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H-2 D^d transgene suppresses Theiler's virus-induced demyelination in susceptible strains of mice

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Theiler's murine encephalomyelitis virus is a picornavirus which induces chronic immune-mediated central nervous system demyelination and virus persistence in susceptible strains of mice. Using murine strains with congeneic recombinant haplotypes, the H-2D region within the class I major histocompatibility complex has been shown to be important in determining susceptibility/resistance to chronic Theiler's murine encephalomyelitis virus infection. We examined the role of H-2D in demyelinating disease with the use of transgenic D8 mice (H-2D^d, resistant haplotype) crossed to susceptible B10.Q (H-2^q) and B10.S (H-2^s) mice. Expression of the H-2D^d transgene dramatically suppressed demyelination and reduced the number of virus-antigen positive cells in the spinal cord 45 days following infection. More complete protection was observed in transgenic B10.Q ($D8^+$) mice than in transgenic B10.S ($D8^+$) mice. These experiments support the hypothesis that the immunologic basis of resistance by H-2D is determined by effective antigen presentation which prevents virus persistence and subsequent demyelination.

Keywords: multiple sclerosis; immunopathology; oligodendrocytes; major histocompatibility complex; picornavirus

Introduction

We have used demyelination in mice infected by Theiler's murine encephalomyelitis virus (TMEV) to investigate the role of class I major histocompatibility complex (MHC) locus gene products in the development of myelin diseases. TMEV is a picornavirus which in susceptible mice (SJL/J, prototypic strain) results in acute encephalitis followed by virus persistence and primary immune-mediated demyelination (Rodriguez *et al*, 1987). In contrast, infection of resistant mice (B10, prototypic strain) results in acute encephalitis followed by virus clearance and absence of demyelination. TMEV infection of mice with *s, f, p, v,* or *q* haplotypes on the C57BL/10 background results in primary demyelination and virus persistence (Rodriguez and David, 1985). In contrast, infection of mice of identical C57BL/10 background, but with *d, b,* or *k* haplotype does not result in chronic pathology in the spinal cord (Rodriguez and David, 1985). We (Rodriguez *et al*, 1986) and others (Clatch *et al*, 1985, 1987; Bureau *et al*, 1992) have used murine strains with congeneic recombinant haplotypes within the D region of MHC to demonstrate that class I MHC H-2D genes are one of the genes responsible primarily for determining susceptibility/resistance of TMEV-induced demyelination B10.$D2^{dm1}$ mice which have a deletion in the 3' end of D^d and the 5' end of L^d and express a hybrid molecule by the 5' of D^d gene and 3' of L^d gene showed prominent demyelination in spinal cord and clinical deficits (Rodriguez *et al*, 1986). The differential effect of virus-induced demyelination between resistant B10.D2 and susceptible B10.$D2^{dm1}$ strongly indicated that genes within the H-2D region are critical in development of demyelinating disease.

In this series of experiments we tested directly whether transgenic expression of H-2D^d (resistant haplotype) would alter susceptibility to TMEV-induced demylination. We used (H-2D^d) transgenic mice created by microinjection of a 8.0 Kb EcoRI fragment from a plasmid containing the H-2D^d gene into fertilized B6 (H-2^b) embryos (Bieberich *et al*, 1987; Yoshioka *et al*, 1987). The expression and distribution of this H-2D^d gene parellels that of the endogenous D^b gene (Bieberich *et al*, 1986) without alteration of the latter. The expressed antigen is rec-

