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The Th1/Th2 Balance Does Not Account for the Difference of Susceptibility of Mouse Strains to Theiler's Virus Persistent Infection[1]

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Theiler's virus causes a persistent infection with demyelination that is studied as a model for multiple sclerosis. Inbred strains of mice differ in their susceptibility to viral persistence due to both *H-2* and non-*H-2* genes. A locus with a major effect on persistence has been mapped on chromosome 10, close to the *Ifng* locus, using a cross between susceptible SJL/J and resistant B10.S mice. We now confirm the existence of this locus using two lines of congenic mice bearing the B10.S *Ifng* locus on an SJL/J background, and we describe a deletion in the promoter of the *Ifng* gene of the SJL/J mouse. We studied the expression of IFN-γ, IL-2, IL-10, and IL-12 in the brains of SJL/J mice, B10.S mice, and the two lines of congenic mice during the first 2 wk following inoculation. We found a greater expression of IFN-γ and IL-2 mRNA in the brains of B10.S mice compared with those of SJL/J mice. Also, the ratio of IL-12 to IL-10 mRNA levels was higher in B10.S mice. However, the cytokine profiles were the same for the two lines of resistant congenic mice and for susceptible SJL/J mice. Therefore, the difference of Th1/Th2 balance between the B10.S and SJL/J mice is not due to the *Ifng* locus and does not account for the difference of susceptibility of these mice to persistent infection.

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The DA strain of Theiler's virus, a murine picornavirus, causes a biphasic neurological disease after intracranial inoculation of genetically susceptible mice. The disease is characterized by an early gray matter encephalomyelitis followed by a persistent infection of the white matter of the spinal cord accompanied by chronic inflammation and primary demyelination. This late disease is studied as a model for multiple sclerosis (1, 2). Genetically resistant strains of mice clear the infection after the first 2 wk following inoculation and do not present with demyelination. Resistance/susceptibility to the persistent infection is a complex phenotype that is under the control of several host genes, including the H-2D region of the MHC (3, 4). Non-*H-2* genes are also implicated, because the SJL/J mouse is more susceptible than the B10.S mouse, although both bear the *H-2*^{s} haplotype. A gene with a major effect on persistence was mapped close to the *Ifng* gene on chromosome 10 by screening the genome of an (SJL/J × B10.S)F_1 × B10.S backcross (5). IFN-γ has a major role in limiting viral persistence and the demyelinating disease, as shown by studying mice that lack the IFN-γR (IFN-$\gamma R^{o/o}$) (6) or by treating mice with a neutralizing anti-IFN-γ Ab (7, 8). These results and the fact that, according to the current view, IFN-γ plays a central role in Th1/Th2 balance and in the outcome of murine infections in general (9–11), made the *Ifng* gene a good candidate gene for the control of Theiler's virus persistence. In the present work, we studied the *Ifng* gene and the cytokine profiles of SJL/J and B10.S mice following inoculation with Theiler's virus. We also used congenic mice to confirm the presence of a susceptibility gene in the *Ifng* region and to test the role of the *Ifng* gene and Th1/Th2 balance in the difference of susceptibility between the SJL/J and B10.S strains.

Materials and Methods

Animals

SJL/J mice were purchased from Janvier (Saint-Berthevin, France); and B10.S-H2S/Sg McdJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Two lines of congenic mice, named SJL.10(70/14) and SJL.10(233/237), were obtained after 10 successive backcrosses of an (SJL/J × B10.S)F_1 toward the SJL/J parent followed by brother/sister mating. These mice were selected because they have the B10.S allelic form of the *D10 Mit70* and *D10 Mit14* markers (line SJL.10(70/14)) or the *D10 Mit233* and *D10 Mit237* markers (line SJL.10(233/237)) (Fig. 4). The sequence of these markers is available at http://www.genome.wi.mit.edu/cgi-bin/mouse/index. A detailed description of these lines will be given elsewhere (F.B., unpublished observations). Mice of 3–4 wk of age were inoculated intracranially with 10^4 PFU of the DA strain of Theiler's virus in 40 μl of PBS buffer.

