loci that affected this qualitative severity and susceptibility trait. In addition, mice that showed at least a 1 in either of the histopathology scores were separately evaluated for the presence of HSK to determine the loci that are involved in progression from infiltration to frank disease.

Genetic analysis. Genomic DNA was prepared from the liver of each animal in the (129/SVEV × C57BL/6) × (129/SVEV × C57BL/6)F_2 population. DNA from the frozen liver was prepared using a DNA tissue extraction kit (Qiagen Inc., Valencia, Calif.). PCR primers, purchased from Research Genetics (Huntsville, Ala.), were employed to perform a genome-wide scan of the mouse. Amplification was conducted using Boehringer Mannheim reagents with the following concentrations: 1× PCR buffer, 0.375 mM deoxynucleoside triphosphates, 0.5 U of of *Taq* polymerase/μl, 0.165 μM (each) primer, and 160 ng of genomic DNA/20 μl. The cycling conditions included a 1-min denaturing at 95°C; 35 to 50 cycles of 1 min at 94°C, 1 min 30 s at 55°C, and 2 min 10 s at 72°C; and a 6-min final extension at 72°C. The PCR products were resolved using 3% Metaphor agarose (FMC, Rockland, Maine) and were visualized through ethidium bromide staining. This method was followed for the majority of polymorphic markers.

Statistics. Genotype data were organized and analyzed through the use of Map Manager QT (27). For quantitative trait analysis, SSLP markers were evaluated individually based on linkage to the phenotypes, and intervals containing likely QTL were identified by interval mapping using the Zmap program in QTL Cartographer model 3 (http://statgen.ncsu.edu/qtlcart/cartographer.htm4). Critical values for the declaration of significance were determined by the permutation test routine in QTL Cartographer, based on a regression model developed by Churchill and Doerge (10, 11). The values were given in terms of a likelihood ratio statistic (LRS) and were based on the following values: $\alpha = 0.37$ (suggestive) (27), $\alpha = 0.1$ (strongly suggestive), $\alpha = 0.05$ (significant), and $\alpha = 0.01$ (highly significant) 7; http://mapmgr.roswellpark.org/mapmgr.html). LRS is equivalent to 4.6 LOD (likelihood of linkage).

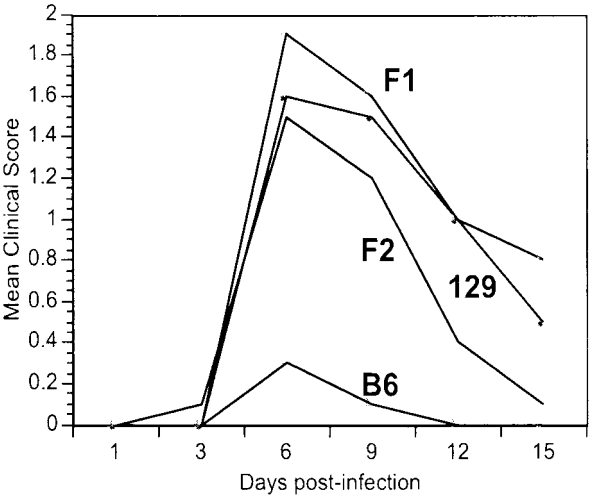

FIG. 1. Kinetics of disease susceptibility. Ten mice each of the parental strains, 129/SVEV (129) and C57BL/6 (B6), and (129/SVEV × C57BL/6)F_1 and F_2 mice were infected with 10^6 PFU of HSV/mouse and followed for clinical symptoms of disease for the next 15 days.

RESULTS

Susceptibility to ocular HSV infection. Corneal scarification was carried out using the KOS strain of HSV-1 at 10^6 PFU/mouse. In the initial susceptibility experiments, male and female C57BL/6 and 129/SVEV mice were infected and followed for disease. Peak disease occurred on day 6, and after this time point, all of the animals recovered (Fig. 1). It can be seen in Fig. 1 that C57BL/6 mice were relatively resistant to disease and that 129/SVEV mice were relatively susceptible to disease.

We next crossed the parental strains, generating (129/SVEV × C57BL/6)F_1 mice, and intercrossed those F_1 mice to generate F_2 mice. Both F_1 and F_2 mice (10 mice each) were infected with virus. The disease kinetics in all groups were similar (Fig. 1), and day 6 was chosen as the time point to analyze disease susceptibility in all further studies.

In contrast, the incidence and disease severity in all of the groups of parental and offspring mice were different from one another. On day 6, less-severe disease was seen in C57BL/6 mice (Fig. 2B), whereas the majority of 129/SVEV mice achieved a disease level of ≥3 (Fig. 2A). Both F_1 (Fig. 2C) and F_2 (Fig. 2D) populations showed an intermediate level of keratitis that was less severe than that shown by the 129/SVEV mice but more severe than that in C57BL/6 mice.

To ensure that the mice had been infected, lymph nodes and spleens were removed from the parental strains after day 15, and antigen-specific proliferative studies showed that all of the mice, both C57BL/6 and 129/SVEV, responded to glycoprotein D of HSV (data not shown), indicating that all of the mice had been infected. Therefore, the relative resistance of C57BL/6 mice seen in Fig. 1 reflects a relatively efficient virus clearance mechanism and/or genetic resistance to the immune consequences of HSV infection and not a failure to induce that infection per se .

