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Infection of Class II-Deficient Mice by the DA Strain of Theiler's Virus

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The DA strain of Theiler's virus causes, in susceptible strains of mice, a persistent infection of the white matter of the spinal cord accompanied by chronic inflammation and primary demyelination. In resistant strains, including all *H-2*^{b} strains, mice clear the infection after 1 to 2 weeks. We inoculated RHAβ$^{o/o}$ mice, an *H-2*^{b} strain which does not express class II molecules. We found that they are susceptible to persistent infection and that they develop foci of chronic inflammation with demyelination. However, these foci are smaller and contain fewer demyelinated axons than those observed in susceptible SJL/J or β2m$^{-/-}$ mice.

The DA strain of Theiler's virus, a murine picornavirus, causes a biphasic neurological disease in susceptible strains of mice such as the SJL/J strain. Following intracerebral inoculation, the virus first infects neurons of the brain and spinal cord and then migrates to the white matter of the spinal cord, where it persists for months in macrophages/microglial cells, oligodendrocytes, and astrocytes (1, 4, 17). This persistent infection is associated with chronic meningitis, mononuclear cell infiltration of the white matter, perivascular cuffing, and focal areas of primary demyelination. Other strains of mice, such as the C57BL/6 strain, eradicate the infection after 1 to 2 weeks and do not present chronic central nervous system disease. Resistance or susceptibility to persistent infection and demyelination is controlled by several loci, including the *H-2* locus (5, 8, 18, 27). All strains with the *H-2*^{b} haplotype are resistant because the *D*^{b} gene confers resistance in a dominant way (2, 5, 22, 24).

The mechanism of the demyelination caused by Theiler's virus is only partially understood. Demyelination is observed in BALB/c nude mice (28–30), and infected oligodendrocytes have a reduced level of mRNA coding for a major structural protein of myelin (21). Therefore, demyelination might be, at least in part, non-immune mediated. On the other hand, a host of experimental data favors an immune-mediated mechanism involving CD4$^+$ T lymphocytes directed against viral epitopes; e.g., treatment with anti-CD4 (14) or anti-Ia monoclonal antibodies (12, 26) reduces the incidence of clinical signs and the extent of demyelination. Also, a delayed-type hypersensitivity reaction against viral antigens is found in susceptible strains of mice only and correlates in time with demyelination (6–8). Immunization against an immunodominant epitope for this delayed-type hypersensitivity, or transfer of a CD4$^+$ T-cell clone specific for this epitope, exacerbates the disease (13–15). Conversely, tolerization against this peptide reduces its severity (16).

We decided to reexamine the role of class II-restricted responses in the pathogenesis of this infection, by taking advantage of the RHAβ$^{o/o}$ mouse. This mouse, which bears the *H-2*^{b} haplotype, does not express the I-E and I-Aβ genes because of, respectively, a deletion in the E-α promoter and the inactivation by homologous recombination of the I-Aβ gene. Its number of CD4$^+$ T lymphocytes is extremely low (9). RHAβ$^{o/o}$ mice and control RHAβ$^{+/o}$ mice were kindly provided by C. Benoist (Strasbourg, France) (9). The genotype of the mice was determined by Southern blot analysis of their DNA. Their phenotype was tested by fluorescence-activated cell sorter analysis of peripheral blood lymphocytes, using a rat anti-mouse CD4 antibody conjugated to phycoerythrin (Tebu RM2404-3). Whereas the number of CD4$^+$ T lymphocytes was normal in control RHAβ$^{+/o}$ and wild-type mice, it represented only 1.3 to 4.1% of the total lymphocytes of RHAβ$^{o/o}$ mice. β2m$^{-/-}$ mice were originally given to us by B. DuBridge (Cell Genesys Inc., Foster City, Calif.) and are now maintained in the animal facility of the Institut Pasteur. SJL/J mice were

