Mutation of a major histocompatibility class I locus, H-2D, leads to an increased virus burden and disease susceptibility in Theiler's virus-induced demyelinating disease

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Genetic studies have demonstrated that susceptibility to Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease is multigenic with linkage to the MHC class I locus, H-2D. We have analyzed the effect of mutations (H-2^{bm13} and H-2^{bm14}) in the H-2D^b gene on central nervous system (CNS) virus replication, virus-specific delayed type hypersensitivity (DTH) and disease induction in mutant [bm14D2F1 and bm13D2F1] and parental B6D2F1 hybrids. The results indicate that substitutions of only a single residue (bm14D2F1) or three residues (bm13D2F1) in H-2D in the mutant leads to a sequence of events culminating in disease susceptibility. Mutation of the H-2D gene is associated with reduced or delayed virus clearance following the acute phase of exponential CNS virus growth and an increased level of virus persistence. Concomittant with the greater virus antigen burden, mutant mice respond with higher levels of virus-specific DTH and develop inflammatory demyelinating lesions.

Keywords: demyelination; major histocompatibility class I locus; picornavirus; virus persistence

Introduction

After intracerebral inoculation (ic), certain Theiler's murine encephalomyelitis virus (TMEV) strains produce a persistent central nervous system (CNS) infection and chronic, inflammatory demyelinating disease in susceptible mice (Lipton 1975; Lehrich *et al*, 1976). Virus persistence is required for the development of demyelination (Chamorro *et al*, 1986; Lipton *et al*, 1991; Pritchard *et al*, 1994), and myelin breakdown appears to be immune-mediated rather than due to a cytolytic effect of the virus on oligodendrocytes, the myelin maintaining cell. Increased cellular immune responses, directed at virus epitopes rather than host neuroantigens (Clatch *et al*, 1986; Miller *et al*, 1987; Miller *et al*, 1989), suggest a role for virus-specific delayed type hypersensitivity (DTH) mediated by major histocompatibility class (MHC) class II-restricted Th1 lymphocytes in demyelination (Gerety *et al*, 1994a; Gerety *et al*, 1994b).

Genetic studies have demonstrated that susceptibility to TMEV-induced demyelinating disease in mice is multigenic with linkage to a MHC class I locus, H-2D (Clatch *et al*, 1985; Rodriguez *et al*, 1986). Previously, the differences between the susceptible DBA/2 (D2; H-2^d (D2; H-2^d) and resistant C57BL/6 (B6) and C57BL/10 (B10) strains (both H-2^b) were shown to be primarily dependent upon H-
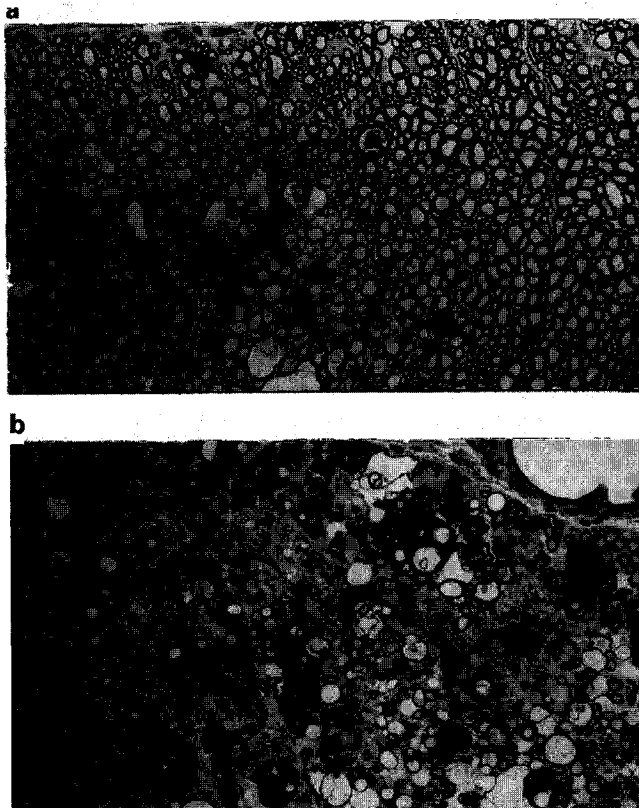
Figure 1 (a) Spinal cord sections from a parental B6D2F1 mouse showing a typical focal lesion characterized by the presence of naked axons and macrophages. In contrast to the bm13D2F1 animals, these mice never showed coalescing lesions, only scattered small foci as depicted in this photograph. (b) Spinal cord section from a mutant bm13D2F1 mouse showing severe disease. A few inflammatory mononuclear cells are present in the perivascular space in the upper right-hand corner. The right half of the photomicrograph shows ongoing demyelination in the presence of numerous large macrophages. The axons in the left half of the picture show extensive remyelination with both central and peripheral-type myelin. 1 μm-thick, Epon-embedded sections stained with toluidine blue about day 90 PI. X225.