Mapping of SSLP markers and significant threshold values. To carry out mapping studies, we generated 132 F_2 mice. All of the mice were infected at 8 weeks of age and scored for disease 6 days after infection. A graph showing disease incidence and

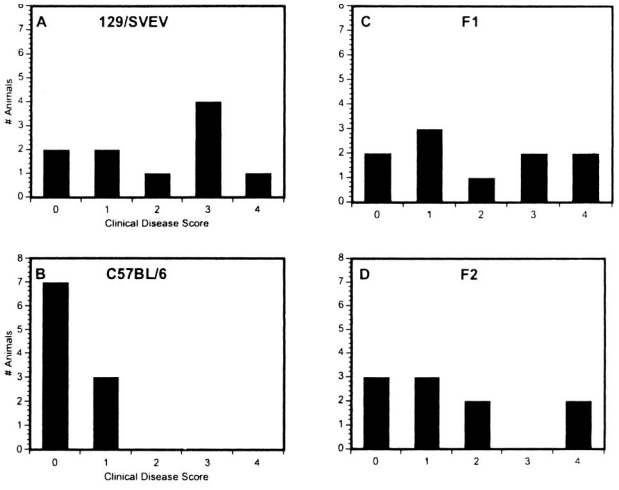

FIG. 2. Severity and incidence of disease. Ten mice were examined on day 6 after HSV infection. The number of mice with an incidence at each clinical disease score is shown. The clinical scores are as follows: 0, normal; 1, light sensitivity; 2, mild blepharitis; 3, severe blepharitis; and 4, ruffled fur and shaking.

severity in the whole F_2 population and also in the males (n = 74) and females (n = 58) can be seen in Fig. 3. The incidence of disease (score, >0) in the total population was 76%. More males (32%) were resistant to disease than females (14%), and their clinical signs were less severe. The mean of the disease level achieved in the total population was 1.7, with females achieving a mean score of 2.0 and males achieving a mean score of 1.5.

All F_2 progeny mice were also scored by ELISA for the presence of antiviral antibody. F_2 mice that did not respond to the virus inoculation either by making specific antibody to HSV (KOS) or by displaying evidence of keratitis (n = 4) were considered possibly uninfected and removed from the analysis.

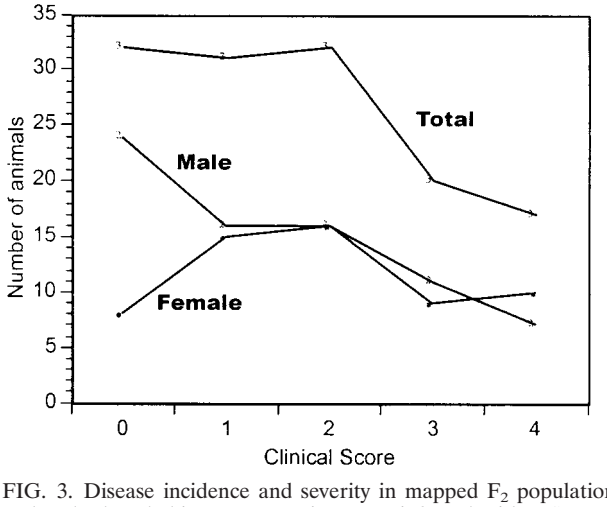

FIG. 3. Disease incidence and severity in mapped F_2 population. One hundred and thirty-two F_2 mice were infected with HSV and examined on day 6 after infection for clinical symptoms as described in the legend to Fig. 2. The data presented are the number of mice at each level of clinical disease achieved. The three populations presented in this graph are the total population, the female group in the total population, and the male group in the total population.

TABLE 1. Locations of susceptibility QTL (clinical HSK)

Marker (cM)	All F_2		Male F_2		Female F_2	
	LRS	*P* value	LRS	*P* value	LRS	*P* value
D4Mit332 (54)	11.1[a]	0.00400	8.0	0.01829	2.5	0.28419
D4Mit278 (55)	10.2	0.00600	7.1	0.02914	1.9	0.39919
D5Mit145 (0)	10.3	0.00580	0.8	0.67749	17.8[c]	0.00013
D5Mit48 (1)	10.3	0.00580	0.8	0.67749	17.8[c]	0.00013
D5Mit1 (5)	6.3	0.04379	0.7	0.71595	10.5	0.00525
D5Mit294 (8)	8.8	0.01221	2.1	0.35630	10.5	0.00525
D12Mit20 (58)	15.1[a]	0.00053	9.9	0.00706	8.7	0.01270
D12Mit134 (59)	15.1[a]	0.00053	9.9	0.00706	8.7	0.01270
D13Mit137 (21)	10.4	0.00544	8.3	0.01599	3.0	0.22396
D13Mit139 (32)	11.2[a]	0.00374	8.8	0.01239	4.7	0.09469
D13Mit13 (35)	13.4[a]	0.00125	8.2	0.01694	7.0	0.03006
D13Mit254 (40)	10.1	0.00647	8.3	0.01607	4.2	0.12360
D13Mit103 (44)	10.9[a]	0.00433	10.1	0.00641	4.2	0.12360
D14Mit228 (46)	11.5[a]	0.00323	2.0	0.36372	15.8[b]	0.00037
D14Mit265 (48)	10.6	0.00502	1.2	0.55730	18.2[c]	0.00011
D14Mit269 (50)	11.4[a]	0.00340	1.9	0.38469	16.1[b]	0.00032
D14Mit197 (52)	12.2[a]	0.00226	2.9	0.23853	14.6[a]	0.00069
D14Mit185 (54)	10.0	0.00658	0.8	0.68400	18.7[c]	8.5e05

[a] Suggestive.
[b] Strongly suggestive.
[c] Significant.

