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Viral Load and a Locus on Chromosome 11 Affect the Late Clinical Disease Caused by Theiler's Virus

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Theiler's virus causes a persistent infection and a demyelinating disease of mice which is a model for multiple sclerosis. Susceptibility to viral persistence maps to several loci, including the interferon gamma locus. Inactivating the gene coding for the interferon gamma receptor makes 129/Sv mice susceptible to persistent infection and clinical disease, whereas inactivating the interferon gamma gene makes C57BL/6 mice susceptible to persistent infection but not to clinical disease. This difference in phenotype is due to the difference in genetic background. Clinical disease depends on high viral load and *Tmevd5*, a locus on chromosome 11. These results have consequences for the identification of viruses which might be implicated in multiple sclerosis.

The DA strain of Theiler's virus, a picornavirus, is responsible for a chronic demyelinating disease in genetically susceptible mice (12, 22). This natural infection is one of the best models for multiple sclerosis. During the first 2 weeks following intracranial inoculation, the virus causes an encephalomyelitis regardless of the genetic background of the mouse. Neurons of the brain and spinal cord are the main cells infected at this stage. All mice recover, although some of them may present with sequellae, such as flaccid paralysis of the hind legs (38). In genetically susceptible strains of mice, such as the SJL/J strain, this early disease is followed by a lifelong persistent infection of the white matter of the spinal cord. At this stage, the virus infects predominantly macrophage and microglial cells but also oligodendrocytes and possibly astrocytes (1, 10, 30, 31, 33). Persistence is associated with chronic inflammation and primary demyelination, which may cause gait disorders and spastic paralysis, particularly when the mice are inoculated with a high dose of virus (10^5 or 2×10^6 PFU). In contrast, genetically resistant mouse strains, such as the C57BL/6 and 129/Sv strains, eliminate the virus after early gray matter encephalomyelitis. Inflammation and demyelination does not occur in these mice.

Persistence of infection and demyelination are under the control of several host genes (3, 4, 37). The haplotype of the major histocompatibility complex class I *H-2D* gene has a major effect on viral persistence (2, 6, 24, 31, 34). *H-2D*^{b} strains, such as C57BL/6 and 129/Sv, are resistant, and their resistance is dominant. The SJL/J strain, however, is more susceptible to viral persistence than predicted by its *H-2D*^{s} haplotype (6). A locus on chromosome 10, close to the *Ifng* locus, explains most of the susceptibility of this strain (5). The role of the interferon gamma pathway in the persistence of the infection was tested by inoculating 129/Sv mice in which the gene coding for the interferon gamma receptor had been inactivated (129/Sv *Ifngr*$^{-/-}$ mice). These mice were highly susceptible to persistent infection and presented with very severe neurological symptoms (16). The role of this pathway can also be tested by using C57BL/6 *Ifng*$^{-/-}$ mice, which are now available. They offer the possibility of testing the role of the cytokine itself in the resistance of an *H-2*^{b} mouse strain. In the present article, we report that these mice were persistently infected, but at a low level, and that they did not show any clinical signs. We also show that most of the difference of susceptibility between the 129/Sv *Ifngr*$^{-/-}$ and the C57BL/6 *Ifng*$^{-/-}$ strains is due to the difference of genetic background. A complete genome scan of two F_2 crosses showed that clinically apparent disease results from two independent factors: high viral load and a locus on chromosome 11, which we called *Tmevd5* (for Theiler's murine encephalomyelitis virus demyelination locus 5). The data indicate that *Tmevd5* modifies the risk of clinical disease in mice infected at a high level.

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Janvier (Le Genest-St-Isle, France), and 129/Sv mice were purchased from the Institut Pasteur animal facility. C57BL/6 *Ifng*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). 129/Sv *Ifngr*$^{-/-}$ mice were provided by Michel Aguet (Institut Suisse de Recherches Expérimentales sur le Cancer, Lausanne, Switzerland). These mice have been described previously (11, 19). F_2 *Ifng*$^{-/-}$ and F_2 *Ifngr*$^{-/-}$ mice (see Results for the definition of these crosses) were bred at the Institut Pasteur animal facility. The mice were genotyped for *Ifng* or *Ifngr* by PCR amplification with DNA extracted from tail biopsies. The following sets of primers were used to distinguish between *Ifng*$^{-/-}$, *Ifng*$^{+/-}$, and wild-type mice: the first (5′-AGAAGTAAGTGGAAGGGCCCAGAAG-3′ and 5′-AGGGAAACT GGGAGAGGAGAAATAT-3′) set of primers amplified a 220-bp product from the *Ifng* gene, and the second (5′-TCAGCGCAGGGGCGCCCGGTTCTTT-3′ and 5′-ATCGACAAGACCGGCTTCCATCCGA-3′) set of primers amplified a 375-bp product from the 2-kb neomycin resistance gene inserted into exon 2 of the *Ifng* gene. Amplification was performed with a Gene Amp kit (Bio-Rad Laboratories, Gaithersburg, Md.) and a 9600 reactor (Perkin-Elmer Cetus, Norwalk, Conn.). After denaturation at 94°C for 2 min, 30 cycles of DNA amplification were performed under the following conditions for the first set of primers: 94°C for 40 s, 60°C for 40 s, and 72°C for 15 s. For the second set of primers, the annealing temperature was 65°C instead of 60°C. The following primers were used to distinguish between *Ifngr*$^{-/-}$, *Ifngr*$^{+/-}$, and wild-type mice: 5′-CCCAT TTAGATCCTACATACGAAACATACGG-3′ and 5′-TTTCTGTCATCATGG AAAGGAGGGATACAG-3′. These primers are located in the *Ifngr* gene, upstream and downstream, respectively, from the neomycin cassette. Amplification was carried out under the following conditions: 94°C for 2 min followed by 40 cycles at 94°C for 40 s, 55°C for 40 s, and 72°C for 2 min.