Gene sequencing

A 3-kb fragment containing the promoter of the *Ifng* gene (GenBank accession number M28381) was amplified by PCR from the genomic DNA of SJL/J and B10.S mice. The DNA fragments were subcloned in the Bluescript KS vector (Stratagene, Cambridge, U.K.) and sequenced using the Sequenase kit (Amersham, Arlington Heights, IL). Two polymorphisms were detected between the two mouse strains: a mutation in an *Sph*I restriction enzyme site of the SJL/J genome (GCATGC→ACATGC) and a 16-bp deletion, which we named *D10Pas4*, just upstream of the TATA box (see *Results*). The following PCR primers were designed to study *D10Pas4*: 5′-GAATCCCACAAGAATGGCACAG-3′ and 5′-CGAAG

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GCTCCTCGGGATTA-3′. PCR reactions with these primers were conducted under standard conditions with buffer (1.5 mM Mg^{2+}) and *Taq* DNA polymerase from Life Technologies (Eragny, France) (at 94°C for 2 min, followed by 40 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 15 s). Amplified DNA was analyzed in 5% agarose gels. Microsatellite allele sizes in inbred strains and subspecies were as follows: ~206 bp for strains 129/Sv, A/J, BALB/cByJIco, B10.S, C3H/HeOuJIco, C57BL/6JIco, C57BR, CB20, CBA/JIco, DBA/2JIco, DDK, DW, FVB/NPas, MAI, MBT, PL/J, PWK/Pas, and SEG; ~190 bp for the MOLD, SJL/JIco, SPR, and STF strains.

The published sequence of the mouse IFN-γ cDNA (12) was used to prepare a specific RT-PCR probe. BAC 232G19 from Research Genetics (Huntsville, AL) was digested with *Eco*RI restriction enzyme and hybridized with the probe. The fragments that hybridized were cloned in the Bluescript vector and used to sequence the exon/intron junctions using primers designed from the published cDNA sequence. The exons of the SJL/J and B10.S strains were amplified from genomic DNA using primers designed from the exon/intron junction sequences. They were cloned with the TOPO TA cloning kit (Invitrogen, San Diego, CA) and sequenced with the Sequenase kit. The following primers were used for genomic DNA amplification: For exons 1 and 2, 5′-AAGTTCTGGGCTTCTCCTCC-3′ and 5′-CATGTCACCATCCTTGGGAA-3′; for exon 3, 5′-TCCCTGTT GTTTCTAATGGG-3′ and 5′-CACCTCCTAGCTTTATCAGC-3′; for exon 4, 5′-GATTTCCATCTTCACTGACC-3′ and 5′-TGGGACAATCT CTTCCCCAC-3′.

Quantification of viral RNA in CNS

The assay has been described in detail previously (13). Briefly, total RNA was extracted from the spinal cord. Five-fold dilutions of the RNA solutions were dotted on Hybond-C extra filters (Amersham). The filters were hybridized with a virus-specific ^{32}P-labeled cDNA probe, washed, and exposed overnight against x-ray-sensitive films. In some experiments, the amount of viral RNA was measured by RT-PCR as described for cytokines (see below).