The markers were distributed on average every 8.0 ± 4.9 centimorgans (cM) over the 19 autosomes.

The HSK susceptibility traits were mapped using the 128 mice from the (129/SVEV × C57BL/6)F_2 intercross. For assessment of the probability of genetic linkage, critical values were calculated from this database using the permutation test (7). Proposed thresholds for highly significant (α = 0.01), significant (α = 0.05), strongly suggestive (α = 0.1), and suggestive (α = 0.37) linkage (27; http://mapmgr.roswellpark.org/mapmgr.html) were assigned to maximum LRS scores, which are indicated in the tables. The order of all markers in this linkage analysis was consistent with the order predicted by the available genomic maps (Whitehead Institute-MIT and Mouse Genome Database, The Jackson Laboratory, Bar Harbor, Maine [http://www.informatics.jax.org]).

Mapping of QTL linked to the HSV clinical disease susceptibility phenotype in the F_2 population. Tables 1 and 2 (All F_2) show the microsatellite markers that were positive for linkage to the HSV susceptibility phenotype. In the F_2 cross, QTL that contribute to this phenotype and are derived from 129/SVEV exist in five primary support intervals on chromosomes 4, 5, 12, 13, and 14. Of these five intervals, the QTL on chromosome 12 had the highest likelihood of linkage to the HSK-related clin-

TABLE 2. Critical values for susceptibility QTL (clinical HSK) in Table 1

Critical value (α)		LRS		
		Total	Male	Female
0.37	Suggestive	10.9	10.3	10.3
0.1	Strongly suggestive	15.4	15.2	15.9
0.05	Significant	17.2	17.4	17.6
0.01	Highly significant	21.2	19.2	20.8

ical susceptibility trait, with a strongly suggestive likelihood of linkage ($P = 0.0005$). The other four QTL are also suggestive and are located on chromosome 4 near *D4MIT332* (54 cM), on proximal chromosome 5 near *D5Mit145* (0 cM), in the middle of chromosome 13 near *D13Mit13* (35 cM), and in the middle of chromosome 14 near *D14Mit197* (52 cM).

Mapping of QTL associated with the HSK clinical susceptibility phenotype in the male and female populations. Tables 1 and 2 also show the analysis of the clinical HSK-related susceptibility trait examined in the male and female populations separately. The suggestive loci on chromosomes 5 and 14 seen in the whole F_2 population reach significance only in the female population; these loci are not significantly linked to clinical susceptibility in the male population. The QTL on chromosome 12 displays about equal significance in both males and females ($P = 0.007$ and 0.013, respectively), but the significance of this linkage reaches a suggestive LRS value only when the combined population is analyzed. In the case of the suggestive locus on chromosome 4, this is seen only in the male population. Finally, in the case of chromosome 13, there is a region at 32 to 35 cM which gives suggestive values in both males and females and perhaps a separate locus at 44 cM which appears to be male associated.

Interval-mapping analyses supported the pointwise linkages to all the traits and enabled the accurate placement of the QTL on their respective chromosomes. In addition, the mode of inheritance was analyzed for each of the QTL, and several of them demonstrated a relationship to the phenotype that was best explained by a dominant or recessive inheritance. For example, the susceptibility allele of the QTL on chromosome 12 is recessive, consistent with its identity as a self antigen encoded by the *Igh* locus, in a model of self-tolerance where heterozygous expression of the deleting allele is sufficient to prevent self-recognition (2, 13, 50). Nevertheless, some females homozygous for the C57BL/6 allele at this QTL were severely affected with clinical HSK-related symptoms. The QTL on chromosome 5 shows recessive protection against clinical symptoms in females with homozygous expression of the C57BL/6 alleles. The QTL on chromosome 14 showed additive inheritance, where heterozygotes on average were characterized by intermediate clinical scores. The most significant intervals containing QTL are shown in Fig. 4. As with the pointwise linkages, the interval analysis showed strong sexual dimorphism in the QTL that underlie clinical disease in these progeny.

HSK histopathology. The analyses discussed above were all based on clinical disease caused by corneal scarification with HSV (KOS). However, we were interested in all loci involved not only in general disease symptoms but in keratitis and other ocular features of disease. This included iritis, uveitis, ulcerations, and neovascularization (Fig. 5).