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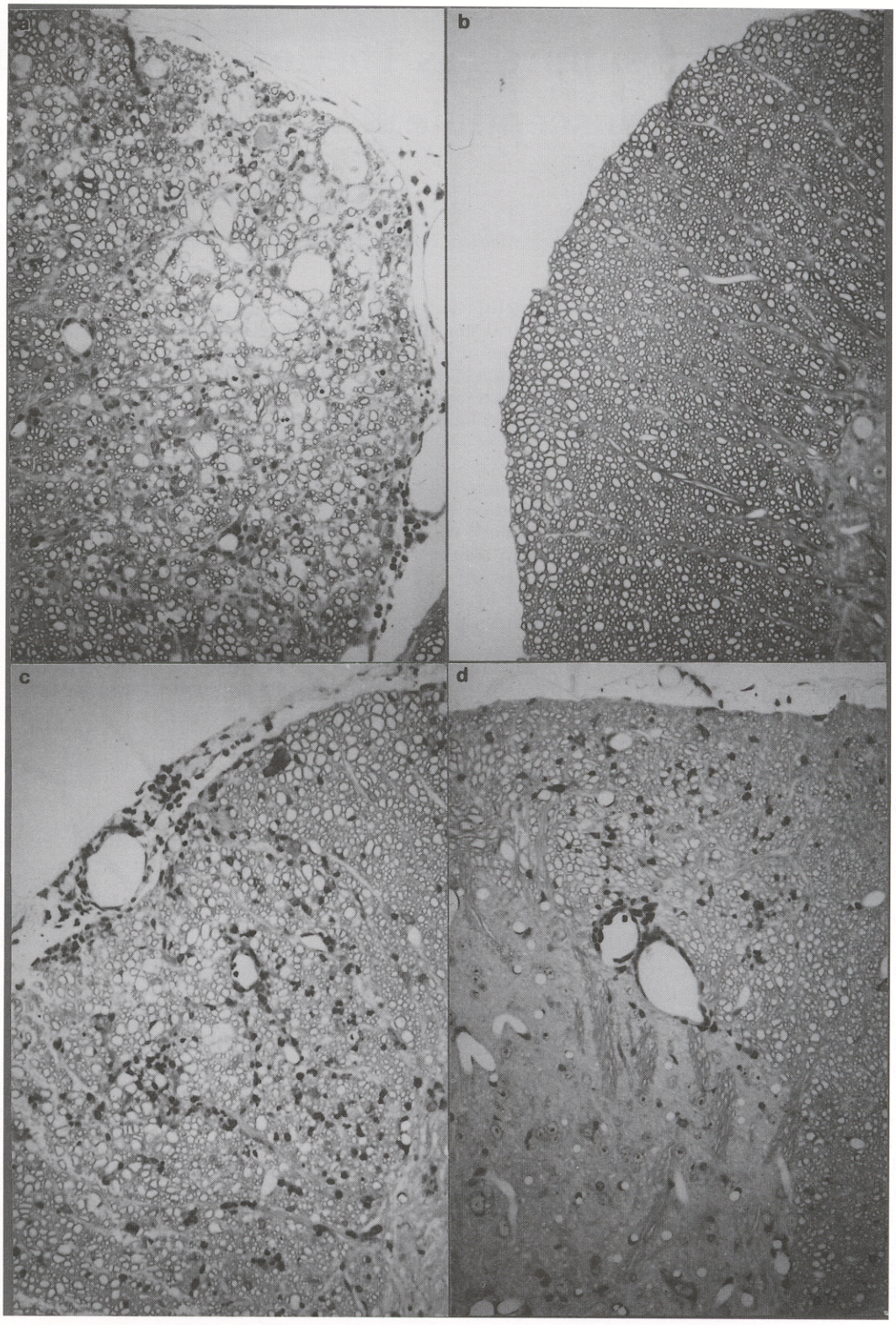

Figure 1 (a) Large area of primary demyelination in the spinal cord of a B10.Q (D8$^-$) mouse infected with Theiler's virus. (b) Normal spinal cord white matter in an infected B10.Q (D8$^+$) mouse. (c) Meningeal perivascular inflammation and adjacent demyelination in the spinal cord of an infected B10.S (D8$^-$) mouse. (d) Small focus of inflammation with minimal demyelination in the spinal cord of an infected B10.S (D8$^+$) mouse. All animals were sacrificed on day 45 following infection. Spinal cord sections were embedded in glycol methacrylate and stained with a modified erichrome cresyl-violet stain (Mag × 250). Sections such as these were used for quantitative analysis.

ognized as self and has been shown to function as a restriction element for T cells (Yoshioka *et al*, 1987). In these experiments, D8 mice were crossed to susceptible B10.Q (H-2^q) and B10.S (H-2^s) mice to determine if expression of resistant H-2D^d gene would abrogate susceptibility to TMEV-induced demyelination.