Viral inoculation. The DA strain of Theiler's virus was produced by transfection of BHK-21 cells with the pTMDA plasmid as described elsewhere (26). Three- to 4-week-old anesthetized mice were inoculated intracranially with 10^4 PFU of the DA strain in 40 μl of phosphate buffered saline (PBS). All mice were observed once or twice a week to record clinical signs and mortality. They were sacrificed at 6, 21, or 45 days postinoculation (p.i.), depending on the experiment.

Histological analysis. Anesthetized mice were perfused through the left ventricle with PBS followed by 4% paraformaldehyde in PBS. The spinal cords were dissected out, postfixed, embedded in paraffin, and sectioned as previously described (1). Detection of Theiler's virus capsid antigen in the paraffin sections was carried out with a primary rabbit hyperimmune anti-capsid serum, a secondary biotinylated goat anti-rabbit immunoglobulin, and the ABC peroxidase de-

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tection system (labelled streptavidin biotin [LSAB] peroxidase; Dako). Slides were counterstained with Mayer hematoxylin.

Extraction of RNA from the spinal cord and quantification of viral RNA. The assay has been described in detail elsewhere (6). Briefly, mice were anesthetized and perfused with 20 ml of PBS. Their spinal cords were removed, and total RNA was extracted by the procedure of Chomczynski and Sacchi (8). For each mouse, series of fivefold dilutions, starting with 10 μg of total RNA, were dot blotted on Hybond C-extra filters (Amersham Corp., Arlington Heights, Ill.). The blots were hybridized with a ^{32}P-labeled cDNA probe (specific activity, greater than 10^8 cpm/μg) specific for the 5′ extremity of the Theiler's virus genome. The hybridized filters were analyzed with a phosphorimager. For each sample, the highest dilution which gave a positive hybridization signal was used to estimate the viral RNA content. The results were expressed as a dilution factor that ranged from 1 to 625 or, to perform statistical analyses, as a score ranging from 0 to 4 (see Fig. 1 and 3 for examples of the relationship between the dilution factor and the score). The data were normalized by two procedures. First, a duplicate blot was hybridized with a β-actin-specific probe to correct, if necessary, for variations in the amount of total RNA. Second, reference samples from previous blots were repeatedly analyzed to adjust for small variations of hybridization efficiency from blot to blot. In most cases, quantification was performed by visual observation of a phosphorimager printout. For the F_2 *Ifng*$^{-/-}$ cross, quantification was also done by image analysis. Both methods gave essentially identical results.

Genetic analysis. DNA was extracted from tail biopsies by standard techniques (5). Mouse genotypes were determined with polymorphic microsatellite markers. The sequences of the PCR primers used are available online (13a). The primers were synthesized at Research Genetics, Genset, or the Institut Pasteur oligonucleotide facility. Amplifications were performed as previously described (28). The optimal annealing temperature varied from 50 to 55°C. The PCR products were visualized on 5% agarose gels stained with ethidium bromide. Genetic maps were constructed with the Mapmanager program (25). Depending on the F_2 cross and the markers, 91 to 151 mice were used to calculate genetic distances. Exclusion mapping and localization of susceptibility loci were performed with the Mapmaker/QTL program (21), and the Kosambi function was used to calculate genetic distances.

Statistical analysis. The mean and standard error of the mean (SEM) of the score of viral persistence were computed for the three genotypes at each locus. Means were compared by using analysis of variance from the Statview F-4.5 package. To account for deviation of the persistence score from the Laplace-Gauss distribution, an empirical significance level was obtained by a Monte Carlo method (data not shown). The F distribution was evaluated in two simulations of 20,000 random replicates, each under the assumption of no linkage between the genotype and the phenotype. For the simulations, persistence scores were those observed in the experiment and genotypes were randomly assigned to members of the F_2 cross. For each of the two crosses, the simulated distribution was very close to the F distribution of the table, and this distribution was used during the remainder of the study. The linkage between clinical signs and each locus was tested by a χ^2 test. Dates of appearance of clinical signs were studied as a function of genotypes with the Logrank test of the nonparametric Kaplan-Meier analysis from the Statview F-4.5 package. Statistical criteria for the genetic analysis were those from Lander and Kruglyak (20). Two levels of significance were used: suggestive linkage, when the probability of linkage by chance was less than 1.6×10^{-3} (decimal logarithm of the odds [LOD] score, >2.8), and significant linkage, when the probability of linkage by chance was less than 5.2×10^{-5} (LOD score, >4.3).