When the analysis of the progeny was restricted to the corneal-pathology phenotype (Tables 3 and 4 [we used the histological scoring group I described in Materials and Methods]) or histopathology in general (Tables 5 and 6 [we used the histological scoring groups I to III]), two major QTL were seen on chromosomes 10 and 12. The C57BL/6-derived allele at the chromosome 12 QTL behaved as a dominant protective gene,

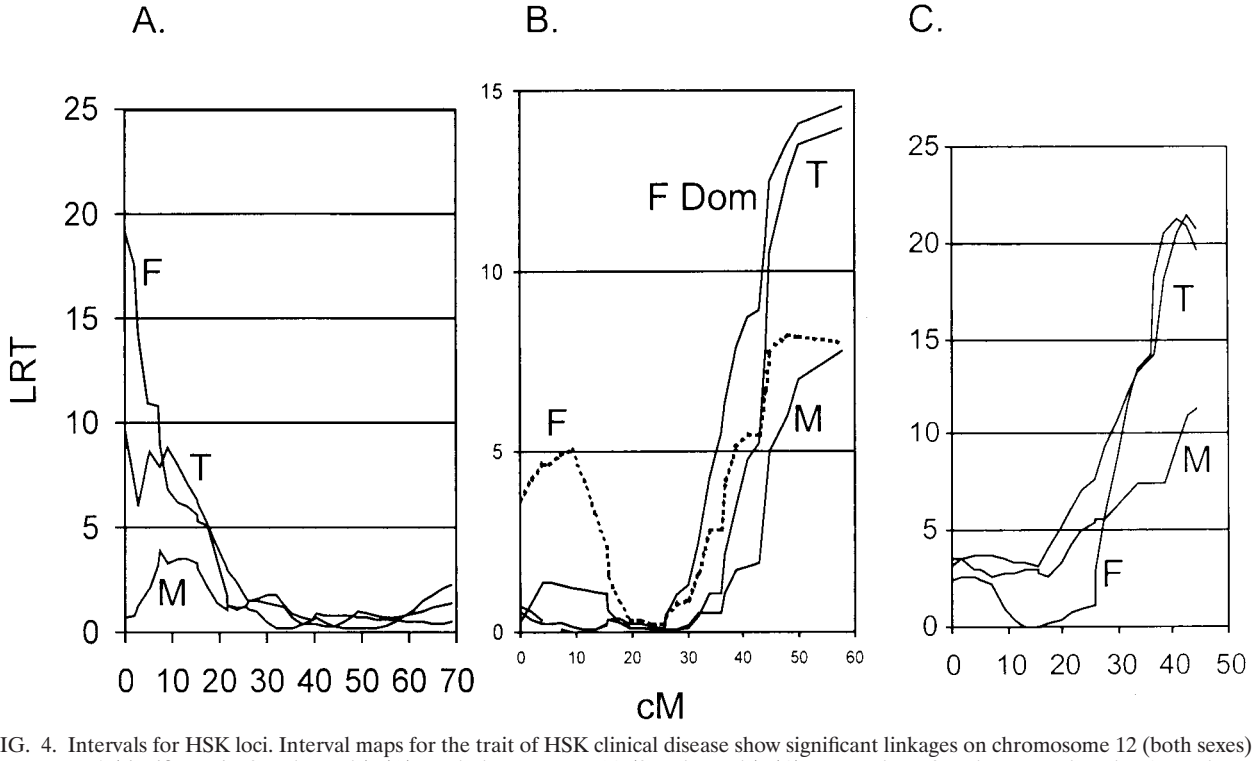

FIG. 4. Intervals for HSK loci. Interval maps for the trait of HSK clinical disease show significant linkages on chromosome 12 (both sexes) (B), chromosome 5 (significant in females only) (A), and chromosome 14 (females only) (C). T, total; F, female; M, male. The QTL shown are calculated with free regression statistics except for chromosome 12 in females, where the dominant inheritance pattern (F Dom) is also depicted. In all three of the intervals shown, a down slope on both sides of the peaks could not be shown; thus, end effects need to be considered.