Detection of cytokine mRNA by RT-PCR

The method is similar to that described by Monteyne et al. (14). Brain tissue and cultured cells were lysed in TRIzol reagent (Life Technologies). The mixture was extracted with chloroform, and the RNA was precipitated with isopropanol, washed in ethanol, and finally resuspended in 50 μl of water. Randomly primed cDNA was prepared from ~5 μg of RNA using 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies) according to the manufacturer's instructions. cDNA was amplified by PCR with a Gene Amp kit (Life Technologies) in a 9600 reactor (Perkin-Elmer Cetus, Norwalk, CT), with 40 cycles for cytokines and 25 cycles for actin and Theiler's virus mRNA. Using these numbers of cycles avoided reaching saturation. The primers used for the PCR reaction were as follows: IL-2, 5′-GTCCTGCAGGCATGTACAGC-3′ and 5′-AGGG CTTGTTGAGATGATGC-3′; IL-10, 5′-CCCAGAAATCAAGGAGC ATTTG-3′ and 5′-CATGTATGCTTCTATGCAGTTG-3′; IL-12, 5′-CAACGTTGCATCCTAGGATCG-3′ and 5′-GCACATCAGACCAGG-3′; IFN-γ, 5′-GACAATCAGGCCATCAGCAAC-3′ and 5′-CGCAAT CACAGTCTTGGCTAA-3′; actin, 5′-ATGGATGACGATATCGCTGC-3′ and 5′-GCTGGAAGGTGGACAGTGAG-3′; Theiler's virus, 5′-TCTA GATCTAGACTATTCAAGTTCGAGAATGGGGA-3′ and 5′-GAATTC GAATTCGAATTCGCCACCATGGGAACGGACAACGCCGAA-3′. The size of the amplified fragment was 500 bp, 250 bp, 1060 bp, 500 bp, 1060 bp, and 800 bp for, respectively, IL-2, IL-10, IL-12, IFN-γ, actin, and Theiler's virus. The integrity of the RNA and the efficacy of reverse transcription were assessed by amplifying the actin cDNA with 25 PCR cycles.

The PCR products were analyzed in 1% agarose gels containing ethidium bromide, transferred to Hybond-N^{+} filters (Amersham), and hybridized overnight at 65°C with internal probes labeled with ^{32}P. The intensity of each hybrid band was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) under conditions for which intensity is proportional to the amount of radioactivity. The ratios between cytokine (or virus) and actin RNA levels were calculated after subtraction of nonspecific background. The sequences of the internal probes were as follows: IL-2, 5′-CCTGAGCAGGATGGAGAATT-3′; IL-10, 5′-GCTGGAA GACCAAGGTGTCTACAAGGC-3′; IL-12, 5′-TGGAATGGCGTCTCT GTCTGC-3′; IFN-γ, 5′-TCGCCTTGCTGTTGCTGA-3′; actin, 5′-CG TACCACAGGCATTGTGATGG-3′; Theiler's virus, 5′-CCTATTA CAAGTGTGACCTTGA-3′. Each experimental point shown in *Results* corresponds to the mean value obtained with at least three mice. The cytokine mRNA/actin mRNA ratio varies from experiment to experiment due to small variations of efficiency of PCR amplification, membrane blotting, and hybridization as well as to variations of the specific activity of the probe. Therefore, only ratios obtained in the same experiment can be compared.

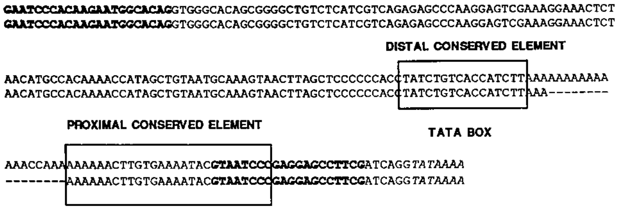

FIGURE 1. Alignment of sequences from the B10.S (*upper line*) and the SJL/J (*lower line*) *Ifng* promoter. Bold letters indicate the PCR primers used to screen for the deletion. Italics indicate the TATA box. The two conserved elements have been defined in Reference 15. The information concerning the *D10Pas4* polymorphism has been deposited in the Mouse Genome Database (The Jackson Laboratory, Bar Harbor, ME) (accession number MGD-CREX-734).

In vitro stimulation of spleen cells and lymphocyte proliferation assay

Fresh spleen cells from SJL/J, B10.S, or congenic mice, cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS, were stimulated with 5% of a cell supernatant containing the 145-2C11 anti-CD3 Ab (a gift of O. Leo, Université Libre de Bruxelles, Rhode Saint-Genèse, Belgium). For cytokine mRNA detection, RNA was extracted from 10^7 cells after 24 h of culture. For the proliferation assay, [^{3}H]thymidine (0.5 mCi/well) was added to the cultures (2.5 10^5 cells/well) for the last 6 h of a 72-h incubation period.