2D genotype and at least one non-H-2 gene, with resistance being dominant to susceptibility (Melvold et al, 1987). In those experiments, the H-2D region had a strong influence and substitution of one or a few amino acids in the H-2D molecule was sufficient to alter the animal's phenotype from resistant to susceptible when placed in appropriate hybrids (Melvold et al, 1987). Similar conclusions were reported by Rodriguez et al, (Rodriguez et al, 1986) using the deletion mutant, H-2^{dml}. Recently, three groups of investigators (Fiette et al, 1993; Pullen et al, 1993; Rodriguez et al, 1993) have reported that disruption by homologous recombination of the β2-microglobulin gene, which causes abortive development of the CD8 lineage, converts mice resistant to TMEV-induced demyelinating disease to susceptible.

Detailed analysis of CNS virus growth is lacking in previous reports of mice with alterations of H-2D; therefore, we have studied the effect of mutations (H-2^{bm13} and H-2^{bm14}) in the H-2D^b gene on CNS virus growth and clearance during the acute phase and on virus persistence in mutant [bm14D2F1 and bm13D2F1] and in parental B6D2F1 hybrids. The H-2^{bm13} mutation involves a single amino acid substitution at residue 70, while H-2^{bm14} contains substitutions at amino acids 114, 116 and 119 (Hemmi et al, 1988). Structural analysis of the human class I HLA-A2 molecule reveals that these four mutated residues line the peptide binding groove (Bjorkman et al, 1987). These substitutions presumably affect antigen presentation and alter CD8+ T lymphocyte responses in the mutant mice.

Results

Potentiation of clinical and pathological demyelinating disease in mutant F1 mice

None of the BeAn virus-inoculated mice developed clinical disease during the first month post infection (PI). However, at 1 month PI, 16/26 (62%) of

Table 1 Incidence of demyelinating disease in parental and mutant F1 hybrid mice after IC inoculation of BeAn virus

F1 hybrid	H-2			No. affected[a]		No. with lesions	
	K	IA	D	A/T	(%)	A/T	Mean severity[b]
B6D2F1	b	b	b	1/12	(8)	3/4	1.03
	d	d	d				
bm13D2F1	b	b	bm13	16/26	(62)	4/4	3.00
	d	d	d				

[a]Demyelinating disease was determined clinically by the appearance of gait spasticity.
[b]Mean lesion severity was determined by grading approximately 10 coronal spinal cord sections from each mouse as 1, several lesions/section; 2, moderate numbers of lesions/section; and 3, extensive, confluent lesions/section
A/T = affected/total

the bm13D2F1 mice compared to 1/12 (8%) of the B6D2F1 progeny developed spastic paralysis indicative of demyelinating disease (Table 1). This difference in clinical disease was significant (X^2 = < 0.01). Similar comparisons for bm14D2F1 mice were precluded by the fact that these H-2D mutant animals were killed for virus titers during the first month (experiment 1, Material and methods).