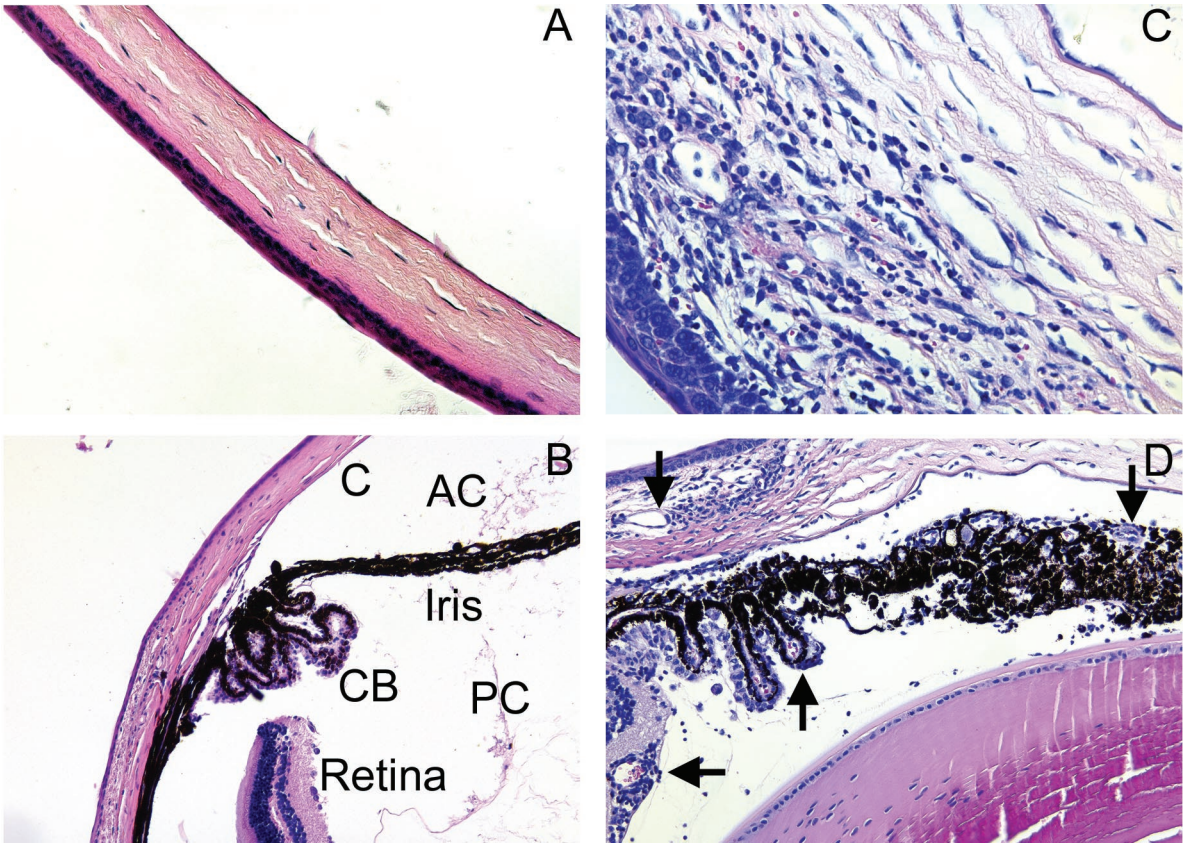

FIG. 5. Histological analysis of the eye after HSV (KOS) infection. Normal uninfected tissue from C57BL/6 mice (A and B) and tissue from infected (129/SVEV × C57BL/6)F_2 mice (C and D) were fixed in buffered 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. Infected mice showed corneal (C) infiltrates and swelling, neovascularization (arrows), and cellular infiltrates in the ciliary body (CB), iris, and anterior (AC) and posterior (PC) chambers. Magnification, ×40 (A, C, and D) or ×20 (B).

with the 129/SVEV alleles associated with severe corneal pathology; protection due to the inheritance of C57BL/6 alleles at the chromosome 10 QTL behaved as a recessive trait. A locus on chromosome 17 (near 46 cM) was also modestly linked to total histopathology of the eye in males.

Interval analysis was performed to position the QTL for the total histopathology index (Fig. 6) and corneal inflammation (not shown). As with the inheritance of clinical disease, an interval on chromosome 12 that encompasses the *Igh* locus showed linkage to the histology scores in a dominant fashion. In addition, two other intervals showed a sexually dimorphic effect.

Incidence and progression to HSK. The F_2 progeny were arbitrarily grouped into three discrete classes based on the presence and severity of keratitis (clinical disease scores 0, 1 to 2, and 3 to 4, respectively), and this discrete phenotype was tested for linkage to the markers used in the genome scan. Table 7 shows that markers on five chromosomes (chromosomes 4, 5, 12, 13, and 14) show linkage (LOD ≥ 3.0) to this incidence and severity trait. In addition, we determined whether genes in these five intervals could act as "progressor" loci, which are genetic loci capable of producing the keratitis trait from underlying inflammation. Of the 128 mice infected with HSV, 38 failed to develop any evidence of ocular inflamma-

TABLE 3. Locations of susceptibility QTL involved in corneal pathology

Marker (cM)	All F_2		Male		Female	
	LRS	*P* value	LRS	*P* value	LRS	*P* value
D10Mit186 (40)	10.8	0.0045	4.0	0.1341	9.2	0.0100
D10Mit42 (44)	11.0[a]	0.0040	3.5	0.1746	9.8	0.0074
D12Mit102 (50)	9.2	0.0099	4.3	0.1165	8.3	0.0157
D12Mit20 (58)	15.2[b]	0.0005	8.3	0.0157	10.7[a]	0.0047
D13MIT142 (37)	5.5	0.0637	5.0	0.0811	7.0	0.0297
D13Mit254 (40)	9.7	0.0077	7.0	0.0305	7.0	0.0306
D13Mit103 (44)	9.8	0.0073	8.1	0.0173	7.0	0.0306
D13MIT191 (45)	6.1	0.0482	8.0	0.0179	3.3	0.1883

[a] Suggestive.
[b] Strongly suggestive.

TABLE 4. Critical values for susceptibility QTL involved in corneal pathology in Table 3

Critical value (α)		LRS		
		Total	Male	Female
0.37	Suggestive	10.2	10.3	10.3
0.10	Strongly suggestive	15.2	15.4	15.4
0.05	Significant	16.7	16.9	17.6
0.01	Highly significant	20.3	22.0	20.8

TABLE 5. Locations of susceptibility QTL involved with total histopathology

Marker (cM)	All F_2		Male F_2		Female F_2	
	LRS	*P* value	LRS	*P* value	LRS	*P* value
D10Mit186 (40)	8.6	0.0136	2.7	0.2620	9.3	0.0095
D10Mit42 (44)	9.0	0.0113	2.3	0.3212	10.2[a]	0.0061
D12Mit102 (50)	15.4[b]	0.0005	9.2	0.0100	10.2[a]	0.0061
D12Mit20 (58)	21.9[c]	1.8e5	14.0[a]	0.0009	12.3[a]	0.0022
D17MIT158 (34)	3.6	0.1650	8.2	0.0164	0.6	0.7515
D17MIT93 (46)	6.3	0.0429	9.8	0.0075	5.5	0.0626
D17MIT155 (56)	4.5	0.1049	6.7	0.0360	0.7	0.6954

[a] Suggestive.
[b] Strongly suggestive.
[c] Highly significant.

tion. Of the remaining 90 mice with histological evidence of ocular disease, 14 had no keratitis signs and another 16 had very modest keratitis scores (<1). The 90 mice with histological evidence of disease were scored separately for the keratitis incidence and severity trait, and these results can be seen in Table 7. Markers on chromosomes 4, 5, and 12 were associated with the progression phenotype, whereas markers on chromosomes 13 and 14 did not contribute to progression. Sexual dimorphism of this trait was also noted.