Results

Expression of D8 (H-2D^d) transgene diminished the extent of demyelination in TMEV-infected mice of susceptible H-2 haplotypes

To test directly the role of H-2D in determining susceptibility/resistance to TMEV-induced demyelinating disease, we utilized transgenic mice which express the H-2D^d gene. Expression of D8 (H-2D^d) transgene markedly inhibited chronic TMEV-induced demyelination in susceptible B10.Q (H-2^q) mice. In the 10 TMEV-infected B10.Q (D8) mice, on average 14% of 518 spinal cord quadrant examined showed demyelination (Table 1). Many demyelinated lesions were large and encompassed most of the spinal cord white matter (Figure 1a). There were multiple focal areas of intense perivascular inflammation exclusively limited to the white matter. In contrast, in the six B10.Q (D8$^+$) mice on average only 1% of 340 spinal cord quadrants examined showed demyelination. Most spinal cord quadrants showed no pathology (Figure 1b). In areas with disease, the abnormalities were minimal and usually consisted of focal inflammation only. This difference in the percent of spinal cord quadrants with demyelination between B10.Q (D8$^+$) and B10.Q (D8$^-$) mice was highly statistically significant (P = 0.01). When demyelination score ≥ 5.0 was used to define susceptibility, then 0/6 (0%) B10. Q (D8$^+$) mice were susceptible whereas 8/10 (80%) B10.Q (D8$^-$) mice were susceptible at 45 days following infection. This difference was highly statistically significant by Pearson's χ^2 test (P = 0.002) in comparing the number of mice with demyelination score of ≥ 5.0. The extent of gray matter disease (Table 1) was not different between mice with and without expression of H-2D^d transgene. This indicated that expression of resistant H-2D^d gene altered the extent of chronic virus-induced demyelination but had no effect on the acute anterior horn cell disease.

We asked whether the protective effect of D8 (H-2D^d) transgene observed in H-2^q mice would be observed in another susceptible strain. We chose B10.S (H-2^s) because this haplotype is highly susceptible to TMEV-induced demyelination (Rodriguez and David, 1985) and SJL/J mice share this haplotype. In 21 B10.S (D8$^-$) mice, on average 19% of 1156 spinal cord quadrants examined showed demyelination. In contrast, in 20 B10.S (D8$^+$) mice, on average 10% of 1144 spinal cord quadrants examined showed demyelination. This difference in the percent of quadrants with demyelination showed a trend, but did not reach statistical significance (P = 0.09). Fifteen of 21 (71.4%) B10.S (D8$^-$) mice and 8 of 20 (40%) B10.S (D8$^+$) mice were classified as susceptible based on having demyelinating scores ≥ 5.0. This difference was statistically significant by Pearson's χ^2 test (P = 0.043). However, some B10.S (D8$^+$) mice had prominent demyelination (ie, scores of 50.0, 41.1, or 33.3) indicating that in these mice the presence of the H-2D^d transgene was not sufficient to prevent chronic demyelination. Therefore the presence of the D8 (H-2D^d) transgene diminished but did not completely inhibit the extent of TMEV-induced demyelinating disease in H-2^s mice.