Results and Discussion

Studies of the Ifng *gene and of the cytokine profiles of susceptible and resistant mice*

Because the *Ifng* gene was a good candidate to explain the difference of susceptibility of the SJL/J and B10.S strains, we cloned and sequenced the gene from both strains. There were no differences in the coding part of the gene (data not shown). However, we found a 16-bp deletion, which we called *D10Pas4*, in the *Ifng* promoter of the SJL/J mouse (Fig. 1). We screened a large number of inbred mouse strains and found the deletion in strains MOLD, SPR, and STF but not in strains 129/Sv, A/J, B10.S, BALB/cByJIco, C3H/HeOuJIco, C57BL/6JIco, C57BR, CB20, CBA/JIco, DBA/2JIco, DDK, DW, FVB/NPas, MAI, MBT, PL/J, PWK/Pas, and SEG.

The *D10Pas4* deletion could have been responsible for the susceptibility of the SJL/J strain for two reasons. First, it is present in the only strain for which the *H-2D* haplotype does not correlate with susceptibility to persistent infection. Second, it is located 44 nt upstream of the TATA box, between two sequence elements that are conserved among mice, rats, and humans (15). This could be a critical position, because both elements contain an AP-1 · cAMP response binding element-activating transcription factor DNA binding domain. These domains are functional because they are involved in the negative regulation of IFN-γ by glucocorticoids (16). The deletion decreases the distance between the two elements by one and and half helix turns and changes their spatial orientation. Because this could affect the activity of the promoter (17, 18), we measured the level of IFN-γ mRNA in the brains of SJL/J and B10.S mice during the first week after inoculation, a time period during which both strains are infected at similar levels. Because the level of viral RNA starts to decline in resistant mice around day 7 postinoculation, we assume that biochemical events leading to clearance would be operating during the first week. We observed

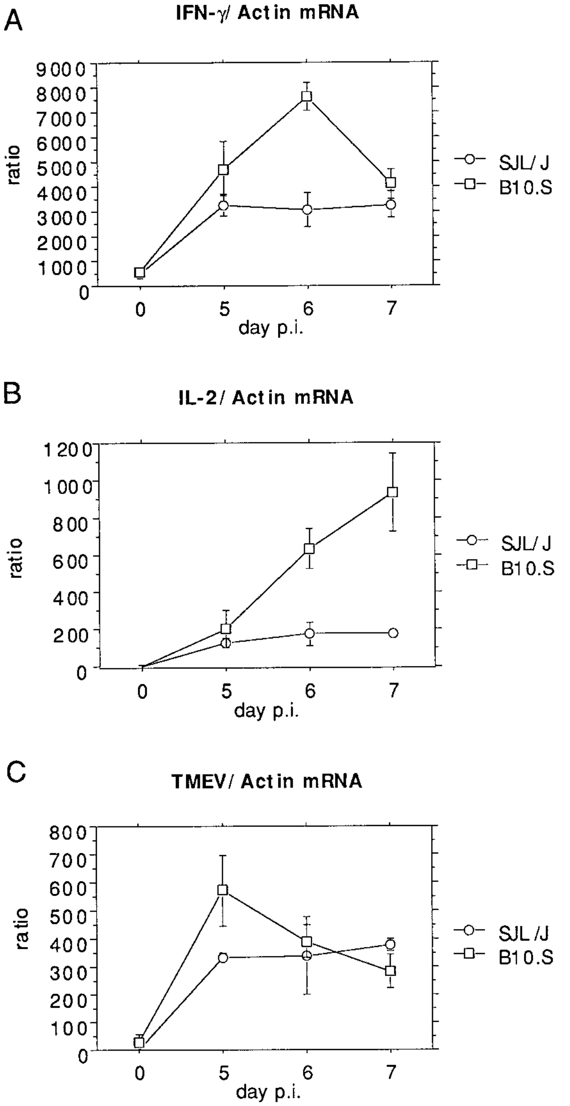

FIGURE 2. Levels of IFN-γ mRNA, IL-2 mRNA and viral RNA in the brain of SJL/J and B10.S mice at different times postinoculation. The level of each RNA was determined with a semiquantitative PCR assay as described in *Materials and Methods*. The figure shows the ratios between the values obtained for cytokine mRNA or viral RNA and actin mRNA. Each point corresponds to the mean and SE of the values obtained with three mice.