Antibody titers in F_2 progeny. As a control for the success of infection in the F_2 progeny, mice were bled on day 15, at the time of sacrifice, and sera were collected for analysis by ELISA for the presence and level of antibodies to HSV (KOS). In addition, these antibody titers were used to determine if a strong anti-HSV (KOS) antibody response was correlated with either HSK susceptibility or corneal-ocular histopathology results. A linear regression between HSK susceptibility scores and the titer of anti-HSV (KOS) antibody (expressed as the optical density of binding at either of two dilutions) was performed and suggested that the two measures of response to HSV were linearly related (high disease scores were correlated with high antibody levels and vice versa: $r = 0.125$; $P = 0.0003$). The correlation was less significant when antibody titers were regressed against HSV-induced ocular or corneal histopathology ($P = 0.11$ and 0.012, respectively). In all cases, the presence of high antibody titers did not correlate with protection against subsequent histopathology or disease but rather correlated with the presence and severity of these pathologies, indicating once again that HSK is the result of excessive viral immunity rather than deficient immune response to the virus.

DISCUSSION

HSV infections. HSK is an infection of the cornea caused by HSV that leads to an inflammatory disease and consequent blindness. Susceptibility to this infectious disease has been shown to be related to both viral factors and factors involved in cellular infectibility; immune responses to the virus, both specific and innate; and immune responses to host antigens (18, 40).

In mice, HSK progression occurs after virus is cleared and requires infiltrates containing neutrophils (44) and T cells (9, 12, 17, 18, 21, 31, 40, 46). The T-cell response is predominantly a TH1 response (33), and it has been reported to be due to a cross-reaction between a corneal self antigen and a determinant on the HSV protein, UL6 (2, 50), as well as to a determinant on the immunoglobulin molecule, immunoglobulin G2. On the other hand, there have been studies showing that T-cell factors in the absence of antigen-specific T cells are sufficient, since a T-cell receptor (TcR) transgenic mouse without cross-reactive T cells can show symptoms of keratitis (15).

Confirmation of a previously identified HSK susceptibility locus. Previous results examining multiple mouse strains for susceptibility to HSV-1 keratitis showed that genes close to the *Igh-1* immunoglobulin allotype locus on chromosome 12 were associated with this trait; the *H-2* haplotype, on the other hand, was not (13). Susceptibility was associated with *Igh-1*^{c}, *Igh-1*^{dM}, and *Igh-1*^{e} alleles; resistance was associated with the *Igh-1*^{a}, *Igh-1*^{b}, and *Igh-1*^{j} alleles. *Igh*-congenic mice on an *H-2*^{d} background show HSK according to their allotype.

In the present study, we used two inbred strains of mice, C57BL/6 and 129/SVEV, which share an MHC haplotype (both are *H-2*^{b}) but differ in their non-MHC genes. That the two strains differ greatly in their susceptibilities to HSV infection, with C57BL/6 being highly resistant, has been previously reported (31, 32, 41). In this study, using HSV-1 (KOS) at 10^6 PFU/mouse, 30% of the C57BL/6 mice showed disease symptoms compared to 80% of the 129/SVEV mice. That this was not due to a difference in infectibility was shown by the fact that in both groups of parental mice given virus, all of the mice responded to HSV antigen in culture. This indicated that our method of inoculation resulted in 100% virus exposure. In the F_2 population that was generated and then mapped, the incidence of disease symptoms was 76%. Of the 32 F_2 mice that did not show disease symptoms, almost half (15 of 32) showed evidence of viral infection histologically, increasing the incidence of measured infectivity to 87%. Finally, all but 4 of the 132 F_2 mice showed titers of anti-HSV antibody that exceeded 1/1,000 dilution (not shown), again indicating the success of infection in all animals.

Carrying out a genome-wide screen at a 10-cM range, we found that in the whole F_2 population, the locus at chromosome 12, close or identical to the *Igh* locus and previously identified, showed the most significant level of linkage with the susceptibility trait as determined by clinical disease symptoms. These included light sensitivity, blepharitis, and ruffled fur. However, we also found additional novel loci on chromosomes 4, 5, 13, and 14 that regulate this trait. Furthermore, in female mice, the level of significance of the QTL on chromosomes 5 and 14 exceeded that of the chromosome 12 QTL for HSK-related clinical disease incidence and severity.