RESULTS

C57BL/6 mice become susceptible to persistent infection with Theiler's virus after inactivation of the *Ifng* gene. C57BL/6 *Ifng*$^{-/-}$ and wild-type C57BL/6 mice were inoculated intracranially with 10^4 PFU of the DA strain and observed for 45 days to record clinical signs and mortality. Some mice were sacrificed at 6, 21, or 45 days p.i. Their brains and spinal cords were dissected out to quantify viral persistence by a dot blot assay or to localize viral antigens by immunocytochemistry.

Six days p.i., the level of infection in the brain was lower in C57BL/6 *Ifng*$^{-/-}$ mice than in wild-type mice (mean value for B6*Ifng*$^{-/-}$ [$m_{\mathrm{B6}Ifng^{-/-}}$] = 1.25 ± 0.2 [$n$ = 12]; m_{B6} = 2.1 ± 0.4 [n = 11]), although the difference was not statistically significant (NS) (P = 0.0524) (Fig. 1). Twenty-one days p.i., the levels of viral RNA in the spinal cord were low and very similar in the two groups ($m_{\mathrm{B6}Ifng^{-/-}}$ = 0.2 ± 0.1 [n = 17]; m_{B6} = 0.4 ± 0.2 [n = 11]; NS) (Fig. 1). However, the locations of infected cells were different in the two groups. Infected cells were found in the white matter of the spinal cord, in the vicinity of the central canal, in three of seven C57BL/6 *Ifng*$^{-/-}$ mice examined. In contrast, a small number of infected cells were found in the gray matter of the spinal cord of two of nine C57BL/6 mice, close to the central canal (data not shown). Forty-five days p.i., the level of infection in the spinal cord was very low in the C57BL/6 control mice, as expected. In contrast, significant amounts of viral RNA were present in the spinal cords of C57BL/6 *Ifng*$^{-/-}$ mice (Fig. 1). The levels of infection were significantly different in the two strains ($m_{\mathrm{B6}Ifng^{-/-}}$ = 1.3 ± 0.4 [n = 15]; m_{B6} = 0.1 ± 0.1 [n = 12]; P = 0.0115). Immunocytochemistry did not detect viral antigens in the spinal cords of three wild-type mice examined, although there was mild inflammation in the gray matter (Fig. 2). In contrast, infected cells were found in the white matter of the spinal cord in 8 of 11 C57BL/6 *Ifng*$^{-/-}$ mice. Furthermore, this infection was associated with inflammation such as perivascular cuffs, meningitis, and diffuse parenchymal infiltration (Fig. 2). None of the C57BL/6 wild-type and C57BL/6 *Ifng*$^{-/-}$ mice showed clinical signs or died during this study.

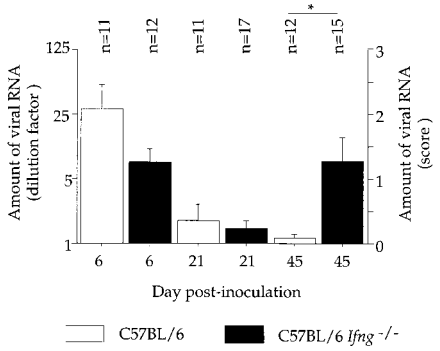

FIG. 1. Amount of viral RNA in the central nervous system at different times p.i. The amount of viral RNA was measured in the brain on day 6 p.i. and in the spinal cord on days 21 and 45 p.i. This amount is expressed as the highest RNA dilution which gave a hybridization signal (left ordinate) or the score of viral persistence (right ordinate). n, number of animals per group. *, $P < 0.05$. The error bars indicate the SEM.

In a previous work, we reported that 129/Sv *Ifngr*$^{-/-}$ mice were highly susceptible to persistent infection with Theiler's virus and died from extensive white matter disease before 45 days p.i. (16). We repeated these experiments to compare C57BL/6 *Ifng*$^{-/-}$ and 129/Sv *Ifngr*$^{-/-}$ mice under the same conditions. Forty-five days p.i., the level of viral RNA was significantly lower in C57BL/6 *Ifng*$^{-/-}$ mice than in 129/Sv *Ifngr*$^{-/-}$ mice ($m_{\mathrm{B6}Ifng^{-/-}}$ = 1.3 ± 0.4 [n = 15]; $m_{129Ifngr^{-/-}}$ = 3.8 ± 0.1 [n = 9]; $P < 0.0001$) (Fig. 3). At that time, seven of nine 129/Sv *Ifngr*$^{-/-}$ mice had developed hind-leg paralysis whereas no symptoms were observed in 26 C57BL/6 *Ifng*$^{-/-}$ mice (Table 1). No mortality was observed in either group of knockout mice.