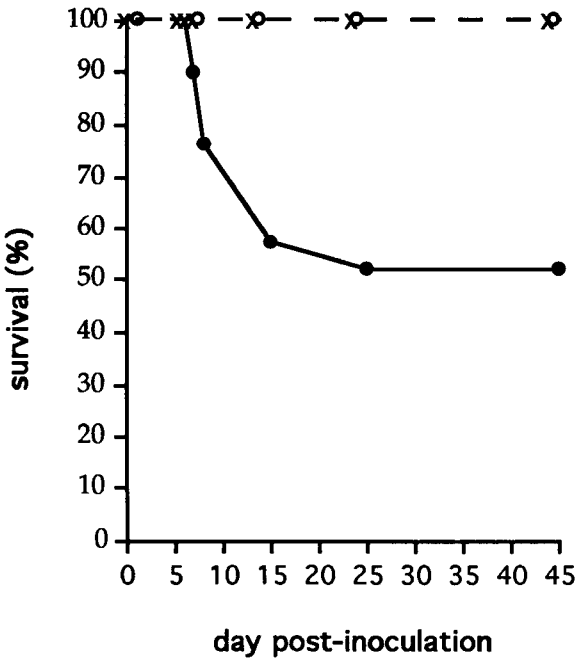

FIG. 1. Mortality as a function of time p.i. Mice were inoculated intracerebrally with 10^6 PFU of the DA strain of Theiler's virus. The number of mice in each group was 21, 7, and 8 for, respectively, RHAβ$^{o/o}$ (●), β2m$^{-/-}$ (○), and SJL/J (×) mice.

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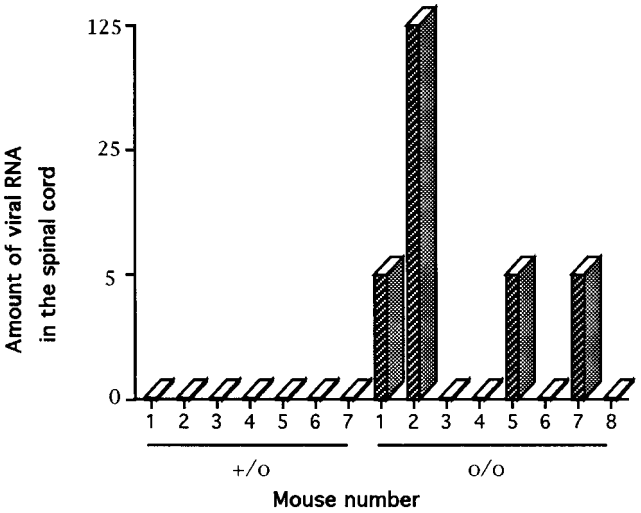


FIG. 2. Amount of viral RNA present in the spinal cords of RHAβ$^{o/o}$ and RHAβ$^{+/o}$ mice 45 days p.i. with 10^4 PFU of Theiler's virus. The viral RNA was quantitated with a dot blot assay as described in the text. The ordinate shows, for each mouse, the highest dilution of the RNA solution which gave a positive hybridization signal.

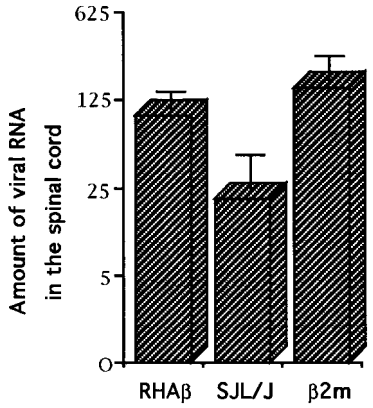


FIG. 4. Amount of viral RNA in the spinal cords of RHAβ$^{o/o}$, β2m$^{-/-}$, and SJL/J mice 45 days p.i. with 10^6 PFU of Theiler's virus. Viral RNA was quantitated with a dot blot assay as described in the text. The ordinate shows, for each group of mice, the average of the highest dilutions of RNA solutions which gave a positive hybridization signal. The number of mice in each group was 11, 7, and 8 for, respectively, RHAβ$^{o/o}$, β2m$^{-/-}$, and SJL/J mice. Error bars show the standard error of the mean. The amounts of viral RNA were compared by the Scheffe test. The only statistically significant difference was observed between the β2m$^{-/-}$ and SJL/J mice ($P < 0.0005$).

obtained from Centre d'Elevage R. Janvier (Le Genest-St-Isle, France).

RHAβ$^{o/o}$ and RHAβ$^{+/o}$ mice were inoculated intracerebrally with 10^4 or 10^6 PFU of the DA strain of Theiler's virus in a volume of 40 μl. In one experiment SJL/J mice and β2m$^{-/-}$ mice were inoculated with 10^6 PFU. Forty-five days postinoculation (p.i.), the mice were perfused under anesthesia with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde–1% glutaraldehyde in PBS. The brain and spinal cord were dissected out and embedded in paraffin for routine histology and immunocytochemistry. Viral antigens were detected on longitudinal paraffin sections of the whole spinal cord by using a rabbit hyperimmune anti-Theiler's virus serum and the avidin-biotin conjugate peroxidase detection system, as described previously (3). For myelin studies, segments of the cervical spinal cord were embedded in Epon and 1-μm-thick sections were prepared and stained with toluidine blue.