Expression of D8 (H-2D^d) transgenes diminished the number of virus-antigen-positive cells in the spinal cord of chronically infected mice

We performed immunoperoxidase staining for virus antigen on frozen spinal cord sections from infected B10.Q or B10.S mice with and without D8 (H-2^d) transgene. The number of virus-antigen-positive cells per total spinal cord areas was determined using morphometric techniques. The total area of spinal cord analyzed per animal was similar in the four groups (Table 2). On average, a 20-fold reduction in the number of virus-antigen-positive cells per area of spinal cord was observed in B10.Q (D8$^+$) mice compared to B.10.Q (D8$^-$) mice. A similar nine-fold reduction was observed in B10.S (D8$^+$) mice compared to B10.S (D8$^-$) mice. The difference in the number of antigen-positive cells per spinal cord in D8$^+$ versus D8$^-$ mice of similar haplotype

Table 1 Effect of D8 transgene (H-2D^d) on Theiler's virus-induced demyelination

Strain	*H-2*	*D8 transgene*	*N*[a]	*Neuronal*[b] *inflammation*	*Meningeal*[b] *inflammation*	*Demyelination*[b]	*P value*[c]
B10.Q	q/q	+	6	0.0 ± 0.0	1.2 ± 0.8	1.2 ± 0.8	—
B10.Q	q/q	-	10	0.4 ± 0.3	10.0 ± 3.5	14.1 ± 4.0	0.01
B10.S	s/s	+	20	1.1 ± 0.7	6.5 ± 3.0	9.8 ± 3.4	—
B10.S	s/s	-	21	1.4 ± 0.8	15.1 ± 4.6	18.8 ± 3.9	0.09

[a]N = Number of mice
All mice were sacrificed on day 45 following infection
[b]Data presented as mean ± SEM
[c]Statistics by Student's t test comparing demyelination scores between mice of identical H-2 haplotype

Table 2 Virus antigen expression in the spinal cord of TMEV-infected mice

Strain	*D8 transgene*	*N*[a]	*Ag-positive*[b] *cells (No.)*	*Spinal cord*[b] *Area (mm²)*	*Cells per*[b] *mm²*	*P*[c]
B10.Q (H-2^q)	-	6	24.8 ± 9.8	10.0 ± 1.8	2.97 ± 1.04	—
B10.Q (H-2^q)	+	6	1.3 ± 0.7	10.8 ± 0.7	0.14 ± 0.08	0.02
B10.S (H-2^s)	-	6	24.3 ± 10.0	14.2 ± 1.0	1.67 ± 0.59	—
B10.S (H-2^s)	+	6	2.3 ± 2.1	12.5 ± 1.8	0.18 ± 0.18	0.03

[a]N = Number of mice
[b]Data presented as mean ± SEM
[c]Statistics by Student's *t* test comparing number of antigen-positive cells/mm^{-2} in mice of identical haplotypes

was highly statistically significant ($P > 0.03$).

All virus-antigen-positive cells at 45 days after infection were found in the white matter. Virus-antigen-positive cells were always in intimate contact with demyelinated axons (Figure 2). No virus antigen-positive cells were observed in areas of normal white matter. We correlated for each mouse the demyelination scores obtained on plastic embedded sections with the number of virus-antigen-positive cells per spinal cord area obtained by immunocytochemistry of frozen sections. There was good correlation between these two parameters (regression $P = 0.01$, correlation coefficient $R = 0.50$). This resulted in a regression equation of:

$$ag/mm^2 = 0.457 + (0.049)\ (\text{demyelination score})$$

B10.Q (D8$^-$) and B10.S (D8$^-$) mice showed the greatest demyelination scores and the most virus-antigen positive cells compared to B10.Q (D8$^+$) mice (Figure 3). In the eight D8$^+$ mice examined without demyelination, four mice showed no antigen-positive cells whereas the other four mice showed only one to four antigen-positive cells in the spinal cord sections examined.