This analysis examined the total response to virus after corneal scarification and included generalized symptoms as well as specific ocular effects seen only by histology. Thus, the virus

TABLE 6. Critical values for susceptibility QTL involved with total histopathology in Table 5

Critical value (α)		LRS		
		Total	Male	Female
0.37	Suggestive	9.9	10.4	10.2
0.10	Strongly suggestive	14.9	15.9	15.4
0.05	Significant	17.0	21.8	21.0
0.01	Highly significant	19.9	21.8	21.0

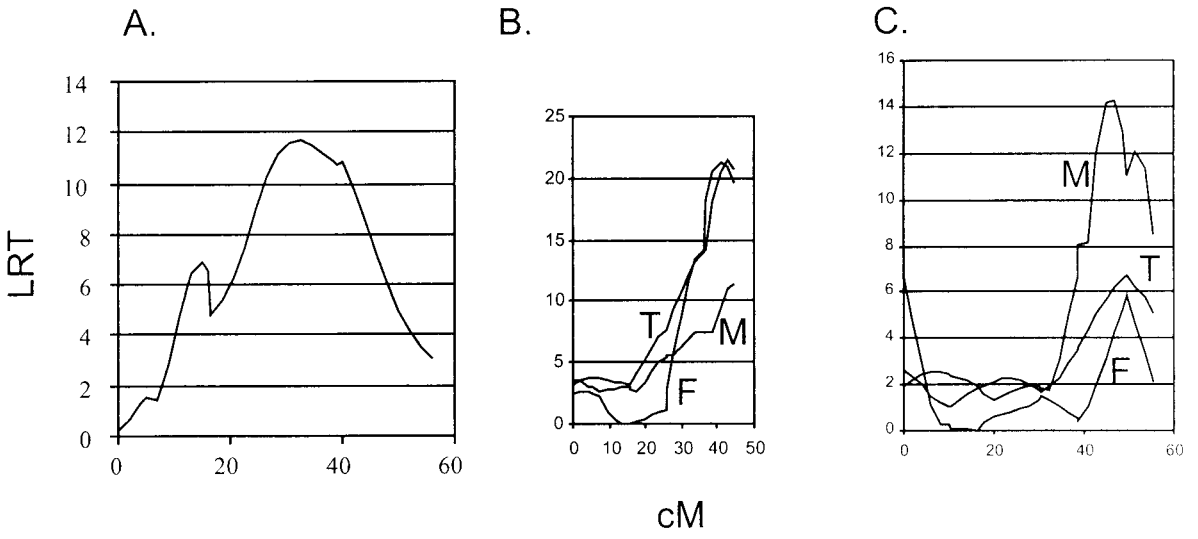

FIG. 6. Intervals for ocular histopathology. Interval maps for the trait of total ocular histopathology show significant linkages on chromosome 10 (the total population) (A), chromosome 12 (both sexes) (B), and chromosome 17 (males only) (C). The QTL shown are calculated with free regression statistics. T, total; F, female; M, male.

infects keratocytes of the eye and induces an inflammatory response in multiple locations and also infects the epithelium surrounding the eye. Susceptibility probably involves factors besides cellular susceptibility to infection, including the immune response, both innate and antigen specific, to virus and to autoantigens.

Therefore, we also used the genome-wide screen to examine the ocular histopathological traits, determining involvement of the cornea as well as other components of the eye. In this case, we identified chromosomes 12 and 13 as loci with nearly suggestive to significant levels of association which were shared with the clinical disease symptoms described above. We also found new loci at chromosomes 10 and 17 which were unique to the analysis involving ocular histopathology.

Loci in males and females: potential modifiers. We also examined the effect of sex on the response to HSV, which was made evident by the fact that the females were more susceptible to HSV disease, although there was no obvious evidence that they had a different disease phenotype.

Examination of the QTL screen in males and females separately revealed two different patterns of responsiveness. In one case, the LRS for the total population was greater than that for either the male or female group, although the QTL was linked to the phenotype at some level in each group. This could be due to the number of mice analyzed. This seems to be true for chromosome 12 and one interval on chromosome 13 at approximately 32 to 35 cM, which were only modestly significant in males and females when they were considered separately.

In the second case, the male or female value was higher than that of the total because the noncontributing male or female population caused the value in the total population to be lower. The QTL on chromosomes 5 and 14 were contributed by either females or males but not both. The QTL on chromosome 10 may be female specific, as it was quite modest in males for predicting histopathology scores.

An interesting interpretation of these data is that the locus on chromosome 12 (and possibly chromosome 13) is the key gene(s) in governing the level and character of the immune response to the virus and that the other QTL cause the disease to progress from inflammatory infiltrates to frank keratitis, with its associated clinical signs. This is consistent with the identity of the chromosome 12 QTL as a self antigen, because tolerance to the self antigen could predict the absence of an immune response to a cross-reactive viral antigen (2, 50). The fact that these two loci are also the loci shared between the

TABLE 7. Locations of loci that control severity and susceptibility to HSK

Marker (cM)	All F_2		Male F_2		Female F_2	
	LOD[a]	Progression to HSK (*P* value)[b]	LOD	Progression to HSK (*P* value)	LOD	Progression to HSK (*P* value)
D4Mit332 (54)	**4.4**		**3.2**		1.2	
D4Mit278 (55)	**4.4**	0.006	2.8	0.004	1.5	NS
D4Mit12 (58)	**4.0**			2.8	1.2	
D5Mit145 (0)	**3.7**		NS		**3.1**	
D5Mit294 (8)	**3.4**	0.04	NS		**3.1**	0.005
D12Mit102 (50)	1.3		NS		NS	
D12Mit20 (58)	**3.0**	0.03	1.8	0.12	NS	0.19
D13Mit13 (35)	3.9	NS[c]	NS	NS	2.9	NS
D13Mit51 (59)	4.2	NS	NS	NS	2.5	NS
D14Mit193 (40)	3.2		NS		2.7	
D14Mit204 (44)	**4.0**	0.27	NS	NS	**3.1**	0.02
D14Mit265 (48)	**4.0**		NS		**3.5**	
D14Mit197 (52)	3.0		NS		2.6	

[a] LOD scores for linkage of the qualitative traits of severity and susceptibility to markers on four chromosomes and their relative strengths of linkage in male and female F_2 mice. Scores of ≥3.0 are in boldface.