Pathologic changes were assessed in four animals from each of the F1 hybrid groups. Sections from three of four unaffected B6D2F1 mice showed only occasional, small demyelinating lesions (Figure 1a); in no section were more than two small lesions found. The sections of the remaining B6D2F1 mouse were normal. In contrast, all of the sections from the diseased bm13D2F1 animals showed severe involvement. The entire lateral and anterior columns of the spinal cord were generally affected with no intervening areas of normal appearance. Demyelinating lesions were of different ages; some were characterized by ongoing myelin breakdown with abundant myelin debris and macrophages,
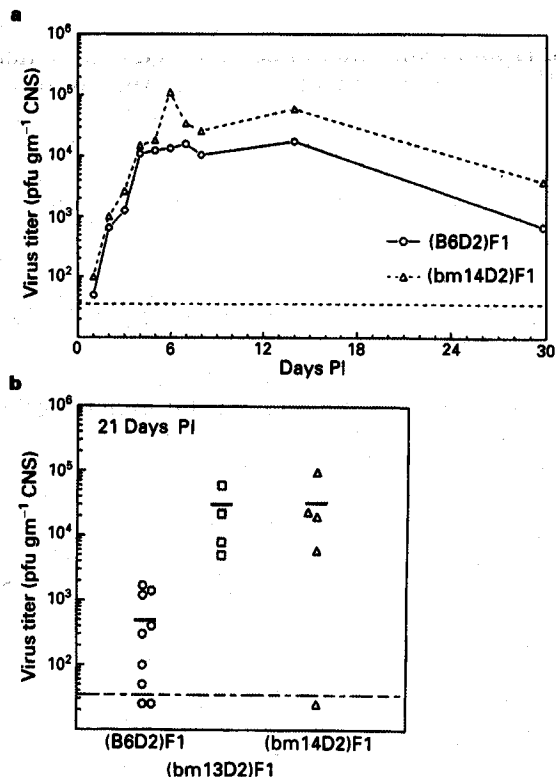

Figure 3 (a) Chronic phase CNS virus titers in individual mice from parental B6D2F1 and mutant bm13D2F1 mice after IC inoculation with BeAn virus. Horizontal solid lines represent mean virus titers. The mean virus titers, recorded as $\log_{10}$ transformed values ± the standard error of the mean were: diseased B6D2F1, 3.52; unaffected B6D2F1, 2.55 ± 0.55; diseased bm13D2F1, 4.09 ± 0.63; unaffected 2.05. Analysis of variance: for clinical condition of diseased mutant compared to either unaffected F1, P = 0.001; for unaffected F1 mice P. 0.05. (b) Virus-specific DTH responsiveness at about 90 days PI in unaffected, parental B6D2F1 and diseased, mutant bm13D2F1 mice. DTH responses in the two groups were significantly different ($P < 0.01$). $N = 4$ for each group.

while other lesions showed extensive remyelination, indicating previous demyelinating activity (Figure 1b). Therefore, there was a striking difference between the pathologic expression of disease in the B6D2F1 mice and mutant bm13D2F1 animals.

Reduced CNS virus clearance and increased virus persistence in mutant F1 mice

Both mutant bm14D2F1 and parental B6D2F1 mice showed a similar exponential phase of CNS virus growth between days 1–5 PI, but the virus content of the mutant mice reached and remained at higher levels between days 6–14 PI and did not decline by as much at day 30 PI (Figure 2a). This trend in the temporal pattern of virus growth in this initial experiment suggested there was reduced virus clearance in the mutant mice. Since these data were