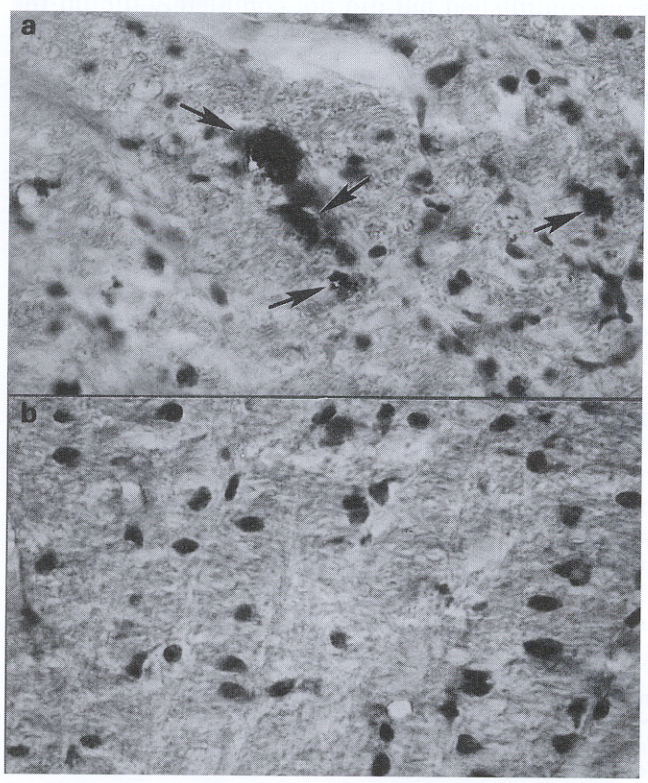

Figure 2 Detection of virus antigen on frozen sections of spinal cords from mice infected with Theiler's virus. Sections were stained by the avidin-biotin immunoperoxidase technique and developed with a solution of p-phenylenediamine-procatechol in the presence of hydrogen peroxide. Sections were counterstained with hematoxylin. (a) Virus Ag-positive cells in an area of inflammatory demyelination from a B10.Q (D8$^-$) mouse. (b) Absence of pathologic abnormalities or virus Ag-positive cells in a B10.Q (D8$^+$) mouse.

Discussion

These experiments demonstrated convincingly that

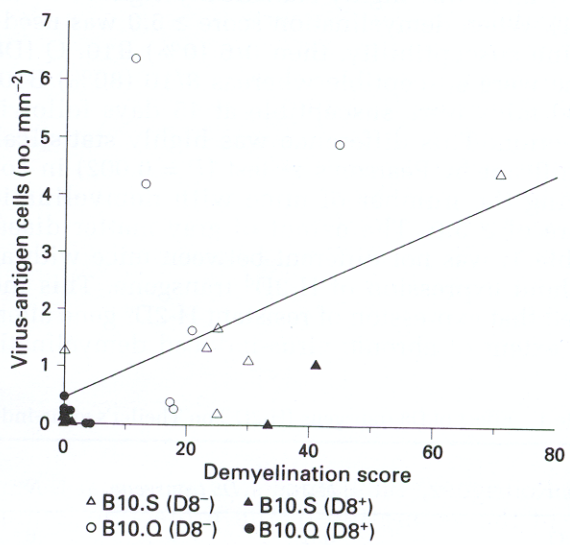

Figure 3 Correlation between the number of virus-antigen-positive cells per mm^2 of spinal cord as detected by immunoperoxidase staining on frozen sections, and demyelination scores detected by erichrome cresyl-violet stain on plastic-embedded sections. D8$^+$ mice are shown by closed circles or triangles. D8$^-$ mice are shown by open circles or triangles.

expression of H-2D^d transgene dramatically suppresses demyelination induced by TMEV. Expression of the H-2D^d transgene was associated with marked reduction in the number of virus-antigen-positive cells in the spinal cord 45 days following infection. The H-2D^d transgene resulted in more complete protection from chronic TMEV infection in H-2^q than in H-2^s mice. The precise role by which a class I MHC H-2D gene product controls virus-induced demyelination is unknown. Two hypotheses have been proposed to explain mapping of H-2D to resistance/susceptibility to TMEV-induced demyelination.