[b] Progression to HSK. Among those mice with histological evidence of HSV-induced ocular pathology, each of the markers in intervals shown to be linked to HSK susceptibility and severity was evaluated for linkage to progression to frank clinical signs of keratitis by analysis of variance, and the probability is indicated in the columns. All of the marker alleles linked to greater severity or progression are derived from the 129/SVEV (susceptible) strain, except those on chromosome 13.

[c] NS, not significant.

TABLE 8. Candidate genes

Chromosome	QT[a]	cM	Gene	Name of product
4	C	52	*Bmfr1*	B-cell maturation factor responsiveness 1
		54.1	*Ckb-rs1*	Creatine kinase; brain; related sequence 1
		57.2	*Grik3*	Glutamate receptor; ionotropic; kainate 3
5	C	19	*Nos3*	Nitric oxide synthase 3
		17	*Il6*	Interleukin-6
		20	*Fgfr3*	Fibroblast growth factor receptor 3
		22	*Hs3st1*	Heparan sulfate 3-*O*-sulfotransferase 1
14	C	45	*Tnfsf11*	TNF superfamily member 11
		53.5	*Irg1*	Immunoresponsive gene 1
		59	*Fgf14*	Fibroblast growth factor 14 (FHF4)
12	C, E	58–59	*Igh*	Immunoglobulin heavy chain complex
13	C, E	33	*Fgfr4*	Fibroblast growth factor receptor 4
		35	*Il9*	Interleukin-9
10	E	40.9	*Mif*	Macrophage migration inhibitory factor
		47	*Timp3*	Tissue inhibitor of MMP3
		48	*Igf1*	Insulin-like growth factor 1
		52	*Cradd*	Caspases Rip adaptor with death domain
		67	*Ifng*	Interferon gamma
17	E	45	*Lamr9*	Laminin receptor 9
		46.5	*Lhcgr*	Choriogonadotropin receptor

[a] QT, qualitative trait; C, clinical disease; E, histopathology in the eye.

different phenotypes also supports the idea that these are the key genes involved in susceptibility and that they act at an early stage. In males, the actions of the chromosome 12 and 13 QTL are apparently insufficient to create the environment necessary for frank keratitis, and a locus on chromosome 4 is necessary for full susceptibility and progression to disease (Tables 1 and 7). In females, the progressor loci are completely different and located on chromosomes 5 and 14. These QTL apparently govern the severity of the clinical signs of keratitis, and we would speculate that they act at a later stage. It should be noted that the clinical score is given on day 6 and the histological score is given on day 15, which may lead to some of the different loci seen.

Candidate genes. The analysis presented in this study does not allow us to identify the exact genes involved in herpes keratitis, but there are many genes of interest related to already-identified factors involved in the severity of HSV infection, as seen in Table 8 and the genetic intervals presented in Fig. 4 and 6, which could provide potential targets for future study.

Perhaps the most intriguing candidate is on chromosome 5: *Hs3st1*, for heparan sulfate 3-*O*-sulfotransferase 1. A receptor for HSV entry, 3-*O*-sulfated heparan sulfate, has recently been described (35, 38). It has been shown that the heparan sulfate 3-*O*-sulfotransferase 3 but not the isoform encoded by *Hst3st1*, which has antithrombin activity (26), is necessary for the activation of this receptor.

Also on chromosome 5 is a gene, *Nos3*, for an endothelial and neuronal nitric oxide synthase. NO has been shown to be important in HSV infectivity after corneal infection (23, 43). Also at this locus is the gene *Fgfr3*. It has been previously reported that a receptor for basic FGF serves as a receptor for HSV-1 (19), and *Fgfr3* and *Fgfr4*, a candidate gene on chromosome 13, could act as such receptors. Not surprisingly, there have also been many studies describing the importance of FGF in keratitis (14, 36). Another candidate gene, *Fgf14* or *Fhf-4*, on chromosome 14, shown to be important in the nervous system (39, 47), could play a role in herpesvirus infectivity.

There is a large literature on cytokine and chemokine (24) involvement in HSV infection, and it includes molecules such as gamma interferon (5, 25). Granulocyte (44) and macrophage infiltrates in keratitis (3, 45) could be affected by interleukin-6 (*Il6* on chromosome 5) and interleukin-9 (*Il9* on chromosome 13), as well as the gene for the macrophage inhibitory factor, a candidate gene found on chromosome 10.

Finally, both tumor necrosis factor (TNF) in keratitis pathogenesis (20) and a member of the TNF receptor family acting as another HSV receptor (48) have been described. In the mapping studies here, the candidate genes include a gene for a novel member of the TNF family called TRANCE, or *Tnfsf11* (chromosome 14), as well as a gene for a molecule involved in TNF receptor death domain adapter protein, or *Cradd* (1) (chromosome 10).

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