Viral RNA in the CNS was quantified by a dot-blot assay described previously (5). Briefly, total RNA was extracted from the spinal cord. Fivefold dilutions of this RNA were dotted on Hybond C-extra filters and hybridized with a Theiler's virus-specific ^{32}P-labelled cDNA probe. Hybridization with a β-actin-specific probe was performed in parallel to control the amount of RNA which had been dotted.

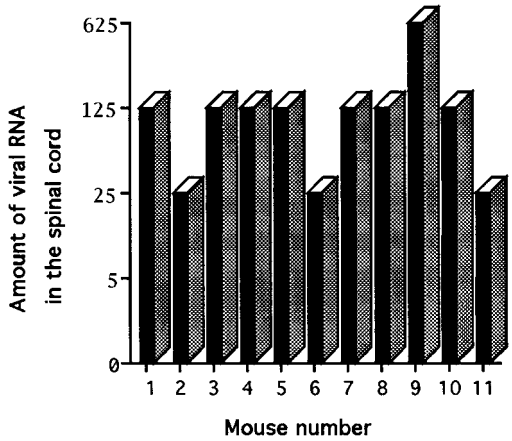
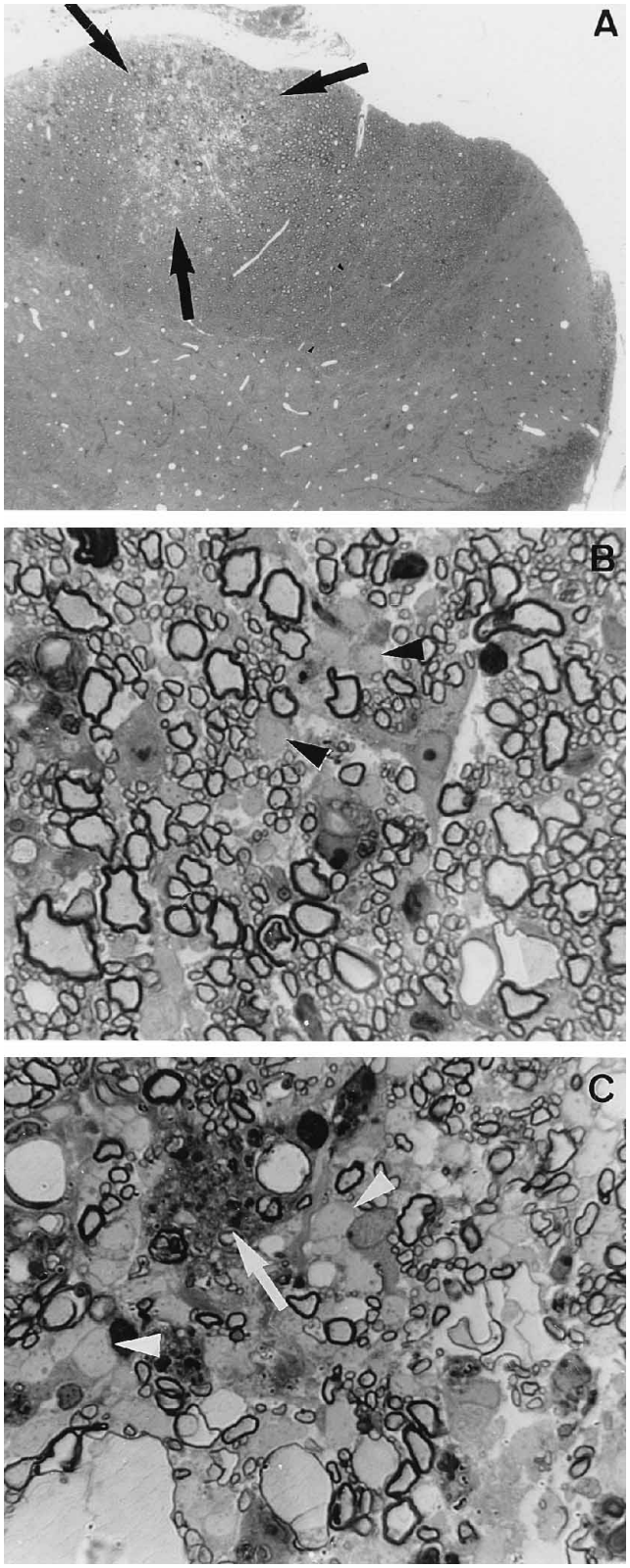


FIG. 3. Amount of viral RNA present in the spinal cords of RHAβ$^{o/o}$ mice 45 days p.i. with 10^6 PFU of Theiler's virus. The viral RNA was quantitated with a dot blot assay as described in the text. The ordinate shows, for each mouse, the highest dilution of the RNA solution which gave a positive hybridization signal.