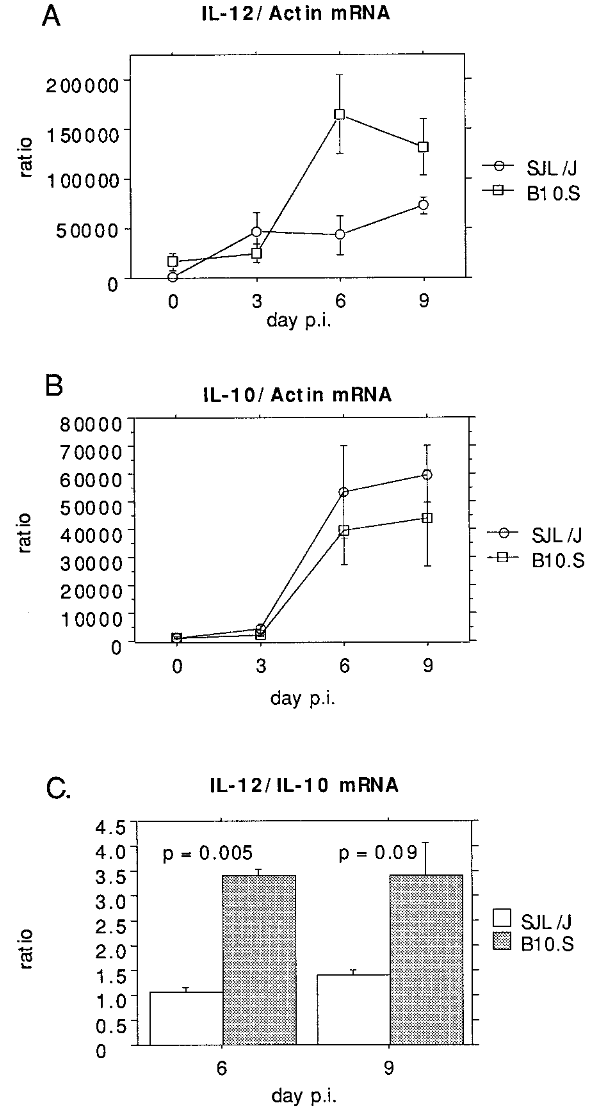

FIGURE 3. Levels of IL-12 and IL-10 mRNA in the brains of SJL/J and B10.S mice at different times postinoculation. *A* and *B*, The level of each mRNA was determined with a semiquantitative PCR assay as described in *Materials and Methods*. The figure shows the ratios between cytokine and actin mRNA. Each point corresponds to the mean and SE of the values obtained with three mice. *C*, Ratio between the levels of IL-12 and IL-10 mRNA at days 6 and 9 postinoculation.

a peak of IFN-γ mRNA expression at day 6 postinoculation in the case of the B10.S mouse. In contrast, the level of IFN-γ mRNA reached a plateau at day 5 postinoculation in the SJL/J mouse (Fig. 2*A*). This result was reproduced in three independent experiments. The difference at day 6 postinoculation was highly significant when comparing 12 mice of each strain (Mann-Whitney *U* test, $p = 0.003$). As shown in Fig. 2*B*, a significantly higher level of IL-2 mRNA was also observed in the brains of B10.S mice (Mann-Whitney *U* test, $p = 0.002$ when comparing 12 mice of each strain at day 6 postinoculation). During the same time period, no significant difference in the amount of viral RNA was observed between the two strains (Fig. 2*C*).