Figure 2 (a) Acute CNS virus titers in parental B6D2F1 and mutant bm13D2F1 mice inoculated IC with BeAn virus. Each symbol represents the mean of two mice. The minimum detectable virus level of the assay, 25 pfu gm⁻¹ of CNS, is indicated by the dashed line. (b) CNS virus titers in individual parental B6D2F1 and mutant bm13D2F1 and bm14D2F1 mice after IC inoculation with BeAn virus. Horizontal solid lines represent mean virus titers for each group.
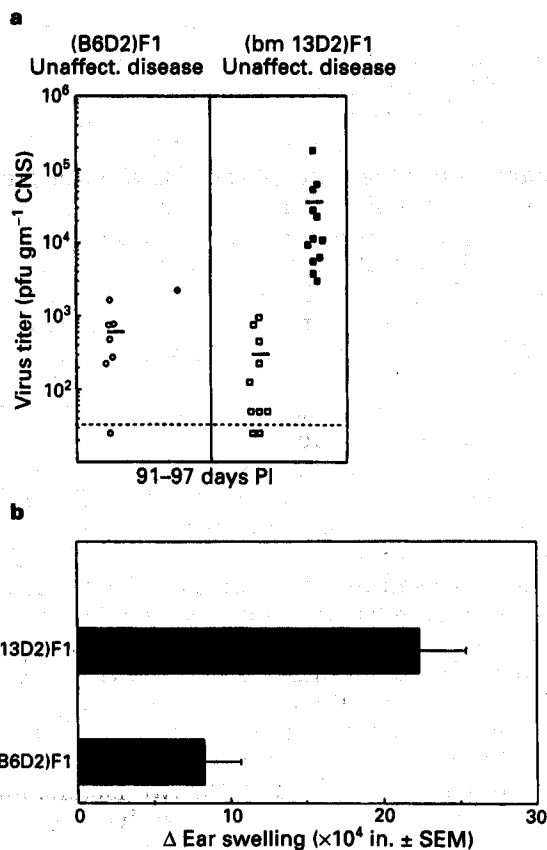
derived from mean titers of two animals at each time point, additional mice representing the three F1 hybrid groups were inoculated with BeAn virus and killed on day 21 PI. This is a time PI that should accurately reflect the efficiency of virus clearance since the acute phase of CNS virus growth peaks at 1 week PI and falls approximately 1 week later. As shown in Figure 2b, the mean CNS virus titers of the mice were significantly higher than those of the parental B6D2F1 mice, indicating reduced or delayed virus clearance (bm13D2F1 vs B6D2F1, P = 0.007; bm14D2F1 vs B6D2F1, P = 0.06).

Since virus persistence is necessary for development of demyelination (Chamorro et al, 1986; Lipton et al, 1991; Pritchard et al, 1994), virus titrations were performed on eight B6D2F1 and 22 bm13D2F1 infected progeny killed between PI days 91–97. Individual and mean virus titers of unaffected and diseased animals for each F1 hybrid are shown in Figure 3a. To determine whether the CNS virus content in both groups of hybrids could be related to the mutational/clinical status of the animals, mean virus titers of the groups were compared by analysis of variance. The fact that there was only one affected B6D2F1 mouse precluded a complete separate analysis of the two factors: mutation and clinical status and, possibly, interaction between the two. The data showed that the diseased mutant mice had a higher mean CNS virus titer than either the unaffected mutant (P = 0.001) or unaffected parental (P = < 0.001) animals. The comparison between the two unaffected F1 hybrids was not significant (P = 0.05). It is of interest that the virus titer of the single diseased parental mouse was slightly higher than the entire range of titers of unaffected mice, both parental and mutant. These results indicate that there is an effect of the H-2D locus on virus persistence. A similar conclusion was drawn by Bureau et al (1992) from quantitation of CNS TMEV RNA levels in a number of susceptible and resistant mouse strains.

Increased virus-specific DTH in mutant F1 mice
Virus-specific DTH, as an index of cellular immunity, was measured in four unaffected B6D2F1 and four diseased bm13D2F1 progeny at approximately day 90 PI. As shown in Figure 3b, the mean DTH response in the bm13D2F1 mice was significantly greater than that found in the B6D2F1 mice. It was not possible to compare virus-specific DTH in unaffected and diseased bm13D2F1 animals because of the limited number of mutant mice. These results are consistent with a greater antigenic stimulus due to higher titer of persisting virus in the mutant F1 mice.