FIG. 5. Longitudinal section of the spinal cord of an RHAβ$^{o/o}$ mouse (45 days p.i.). The field illustrates diffuse parenchymal inflammation of the white matter and the presence of cells containing viral capsid antigens (arrows). Final magnification: ×426.



In a first experiment, 12 RHAβ$^{o/o}$ mice were inoculated intracerebrally with 10^4 PFU of the DA strain of Theiler's virus and observed for 45 days. No mouse died; however, three mice showed paresis of the hind legs 3 to 4 weeks p.i. In another experiment, 21 RHAβ$^{o/o}$ mice, as well as 7 β2m$^{-/-}$ and 8 SJL/J control mice, were inoculated with 10^6 PFU of the DA strain of Theiler's virus. Ten of the RHAβ$^{o/o}$ mice were dead by day 25 p.i. No mortality occurred in the control mice (Fig. 1). One surviving RHAβ$^{o/o}$ mouse showed paresis of the hind legs and one β2m$^{-/-}$ mouse had paralysis of both front legs at 45 days p.i.

To study viral persistence, the amount of viral RNA present in the spinal cords of eight RHAβ$^{o/o}$ and seven RHAβ$^{+/o}$ mice which had been infected with 10^4 PFU was measured 45 days p.i. None of the RHAβ$^{+/o}$ mice had detectable viral RNA. In contrast, variable amounts of viral RNA were detected in four of the eight RHAβ$^{o/o}$ mice (Fig. 2). In another experiment, 11 RHAβ$^{o/o}$ mice were inoculated with 10^6 PFU of Theiler's virus. In this case, high levels of viral RNA were found in the spinal cords of all the mice (Fig. 3). The level of viral RNA was also measured in the spinal cords of 11 RHAβ$^{o/o}$, 7 β2m$^{-/-}$, and 8 SJL/J mice 45 days p.i. with 10^6 PFU of Theiler's virus. As shown in Fig. 4, the three groups of mice were persistently infected. The amount of viral RNA was not statistically different between them, except when comparing the β2m$^{-/-}$ and SJL/J mice.

We used an enzyme-linked immunosorbent assay, with purified virions as the antigen and a 1/60 dilution of the serum, to look for virus-specific antibodies in RHAβ$^{o/o}$ and RHAβ$^{+/o}$ mice which had been infected with 10^4 PFU of Theiler's virus. Six of ten RHAβ$^{+/o}$ mice, but none of seven RHAβ$^{o/o}$ mice, had antibodies.

Histological studies were performed on longitudinal sections of the spinal cords of 12 RHAβ$^{o/o}$ and 9 RHAβ$^{+/o}$ mice 45 days after inoculation with 10^4 PFU of Theiler's virus. Very discrete inflammation, similar to that usually found in mice of the *H-2^b* haplotype, was seen in the spinal cords of five of the nine RHAβ$^{+/o}$ mice. In contrast, in 11 of 12 RHAβ$^{o/o}$ mice, there was conspicuous, focal inflammation of the white matter of the spinal cord, with mononuclear cells in the meninges, the perivascular spaces, and the parenchyma (Fig. 5). Adjacent sections were used to look for viral antigens by immunocytochemistry. Rare positive cells were seen in sections from RHAβ$^{+/o}$ mice. In contrast, numerous infected cells were identified in the white matter of the spinal cords of RHAβ$^{o/o}$ mice. These cells were located within, or close to, the inflammatory infiltrates (Fig. 5).

Demyelination was studied in semithin transverse sections of the cervical spinal cords of eight RHAβ$^{o/o}$ and eight β2m$^{-/-}$ mice, 45 to 53 days p.i. with 10^4 PFU of Theiler's virus (Fig. 6). The β2m$^{-/-}$ mice provided a useful control since they are susceptible to demyelination and have the same genetic background as the RHAβ$^{o/o}$ mice (11). Both are derived from C57BL/6 × 129Sv crosses and are of the *H-2^b* haplotype. Le-

FIG. 6. Semithin transverse section of Epon-embedded spinal cord. (A) Low-magnification view of a section from an RHAβ$^{o/o}$ mouse infected with 10^4 PFU of Theiler's virus and sacrificed 45 days p.i. The arrows point to a lesion in a lateral column. (B) Representative field in a lesion observed in a RHAβ$^{o/o}$ mouse infected with 10^4 PFU of Theiler's virus and sacrificed 45 days p.i. The arrowheads point to demyelinated axons. Note the large number of normally myelinated axons. Final magnification: ×967.3. (C) Representative field in a lesion observed in a β2m$^{-/-}$ mouse infected with 10^4 PFU of Theiler's virus and sacrificed 45 days p.i. The arrow points to a "foamy" macrophage. The arrowheads point to demyelinated axons. Note the vacuolization of the white matter parenchyma. Final magnification: ×967.3.