These results suggested that the response of the B10.S mouse to Theiler's virus CNS infection was more of the Th1 type than that of the SJL/J mouse. Therefore, we examined the levels of IL-12 and IL-10 mRNA in the brains of B10.S and SJL/J mice inoculated with Theiler's virus. The expression of IL-12 was higher in B10.S mice than in SJL/J mice, with a peak of expression on day 6 postinoculation (Fig. 3*A*). No significant difference was found for IL-10, although its expression tended to be higher in SJL/J mice. Despite some variation of cytokine mRNA levels from experiment to experiment, the ratio of IL-12 to IL-10 mRNA levels was significantly higher in B10.S mice than in SJL/J mice on day 6 postinoculation (Mann-Whitney *U* test, $p = 0.005$) (Fig. 3*C*). Interestingly, we could not detect IL-4 mRNA by RT-PCR in the brains of either B10.S or SJL/J mice during the first 2 wk following inoculation, although it was found in the spinal cord of SJL/J mice at 45 days postinoculation (data not shown).

Studies with congenic mice

Two lines of congenic mice, named SJL.10(70/14) and SJL.10(233/237), were obtained as described in *Materials and Methods*. They possess the *Ifng* gene and varying amounts of the surrounding region of the B10.S parent on an SJL/J background

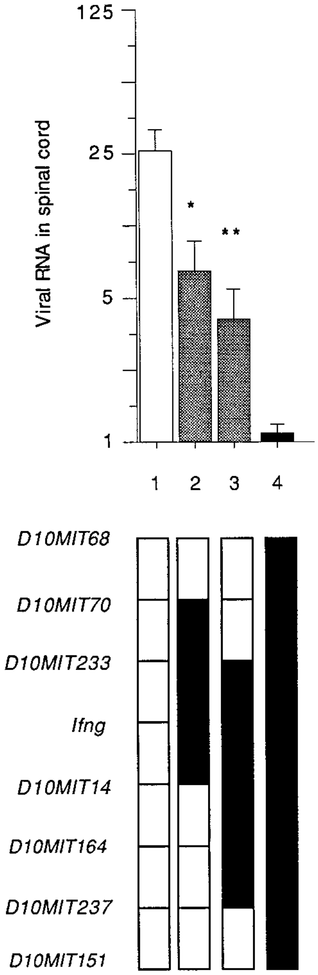

FIGURE 4. Phenotype and genotype of inbred strains of mice. The phenotype is the amount of viral RNA present in the spinal cord at 45 days postinoculation as measured with a dot blot hybridization assay (see *Materials and Methods*). *Lane 1*: strain SJL/J ($n = 28$); *lane 2*: congenic strain SJL.10(70/14) ($n = 21$); *lane 3*: congenic strain SJL.10(233/237) ($n = 17$); *lane 4*: strain B10.S ($n = 22$). n is the number of mice per group. *, $p = 0.002$ for the difference between *lanes 1* and *2*. **, $p \leq 0.0001$ for the difference between *lanes 1* and *3*. The genotype of each strain is shown in the lower part of the figure. The distance shown between genetic markers is arbitrary.

(Fig. 4) (F.B., unpublished observations). Both lines were significantly more resistant to persistent infection than the SJL/J parent (Fig. 4), which demonstrates the existence of a susceptibility locus in the *Ifng* region. Fig. 4 also shows that this region accounts for 80% of the difference of susceptibility between the SJL/J and B10.S strains. Because *Ifng* is the only locus for which there is a significant linkage with susceptibility in a (SJL/J × B10.S)F_1 × B10.S backcross (5), it is unlikely that the rest of the susceptibility is due to a single locus. Rather, it probably involves several genes and/or interactions between genes.

To determine whether a locus in the *Ifng* region is responsible for the difference of Th1/Th2 balance described above for the SJL/J and B10.S strains, we examined the levels of IFN-γ, IL-2, IL-10, and IL-12 mRNA in the brains of SJL/J mice and of the two lines of resistant congenic mice during the first week postinoculation. As shown in Fig. 5, the levels of IFN-γ and IL-2 mRNA and the ratio of IL-12 to IL-10 mRNA were the same for the SJL/J and SJL.10(70/14) mice. The same result was obtained with SJL.10(233/237) congenic mice (data not shown). Therefore, the