Modifier genes controlling virus load and clinical signs explain differences between C57BL/6 *Ifng*$^{-/-}$ and 129/Sv *Ifngr*$^{-/-}$ mice. As discussed above, C57BL/6 *Ifng*$^{-/-}$ mice are less susceptible to persistent infection with Theiler's virus and to the associated clinical symptoms than 129/Sv *Ifngr*$^{-/-}$ mice. This could be due to the fact that the gene which is inactivated codes for the cytokine in the former strain whereas it codes for the cytokine receptor in the latter. Alternatively, it could be due to the difference in genetic background between the two strains. Because null mutants with the same genetic background were not available, we tested these hypotheses by comparing two F_2 crosses (C57BL/6 *Ifng*$^{-/-}$ × 129/Sv) F_2 *Ifng*$^{-/-}$ and (129/Sv *Ifngr*$^{-/-}$ × C57BL/6) F_2 *Ifngr*$^{-/-}$, for level of viral persistence and appearance of symptoms. (For the sake of

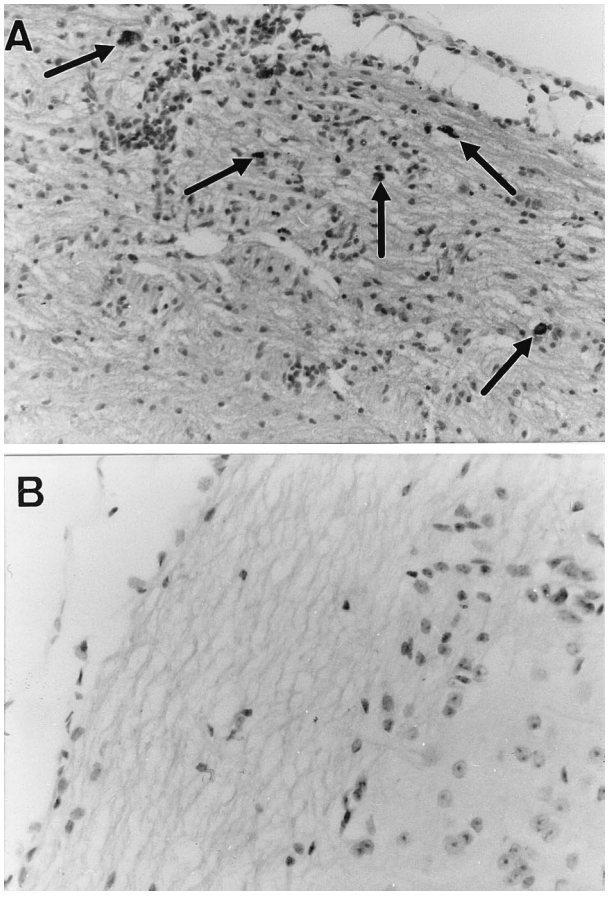

FIG. 2. Longitudinal sections of spinal cord from a C57BL/6 *Ifng*$^{-/-}$ mouse (A) and a wild-type C57BL/6 mouse (B) with Theiler's virus 45 days p.i. The arrows indicate infected cells detected by immunocytochemistry. Magnification, ×200 (A) and ×320 (B).

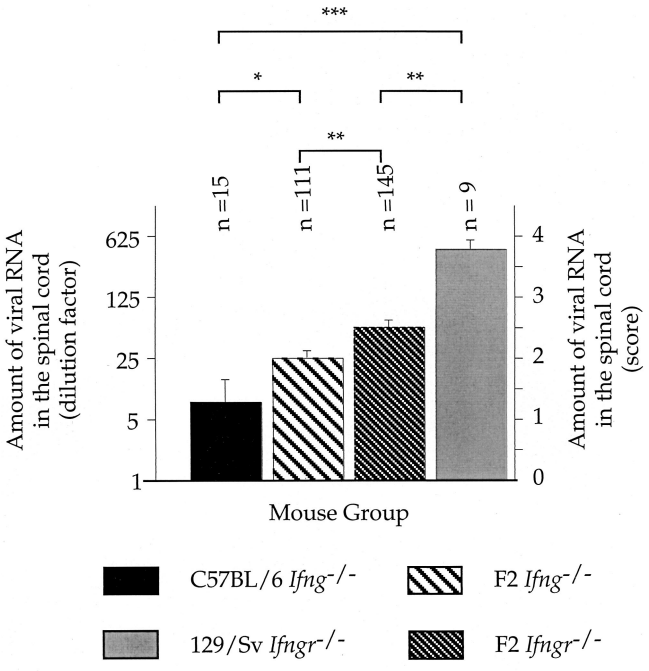

FIG. 3. Amount of viral RNA 45 days p.i. in the spinal cord of the two F_2 crosses and their parental strains. The amount of viral RNA is expressed as the highest RNA dilution (+ SEM) which gave a hybridization signal (left ordinate) or the score of viral persistence (right ordinate). *n*, number of animals per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

simplicity, these crosses will be designated F_2 *Ifng*$^{-/-}$ and F_2 *Ifngr*$^{-/-}$, respectively, in the rest of this article). If the difference in susceptibility were due to the functional differences between interferon gamma and its receptor, the F_2 *Ifng*$^{-/-}$ and C57BL/6 *Ifng*$^{-/-}$ mice should have the same phenotype and the F2 *Ifngr*$^{-/-}$ and 129/Sv *Ifngr*$^{-/-}$ mice should also have the same phenotype. If, on the other hand, the difference in susceptibility were due to the difference in genetic background, both crosses should have similar phenotypes, with large variations between animals.