Discussion

We have shown that mutation of the H-2D gene in a genetically resistant strain of mouse to TMEV-induced demyelinating disease leads to a sequence of events culminating in disease susceptibility. The results indicate that substitutions of only a single residue (bm14D2F1) or three residues (bm13D2F1) in H-2D are associated with reduced or delayed virus clearance following the acute phase of exponential CNS virus growth, and subsequently, an increased level of virus persistence. Presumably because of the greater virus antigen burden, the mutant mice respond with higher levels of virus-specific DTH and develop inflammatory demyelinating lesions. Thus, in this system an increasing virus burden appears to drive the immunopathology, which produces clinical signs of demyelination upon exceeding the pathologic threshold.

The H-2D gene may influence susceptibility (or resistance) to TMEV-induced demyelinating disease by affecting MHC class I T cells in either of two ways. The first is via the generation of CD8+ cytolytic T lymphocytes (CTL) which may contribute to CNS virus clearance (Lindsley et al, 1991; Rossi et al, 1991b). As yet neither comparisons of CD8+ CTL activity in susceptible and resistant strains of mice nor temporal analyses of CTL activity in TMEV-induced demyelinating have been reported. Borrow et al (1992) found that mice rendered CD8-deficient by thymectomy and in vivo depletion with a rat monoclonal antibody were less efficient at clearing CNS virus compared to intact animals, and developed more severe demyelination. Gerety et al (1994b) have confirmed the failure of depletion of CD8+ T lymphocytes to diminish demyelination. In contrast, Rodriguez and Sriram (1988), who also treated infected mice with a monoclonal antibody against CD8+ cells, reported that CD8 depletion reduced demyelination in mice. Whereas Borrow et al (1992) and Gerety et al (1994b) treated TMEV-infected mice chronically, in the experiments of Rodriguez and Sriram (1988), mice only received short-term treatment (3 days). Based on suppression of murine lupus by inhibition of CD4+ T lymphocytes, chronic administration of monoclonal antibodies appears to be necessary to achieve sustained functional inhibition of T cells, even in thymectomized mice (Connolly et al, 1992). It is also possible that other immune responses, such as neutralizing antibodies, ADCC and NK activity participate in clearance of persisting TMEV. Rossi et al (1991a) have reported that passive transfer of serum from infected, resistant B6 mice to infected, susceptible SJL mice during the first month PI results in a marked decrease in infected white matter cells. Additional studies are needed to determine how TMEV is cleared during the acute and persistent phases of the infection.

A second way that MHC class I T cells may affect susceptibility is via the activity of CD8+ regulatory T cells (Olsberg et al, 1993). In this instance, the putative loss of CD8+ regulation of virus-specific

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DTH in H-2D mutant mice as in this study or in resistant mice given low dose cyclophosphamide (Olsberg et al, 1993) or low dose irradiation (Rodriguez et al, 1990; Olsberg et al, 1993), would lead to increased virus-specific DTH and development of demyelination. The recruitment of monocytes into the CNS would provide target cells (macrophages) for continued virus growth (Clatch et al, 1990; Lipton et al, 1994) as well as effector cells involved in myelin damage (see below). Thus, the net effects of the loss of class I function, whether this effect is on CD8+ CTL or regulatory T lymphocytes, would be increased levels of virus persistence.

The present findings, the results from disruption by targeting the β2-microglobulin gene which converts mice resistant to TMEV-induced demyelinating disease to susceptible (Fietta et al, 1993; Pullen et al, 1993; Rodriguez et al, 1993), and the fact that mice rendered CD8-deficient by thymectomy and/or in vivo administration of monoclonal antibodies suffered TMEV-induced demyelinating disease of earlier onset and increased severity compared to intact animals (Borrow et al, 1992; Gerety et al, 1994b) seriously question the notion that the Theiler's immunopathology is mediated by CD8+ CTL. In contrast, the present data are entirely consistent with a virus-specific DTH mechanism of myelin breakdown induced by CD4+ Th1 cells (Clatch et al, 1986; Peterson et al, 1992; Peterson et al, 1993). A similar immune effector mechanism appears to be responsible for demyelination in experimental allergic encephalomyelitis (EAE) (Swanborg, 1983; Brostoff and Mason, 1986; Hayofh et al, 1989). Therefore, in TMEV-induced demyelinating disease the macrophage may serve as both a cell that perpetuates the infection, and, as in EAE, initiates the breakdown of myelin.