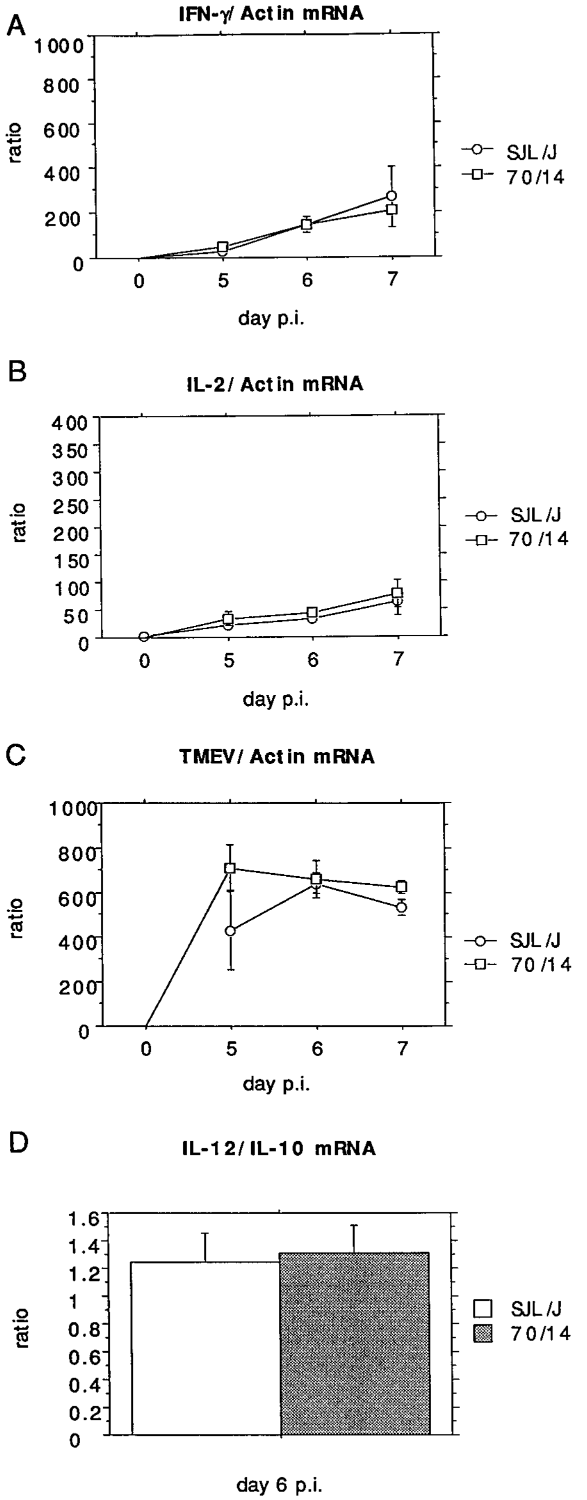

FIGURE 5. Levels of IFN-γ mRNA (*A*), IL-2 mRNA (*B*), and viral RNA (*C*) in the brains of SJL/J and SJL.10(70/14) strains of mice at different times postinoculation. The level of each RNA was determined with a semiquantitative PCR assay as described in *Materials and Methods*. The figure shows the ratios between cytokine or viral mRNA and actin mRNA. Each point corresponds to the mean and SE of the values obtained with three mice. *D*, Ratio of IL-12/IL-10 mRNA levels in the brains of SJL/J and SJL.10(70/14) mice at 6 days postinoculation.

Ifng region is not responsible for the difference of Th1/Th2 balance observed between the SJL/J and B10.S strains, although it accounts for most of the difference of susceptibility between these strains (Fig. 4). These results demonstrate that the Th1/Th2