One hundred and seventeen F_2 *Ifng*$^{-/-}$ and 151 F_2 *Ifngr*$^{-/-}$ animals were inoculated and monitored for clinical symptoms. After 45 days, the level of viral persistence was measured for each mouse. As shown in Fig. 3, both groups of F_2 mice were infected at a significantly higher level than the C57BL/6 *Ifng*$^{-/-}$ strain ($P = 0.0405$ and $P = 0.0014$, respectively, for F_2 *Ifng*$^{-/-}$ and F_2 *Ifngr*$^{-/-}$) and at a significantly lower level than the 129/Sv *Ifngr*$^{-/-}$ strain ($P < 0.0001$ and $P = 0.0077$, respectively, for F_2 *Ifng*$^{-/-}$ and F_2 *Ifngr*$^{-/-}$). In contrast to the C57BL/6 *Ifng*$^{-/-}$ mice, which remained asymptomatic, 14.5 and 26% of the F_2 *Ifng*$^{-/-}$ and F_2 *Ifngr*$^{-/-}$ mice, respectively, showed paralysis. Mortality was 2.5 and 4%, respectively (Table 1). The incidence of clinical disease was lower for both F_2 crosses than for the 129/Sv *Ifngr*$^{-/-}$ strain (78%). Taken together, these results imply that the difference in susceptibility between the C57BL/6 *Ifng*$^{-/-}$ and the 129/Sv *Ifngr*$^{-/-}$ mice is due mainly to the difference in genetic background. We cannot rule out a contribution of the difference in mutation, since the F_2 *Ifngr*$^{-/-}$ mice were infected at a slightly, but significantly, higher level than F_2 *Ifng*$^{-/-}$ mice ($P = 0.0039$) and showed more clinical symptoms ($P = 0.0241$). Only males contributed to the difference in susceptibility between the F_2 crosses ($m_{F_2 Ifngr^{-/-}\text{male}} = 2.7 \pm 0.1$ [$n = 81$]; $m_{F_2 Ifng^{-/-}\text{male}} = 2.0 \pm 0.20$ [$n = 57$]; $P = 0.0008$; $m_{F_2 Ifngr^{-/-}\text{female}} = 2.2 \pm 0.2$ [$n = 53$]; $m_{F_2 Ifng^{-/-}}$female $= 2.1 \pm 0.2$ [$n = 53$]; NS).

Clinical symptoms appeared at the same time in the two groups of F_2 mice (mean time of appearance, 37 days p.i.). Interestingly, in both groups, clinical signs were associated with a high level of persistent infection: 42 of 46 mice with paralysis had a persistence score higher than 3 (Fig. 4).

Localization of loci controlling viral persistence. The 145 mice of the F_2 *Ifngr*$^{-/-}$ cross and the 111 mice of the F_2 *Ifng*$^{-/-}$ cross were genotyped in an attempt to map loci controlling viral persistence. The entire genome was screened with 99 and 108 microsatellite markers, respectively. Two to eight loci were analyzed for each chromosome, depending on the size of the chromosome and on the presence or absence of a putative linkage. The distance between adjacent loci varied from 1 centi-Morgan (cM) to 27 cM. No marker was linked with viral

TABLE 1. Clinical disease for the different groups of null-mutant mice

Mouse group	No. of mice	Morbidity [no. (%)]	Mortality [no. (%)]
129/Sv *Ifngr*$^{-/-}$	9	7 (78)	0 (0)
C57BL/6 *Ifng*$^{-/-}$	26	0 (0)	0 (0)
F_2 *Ifng*$^{-/-a}$	117	17 (14.5)	3 (2.5)
F_2 *Ifngr*$^{-/-b}$	151	39 (26)	6 (4)

[a] F_2 *Ifng*$^{-/-}$ is short for (C57BL/6 *Ifng*$^{-/-}$ × 129/Sv) F_2 *Ifng*$^{-/-}$.
[b] F_2 *Ifngr*$^{-/-}$ is short for (C57BL/6 × 129/Sv *Ifngr*$^{-/-}$) F_2 *Ifngr*$^{-/-}$.