The first hypothesis is that class I MHC H-2D functions as a restricting molecule for a virus peptide recognized by CD8$^+$ T cell that functions as cytotoxic lymphocytes (CTL) to clear virus infection. B10.Q and B10.S mice would be susceptible to TMEV infection possibly as a consequence of a defect in clearing early virus infection. In support of the protective CTL hypothesis is the observation that genetic crosses (F1 progeny) of congeneic mice on identical C57BL/10 background with different MHC haplotypes derived from susceptible and resistant mice exhibit minimal or no demyelination and clear virus infection (Patrick *et al*, 1990). This is consistent with the hypothesis that the immunologic basis of resistance is determined by effective antigen presentation to the immune system. Also, transgenic expression of a human class I MHC gene (human leukocyte antigen B27) decreases demyelination and virus antigen expression in highly susceptible H-2^s and B10.D2^{dm1} mice (Rodriguez *et al*, 1991a, 1993). The abrogation of resistance to TMEV-induced demyelination in H-2^b mice deficient in β2 microglobulin also supports the role of a class I immune response mediated by virus-specific CD8$^+$ T cells in protection (Fiette *et al*, 1993; Pullen *et al*, 1993; Rodriguez *et al*, 1993).

In support of the protective CTL hypothesis is the demonstration of virus-specific cytotoxic CD8$^+$ T cells in the CNS (Lindsley *et al*, 1991) and spleens (Lindsley *et al*, 1991; Pena Rossi *et al*, 1991) of mice infected with TMEV. Preliminary experiments from our laboratory indicate that these CTLs are H-2D restricted in mice of C57BL/10 background. A rebutal to this hypothesis is the observation that cytotoxic lymphocytes (CTL) can be detected in the CNS of both susceptible and resistant strains of mice (Lindsley *et al*, 1991). However, CD8$^+$ T cells are detected earlier in the CNS of resistant (Lindsley and Rodriguez, 1989) mice suggesting that the timing of the generation of the protective immune response may be important in clearing virus from the CNS.

To test directly the role of H-2D region in clearing virus infection and preventing subsequent chronic demyelination, Bureau *et al* (1992) studied the amount of viral RNA in the spinal cords of 17 inbred strains of mice and their F1 crosses with the SJL/J strain. Viral persistence correlated strongly with the presence of susceptible alleles in H-2D, thus supporting the role of class I MHC in clearing infection. A second allele, outside of the H-2D region, contributed to virus persistence in the SJL/J (H-2^s) strain and was dominant. This may explain the partial protection from TMEV-induced demyelination and virus-antigen persistence in H-2^s mice expressing D8 (H-2D^d) transgene.

The second hypothesis is that class I MHC H-2D gene functions as a regulatory factor to control class II-mediated delayed-type hypersensitivity (DTH) responses important in TMEV-induced disease. In support of this hypothesis is the strong correlation between skin DTH responses to virus and susceptibility to chronic disease (Clatch *et al*, 1985: Gerety *et al*, 1991). Resistance can be restored to mice treated with low dose cyclophosphamide by adoptive transfer of T cell-enriched splenic cells (Olsberg *et al*, 1993). This ability to induce TMEV-induced demyelinating disease in otherwise resistant mice by an immunosuppressive regimen that preferentially abrogates suppressive mechanisms may be consistent with a protective role of a regulatory cell. In this model, H-2D region genes would function to 'down-regulate' a pathogenic CD4$^+$ -mediated immune response.

Examples in which susceptibility and resistance do not correlate with virus titers by plaque assay have been reported (Clatch *et al*, 1985, 1987). However, this does not exclude the possibility that H-2D is functioning primarily to limit virus infection. Virus persistence may be necessary but not sufficient for development of demyelination. In this scenario, other genes would determine the process of inflammation and subsequent demyelination. There is evidence that T cell receptor (TCR) V^β genes (Melvold *et al*, 1987; Kappel *et al*, 1991; Rodriguez *et al*, 1992) and non H-2 genes which do not involve the TCR (Melvold *et al*, 1990; Bureau *et al*, 1992) influence disease.