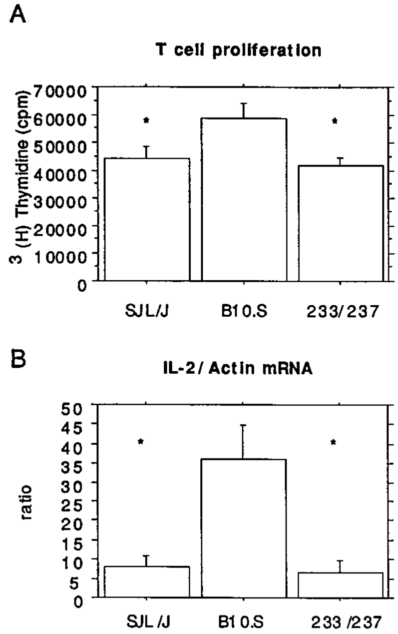

FIGURE 6. *A*, T cell proliferation after in vitro stimulation of spleen cells from SJL/J, B10.S, and SJL.10(233/237) mice with anti-CD3 mAb. Each bar shows the mean of values obtained in triplicate for three different animals. Difference between the SJL/J and B10.S strains: $p = 0.04$. Difference between the B10.S and SJL.10(233/237) strains: $p = 0.01$. No significant difference was found between strains SJL/J and SJL.10(233/237). *B*, Level of IL-2 mRNA in splenocytes from SJL/J, B10.S, and SJL.10(233/237) mice after 24 h of stimulation with anti-CD3 mAb. The level of IL-2 and actin mRNA was determined with a semiquantitative PCR assay as described in *Materials and Methods*. The figure shows the ratios between levels of IL-2 and actin mRNA. Each bar shows the mean and SE of the values obtained with the splenocytes of three mice. Difference between SJL/J and B10.S mice: $p = 0.03$. Difference between B10.S and SJL.10(233/237) mice: $p = 0.03$. No significant difference was found between strains SJL/J and SJL.10(233/237).

balance is not the major factor in resistance/susceptibility to persistent infection in this system.

In the past, the fact that IFN-γ limits an infection and that there is a correlation among mouse strains between Th1/Th2 responses and resistance/susceptibility has been taken as evidence for the role of Th1 responses in resistance. Our results illustrate how such an assumption can be improper in the absence of direct genetic proof, such as that provided by congenic animals.

IFN-γR$^{o/o}$ animals (6) and treatment of resistant mice with an anti-IFN-γ Ab (7) showed that IFN-γ restricts Theiler's virus expression during persistent infection. This finding is not in contradiction with the results reported here. In the present case, the effect of IFN-γ on late disease is the same for the SJL/J and the B10.S strains. Knockout mice and immunological manipulations such as treatments with neutralizing mAbs uncover genes and biochemical pathways with essential roles in pathogenesis but which are not necessarily responsible for differences of susceptibility between strains.

T cell proliferation and IL-2 production after in vitro stimulation

The proliferative response of T lymphocytes after stimulation with anti-CD3 mAbs is controlled by a gene that has been mapped to the region containing the *D10Mit14* marker (19). To examine whether this gene was involved in the control of the persistence of Theiler's virus, we compared the proliferative response of T cells from the SJL/J, B10.S, SJL.10(70/14), and SJL.10(233/237) strains. As shown in Fig. 6, T cells from B10.S mice proliferated more and secreted more IL-2 than T cells from the SJL/J or SJL.10(233/237) strains; there were no differences when comparing T cell proliferation and IL-2 mRNA expression for the SJL/J and SJL.10(233/237) mice. The same result was obtained with the SJL.10(70/14) congenic strain (data not shown). Therefore, the gene that controls the T cell-proliferative response is not in the *Ifng* region and must be different from that which controls the susceptibility to Theiler's virus persistent infection.

In summary, the *Ifng* gene was a good candidate to explain the difference of susceptibility of the SJL/J and B10.S mice to persistent infection by Theiler's virus. We found a strategically placed deletion in the promoter of the gene of the susceptible SJL/J strain and observed that the response of the resistant B10.S strain to the infection was more of a Th1 type than that of the susceptible SJL/J strain. However, the study of mice congenic for the region ruled out a role for the *Ifng* gene and the Th1/Th2 balance in the difference of susceptibility between these strains. These studies emphasize how the existence of a correlation between Th1/Th2 responses and resistance/susceptibility to an infectious agent does not demonstrate a causal relationship.

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