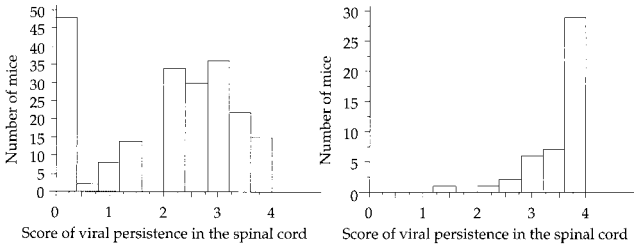

FIG. 4. Amount of viral RNA at 45 days p.i. in the spinal cords of asymptomatic (left) and symptomatic (right) mice.

persistence, even at the suggestive-linkage level, in either cross. However, in the F_2 *Ifng*$^{-/-}$ cross, weak linkage with viral persistence was observed on chromosome 19, close to *D19Mit123* ($P = 0.0028$), on chromosome 15 between *D15Mit154* ($P = 0.0059$) and *D15Mit156* ($P = 0.0043$), and on chromosome 12 between *D12Mit218* ($P = 0.0029$) and *D12Mit106* ($P = 0.0094$). These results were not confirmed with the F_2 *Ifngr*$^{-/-}$ cross.

Localization of loci controlling clinical disease. The number of clinically affected mice ($n = 39$) was large enough in the F_2 *Ifngr*$^{-/-}$ cross to warrant looking for linkage with polymorphic markers. This was not the case for the F_2 *Ifng*$^{-/-}$ cross ($n = 17$). The F_2 *Ifngr*$^{-/-}$ mice were separated into two groups, affected and nonaffected, regardless of the time of appearance of the symptoms. Linkage was tested at each locus by comparing the numbers of affected and nonaffected mice as a function of genotype with a χ^2 test. There was significant linkage with clinical disease on chromosome 11 for marker *D11Mit179* ($P < 0.0001$; LOD score = 4.8) and suggestive linkage with the two flanking markers, *D11Mit4* and *D11Mit99* ($P = 0.0008$, LOD score = 3.1, and $P = 0.0012$, LOD score = 2.9, respectively) (Table 2). In the nonaffected group, alleles segregated randomly. Susceptibility to clinical disease was conferred by the C57BL/6 allele at the *D11Mit179* locus. Indeed, among the 39 affected mice, 26 were homozygous for the C57BL/6 allelic form, 5 were homozygous for the 129/Sv allelic form, and 8 were heterozygous. This result was confirmed with the Logrank test of the nonparametric Kaplan-Meier survival analysis ($\chi^2 = 24.468$ [2 df]; $P < 0.0001$).

The results reported in a previous section of this paper suggested the existence of a relationship between clinical symptoms and a high level of viral persistence. To examine the relationship between clinical signs, viral persistence, and genotype at the *D11Mit179* locus, the amounts of viral RNA in the nonaffected and affected mice were compared as a function of genotype (Fig. 5). Clinical signs were associated with a high level of persistent infection regardless of the genotype. However, when one compares mice with high viral loads (with scores equal or superior to 3.5), 77% of the C57BL/6 homozygous mice were affected as opposed to 30% of the heterozygous mice and 36.5% of the 129/Sv homozygous mice (Fig. 5). Interestingly, there was only weak linkage between viral persistence and the *D11Mit179* marker ($P = 0.0167$). In conclusion, our results strongly suggest the presence on chromosome 11 of a locus, which we named *Tmevd5*, which controls the severity of clinical disease but not the viral load.

TABLE 2. Association of chromosome 11 markers with clinical disease

Locus		No. of affected mice[b]			No. of nonaffected mice[b]			*P* value	LOD score[c]
Name	Location (cM)[a]	B6	B6/129	129	B6	B6/129	129		
D11Mit62	1	11	21	7	19	56	27	0.7300	
D11Mit21	21.5	15	21	3	27	62	23	0.0852	
D11Mit4	33.4	23	10	6	29	57	26	0.0008	3.1
D11Mit179	41.1	26	8	5	28	58	26	<0.0001	4.8
D11Mit99	47.7	22	13	4	28	55	29	0.0012	2.9
D11Mit258	52.6	20	15	4	29	47	36	0.0040	

[a] Distance from the *D11Mit74* marker.
[b] B6, homozygous C57BL/6 allele; B6/129, heterozygous allele; 129, homozygous 129/Sv allele.
[c] The LOD score is indicated only for suggestive or significant linkage.