During the preparation of this manuscript Azoulay *et al* (1994) reported that transgenic expression of H-2D^b gene resulted in resistance to TMEV infection in susceptible FVB (H-2^q) mice. Transgenic expression of H-2D^b (probably the strongest resistance allele) cleared virus infection and inhibited demyelination. The experiments by Azoulay *et al* (1994) (using H-2D^b) in concert with the data presented in this manuscript (using H-2D^d) strongly support the hypothesis that H-2D genes play a critical role in preventing TMEV persistence and subsequent immune-mediated demyelination.

Materials and methods

Virus

The Daniel's strain (DA) of Theiler's virus was used in all these experiments. The passage history of this virus has been described previously (Rodriguez *et al*, 1983).

Mice

B6/D8 transgenic mice were provided by Dr M Bennett (Dallas, TX) with permission from Dr G Jay (Bethesda, MD) and maintained in the Mayo Mouse Immunogenetics Facility. D8 mice were backcrossed to B10.Q (H-2^q) and B10.S (H-2^s) to transfer the transgene into these strains. The D8 transgenic mice used in these studies were in the N4 backcross generation. D8$^+$ and D8$^-$ littermates were used to minimize for unknown differences.

Mice infection, harvesting, and morphology of spinal cord

Animals were injected intracerebrally with 2×10^5 plaque forming units of DA strain of TMEV in 10 µl of volume. On day 45 after infection, mice were perfused by intracardiac puncture with Trump's fixative (phosphate-buffered 4% formaldehyde with 1.5% glutaraldehyde, pH 7.2) and spinal cords were processed to provide 2-µm-thick glycolmethacrylate-embedded sections. A detailed, non-biased morphological analysis was performed on each of 10–15 coronal spinal cord sections from each of 57 mice, producing analysis of 3158 spinal cord quadrants. Independent pathological scores (maximum 100) for neuronal inflammation, meningeal inflammation, or demyelination were obtained for each animal as described (Rodriguez *et al*, 1991a, 1991b, 1992). Each quadrant from every coronal section from each mouse was scored for the presence or absence of the pathologic abnormality. The score was expressed as a percent over the total number of quadrants examined. The maximum pathologic score, 100, indicated the presence of the pathologic abnormality in every quadrant of all spinal cord sections of one mouse. Student's unpaired *t* test was used to compare mean values of scores for neuronal inflammation, meningeal inflammation and demyelination between D8$^+$ and D8$^-$ littermates with identical H-2.

Immunoperoxidase staining for TMEV antigen

Spinal cord blocks (2 mm thick) from animals perfused with Trump's fixative were stored in 0.1 M phosphate buffer. Blocks were rinsed in 0.1 M Tris buffer with 25 mM hydroxylamine, pH 7.4, treated with 10% dimethyl sulfoxide in the same buffer for 1 h and quick-frozen in isopentane chilled in liquid nitrogen. Frozen sections were reduced with 1% sodium borohydride in 0.1 M Tris buffer with 25 mM hydroxylamine, pH 7.4, at 4°C and refixed with 95% alcohol/5% glacial acetic acid. Sections were immunostained with rabbit polyclonal antiserum to purified Daniel's virus virions (Rodriguez *et al*, 1983) by avidin-biotin immunoperoxidase technique (Vector Laboratories, Burlingame, CA).

Morphometry of the number of virus-antigen-positive cells in spinal cord.

For quantitative analysis of number of virus antigen-positive cells, 5–8 spinal cord coronal sections from mice were processed for immunocytochemistry. A Zeiss (Oserkochen, West Germany) IBAS 2000 image analysis system with host computer was used to determine the total area of spinal cord sections. We analyzed a total 284.5 mm^2 of spinal cord tissue. The number of virus-antigen-positive cells for each mouse was counted and expressed per area of spinal cord. This allowed us to correlate the number of virus-antigen-positive cells with the extent of demyelination.

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