sions were observed in seven of eight RHAβ$^{o/o}$ mice in 28 of a total of 44 levels of spinal cord examined. These lesions were small, and in most cases there was only one lesion per section of spinal cord (Fig. 6A). On rare occasions there were two. Although the white matter was often vacuolated and contained myelin-laden macrophages, the number of demyelinated axons in the lesions was small (Fig. 6B). Demyelinating lesions were observed in seven of eight β2m$^{-/-}$ mice in 17 of 45 levels of spinal cord examined. These lesions were generally larger than those observed in RHAβ$^{o/o}$ mice. Multiple lesions in the same section of spinal cord were common. As illustrated in Fig. 6C, they contained myelin-laden macrophages and a large number of demyelinated axons. Therefore, although a detailed quantitative analysis of axon demyelination was not performed, it appeared that demyelination was more pronounced in β2m$^{-/-}$ mice than in RHAβ$^{o/o}$ mice. The lesions found in RHAβ$^{o/o}$ mice were also compared with those observed in susceptible SJL/J mice inoculated and sacrificed under the same conditions. The lesions in SJL/J mice were much larger and contained a high proportion of demyelinated axons (not shown).

The disease caused by Theiler's virus consists of an early infection of brain and spinal cord neurons followed by a late persistent infection of glial cells in the white matter of the spinal cord. The early phase is exacerbated in class II-deficient RHAβ$^{o/o}$ mice, as shown by a high mortality rate (Fig. 1). This indicates that class II-restricted immune responses, most likely antibodies, are important in limiting viral replication in the grey matter and is consistent with the observation that the antibody response against Theiler's virus is T cell dependent (28, 29). Interestingly, early grey matter disease is not exacerbated in class I-deficient β2m$^{-/-}$ mice (Fig. 1), which develop a normal antibody response (11, 23, 25).

Previous work showed that class I-restricted responses, most likely CD8$^+$ cytotoxic T lymphocytes, are important for the resistance of *H-2^b* mice to persistent infection. In particular, β2m$^{-/-}$ (*H-2^b*) mice are susceptible (11, 23, 25), and susceptible FVB (*H-2^q*) mice become resistant when they are transgenic for the *D^b* gene (2). Therefore, the susceptibility of RHAβ$^{o/o}$ mice, which express the *D^b* gene, might seem surprising at first. There could be at least two reasons for this paradox. First, both cytotoxic T lymphocytes and the antibody response might be required for the resistance of *H-2^b* mice. If this were the case, the absence of one or the other would make the animal susceptible. Second, the lack of help from CD4$^+$ T lymphocytes could affect the antiviral CD8$^+$ cytotoxic T-lymphocyte response. Admittedly, the role of CD4$^+$ T cells in these responses is still debated and seems to depend on the virus under study and the experimental protocol (10). No information is available in the case of the infection by Theiler's virus.

Although this point may require a careful quantitative analysis, there was less demyelination in RHAβ$^{o/o}$ mice than in SJL/J or β2m$^{-/-}$ mice. The size of the lesions and the proportion of demyelinated axons were both smaller (Fig. 6A and B). The comparison between the RHAβ$^{o/o}$ and the β2m$^{-/-}$ mice was particularly telling since both have the same genetic background. Furthermore, the amounts of virus persisting in the spinal cord were similar in both, or possibly lower in the β2m$^{-/-}$ mice (Fig. 4). Therefore, our data show that some demyelination occurs in the absence of class II-restricted responses, possibly because of a direct effect of the infection of oligodendrocytes, but that intense demyelination, as seen in susceptible SJL/J or β2m$^{-/-}$ mice, requires the expression of class II molecules. This is consistent with the role of a virus-specific delayed-type hypersensitivity in demyelination (13, 14, 19). Interestingly, this delayed-type hypersensitivity was found in β2m$^{-/-}$ mice (23, 25) but not in RHAβ$^{o/o}$ mice (20).

In conclusion, we found that, in spite of their *H-2^b* haplotype, RHAβ$^{o/o}$ mice are susceptible to persistent infection and develop foci of chronic inflammation with demyelination. Compared with those of susceptible SJL/J or class I-deficient β2m$^{-/-}$ mice, these lesions are small and contain only a small number of demyelinated axons. A recent publication by Njenga et al. describes similar findings (20). Therefore, our results show that class II-restricted responses are important in the resistance of mice of the *H-2^b* haplotype. They also favor a role for these responses in myelin destruction.

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