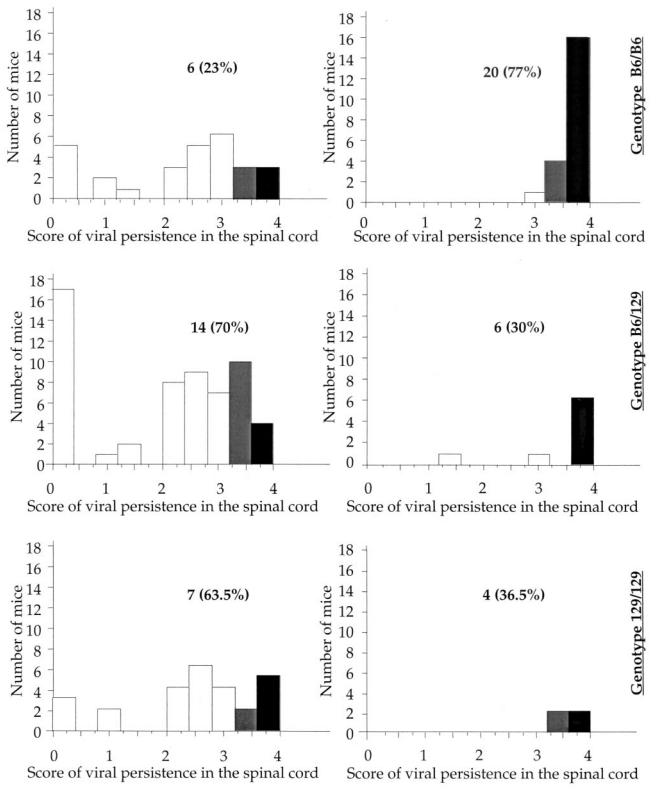

FIG. 5. Amount of viral RNA 45 days p.i. in the spinal cords of asymptomatic (left-hand graphs) and symptomatic (right-hand graphs) mice as a function of genotype at the *D11Mit179* locus. For each group of animals, the absolute number and the percentage of mice with a score of viral persistence equal or superior to 3.5 are given. These animals are indicated by hatched and solid boxes, respectively. Six asymptomatic mice died before 45 days p.i. (five were C57BL/6 homozygous, and one was 129/Sv homozygous for the *D11Mit179* marker).

In the F_2 *Ifngr*$^{-/-}$ cross, males had 2.2 times more viral RNA in their spinal cords than females. Therefore, we decided to study susceptibility to clinical signs in males and females separately. When doing so, two markers, *D19Mit65* and *D19Mit119*, had suggestive linkage with disease only in females ($P = 0.0011$ and $P = 0.0013$, respectively) (Table 3). The segregation of alleles in males and in nonaffected females was random, but in affected females there was a deficit in heterozygous animals. Therefore, there might be another locus, on chromosome 19, which controls clinical symptoms.

DISCUSSION

In a previous work, we used a cross between the SJL/J and B10.S strains to identify a locus, close to *Ifng*, which controls the persistence of Theiler's virus in the spinal cord (5). Because

TABLE 3. Association of chromosome 19 markers with clinical disease

Sex of mouse	Locus		No. of affected mice[b]			No. of nonaffected mice[b]			*P* value	LOD score[c]
	Name	Location (cM[a])	B6	B6/129	129	B6	B6/129	129		
Female	*D19Mit16*	15	5	6	4	5	31	15	0.0758	
	D19Mit65	35.9	10	1	4	10	24	17	0.0011	2.9
	D19Mit119	38.2	10	1	4	10	22	19	0.0013	2.9
	D19Mit83	42.8	9	1	5	9	22	20	0.0023	2.6
	D19Mit123	46.8	9	2	4	10	23	18	0.0070	
	D19Mit1	59.8	9	2	4	12	22	17	0.0199	
	D19Mit137	56.6	8	3	4	12	24	15	0.0638	
Male	*D19Mit16*	15	7	11	6	15	25	21	0.6988	
	D19Mit65	35.9	7	9	8	14	28	19	0.7503	
	D19Mit119	38.2	8	8	8	14	28	19	0.5002	
	D19Mit83	42.8	8	8	8	15	28	18	0.5457	
	D19Mit123	46.8	7	12	5	13	30	18	0.6272	
	D19Mit1	49.8	7	13	4	12	31	18	0.4007	
	D19Mit137	56.6	6	14	4	11	36	14	0.6905	

[a] Distance from the *D19Mit32* marker.
[b] B6, homozygous C57BL/6 allele; B6/129, heterozygous allele; 129, homozygous 129/Sv allele.
[c] The LOD score is indicated only for suggestive or significant linkage.

of the antiviral and immunoregulatory properties of interferon gamma, the *Ifng* gene was a good candidate for the control of viral persistence. Studies with 129/Sv mice in which the gene coding for the interferon gamma receptor had been inactivated demonstrated the role of the interferon gamma pathway in resistance to persistent infection (16, 35). The recent development of a C57BL/6 strain whose gene coding for interferon gamma has been inactivated allowed us to test the role of the cytokine directly. Unexpectedly, these mice were less susceptible to viral persistence and to clinical signs than the 129/Sv *Ifngr*$^{-/-}$ mice. The difference in susceptibility between the 129/Sv *Ifngr*$^{-/-}$ and C57BL/6 *Ifng*$^{-/-}$ strains could have been due to the functional differences existing between the cytokine and its receptor or to the difference in genetic background between the two mouse strains. By comparing the phenotypes of the F_2 *Ifng*$^{-/-}$ and the F_2 *Ifngr*$^{-/-}$ crosses (see Results for the definition of these crosses), we showed that 80% of the difference in susceptibility to viral persistence was due to the difference in genetic background. Since there is a small, but statistically significant, difference in viral load between the F_2 *Ifng*$^{-/-}$ and F_2 *Ifngr*$^{-/-}$ lines (Fig. 3), part of the difference in susceptibility between the parental strains might be due to the fact that the inactivated gene was different for each of them. However, we cannot rule out the possibility that some 129/Sv susceptibility loci were still present in the C57BL/6 *Ifng*$^{-/-}$ strain, since it was obtained from a 129/Sv ES cell line after only 10 backcrosses. 129/Sv *Ifng*$^{-/-}$ mice have been described by Cantin et al. since this article was submitted for publication (7). They will be invaluable to compare the roles of gamma interferon and its receptor in the pathogenesis of this model disease.

The comparison of the phenotypes of the parental strains and of the two F_2 crosses showed that the level of viral persistence was genetically controlled (Fig. 3). In spite of this, we did not find a locus linked to viral persistence when a complete genome scan of both crosses was performed. This is most likely because the number of loci involved is too high to allow the detection of linkages with a cross of approximately 100 animals. It has been reported that the C57BL/6 strain is more resistant to demyelination than other *H-2D*^{b} strains (32). The existence of a large number of non-*H-2* resistance genes in the C57BL/6 strain might put limits on the use of null-mutant mice with this background to study the pathogenesis of Theiler's virus infection. The phenotypes of the mutant mice might be less pronounced than on another resistant background, and also more difficult to interpret.

The main conclusion of the present work is that the appearance of symptoms in this model disease depends on at least two independent factors: high viral load and a locus on chromosome 11 which we named *Tmevd5*. In previous publications, we noted that the mouse strains with high persistent viral load, as measured with a dot blot assay, were usually the strains identified by other groups as susceptible to clinical disease (6, 27). In the present study we confirmed the association between high viral load and clinical disease among mice from two different F_2 crosses (Fig. 4 and 5). However, others who measured viral load with an infectivity assay did not find a correlation between viral persistence and clinical disease (9, 23, 31). The reason for this discrepancy is not clear at present. It could be due in part to trivial technical difficulties with the infectivity assay (the DA virus remains extremely membrane associated in tissue extracts).

The *Tmevd5* locus is significantly linked to clinical disease but not, or only weakly, to viral persistence. This locus could act in two different ways: (i) it could change the threshold of viral load required for the appearance of symptoms, and (ii) it could change the likelihood of appearance of symptoms for mice with a given viral load. The data shown in Fig. 5 clearly favor the second possibility: all symptomatic mice have a large viral load, regardless of genotype. However, the fraction of animals with a large viral load which are symptomatic is much higher when the animals are C57BL/6 homozygous at the *Tmevd5* locus. The characterization of the *Tmevd5* locus is now an important step in the study of the pathogenesis of this model disease. Interestingly, *Idd4*, a locus involved in the insulin-dependent diabetes of the nonobese diabetic mouse, is located in the same region (39). The *Tmevd5* C57BL/6 allele confers susceptibility, although the C57BL/6 strain is resistant. Although this may seem surprising at first, similar results have been reported with other polygenic diseases (13, 17, 29). It is likely that, in the C57BL/6 strain, the effect of a susceptible allele at one locus is overcome by the effects of resistant alleles at other loci. As shown by our work, the C57BL/6 allele at *Tmevd5* affects clinical disease only in mice with a high viral

burden. The level of viral infection was probably too low to observe the effect of this locus in the C57BL/6 *Ifng*$^{-/-}$ mice.

Our findings may have implications for the study of diseases of humans. Persistent viral infections may be asymptomatic or associated with severe diseases in humans. Infection by human T-cell leukemia virus type I is a case in point. With other viruses, such as human immunodeficiency virus, the duration of the asymptomatic phase can be highly variable from individual to individual. It is usually assumed that, in these situations, the main determinant of clinical disease is high viral burden. Our work with Theiler's virus shows that the situation might be more complex and that host genetic factors can influence the occurrence of clinical disease in individuals with the same high viral load.

Multiple sclerosis is a multifactorial disease in which environmental, presumably infectious, and genetic factors (14, 15, 18, 36) interact to cause white matter damage. In mice, several common viruses, including Theiler's virus and strains of coronaviruses, cause diseases which resemble multiple sclerosis in genetically susceptible inbred strains. By analogy with the murine models, one has to consider the possibility that multiple sclerosis results from infections by common human viruses in individuals with a specific genetic background which renders them susceptible to demyelination by a particular virus. If such were the case, the epidemiological studies performed so far to test the viral hypothesis of multiple sclerosis would be unable to establish a causal relationship between a given virus and the disease.

In conclusion, high viral load is necessary but not sufficient to cause clinical disease in mice persistently infected with Theiler's virus. The *Tmevd5* locus on chromosome 11 modifies the risk of disease in mice with a high viral burden. These results might have important implications for the study of multiple sclerosis and other multifactorial human diseases.

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