Materials and methods

Cell culture
BHK-21 cells were grown in 100-mm diameter plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 0.22% tryptose phosphate broth (Sigma), 100 µg ml⁻¹ of streptomycin, 100 U ml⁻¹ of penicillin, and 10% iron-supplemented bovine calf serum (Hyclone).

Virus
A plaque-purified stock of the BeAn 8386 virus was grown in BHK-21 cell monolayers. This virus stock produced small plaques and had a titer of 6.6×10^8 pfu ml⁻¹.

Animals and animal inoculations
Parental C57BL/6ByJ and (C57BL/6ByJ × DBA/2J) F1 hybrids (abbreviated B6 and B6D2, respectively) were purchased from The Jackson Laboratory, Bar

Harbor, ME. The (B6.C-H-2^{bm13} × DBA/2J)F1 and (B6.C-H-2^{bm14} × DBA/2J hybrids (abbreviated bm13D2 and bm14D2, respectively) were bred in the Center for Experimental Animals at Northwestern University. The mice were caged and maintained in accordance with AALAS standards and received standard mouse chow and acidified water ad libidum. Mice, 6 to 10 weeks of age, were anesthetized with methoxyfluorane (Metafane^R), inoculated in the right cerebral hemisphere with 1.3 $\times 10^6$ pfu of virus, and examined weekly thereafter for development of neurologic signs.

Preparation of CNS homogenates and virus assay
After mice were killed under anesthesia, each spinal cord was removed by forced flushing of the spinal canal with DMEM, homogenized as a 10% suspension with a Polytron (Brinkman Instruments Inc, Westbury, New York), further disrupted with a Branson sonifier (no. 2 setting for 30 s on ice), clarified by low speed centrifugation, and stored at −70°C. The virus content of clarified CNS samples was determined by standard plaque assay on BHK-21 cell monolayers as described previously (Lipton and Melvold, 1984).

Virus-specific delayed type hypersensitivity (DTH)
At approximately day 90 PI, the ears of infected parental F1 hybrid, H-2D mutant F1 hybrid, and control, uninfected B6 mice were measured with a Mitutoya Model 7326 micrometer (Schlesinger Tools, Brooklyn, New York). The dorsal side of each ear received an intradermal injection of 5 µg of purified BeAn virus, and each ear was again measured to determine the increase in thickness 24 h after challenge. The increases in thickness were expressed in units of 10^{-4} inches.

Histopathology
Mice were anesthetized and killed by perfusion through the left ventricle with 10 ml phosphate buffered saline (pH 7.3), followed by 100 ml chilled 3% glutaraldehyde in phosphate buffer (pH 7.3). Spinal cords were dissected from the vertebral canal, sectioned at 1-mm intervals, post-fixed in 1% osmic acid for 1 h and processed for Epon embedding. Ten 1-µm sections from each cord were stained with toluidine blue and examined by light microscopy.

Experimental design
Experiment 1, acute phase Using small numbers of mice, Melvoid et al (1987) previously reported that a high percentage of bm14D2F1 (63%) and bm13D2F1 (100%) hybrids developed clinical demyelinating disease after IC inoculation with BeAn virus. In the present study, all of the virus-inoculated bm14D2F1 mice were arbitrarily taken during the first month PI to determine the temporal course of CNS virus growth and clearance. Twenty

'parental' B6D2F1 mice, inoculated IC with BeAn virus, were designated at the beginning of the study for acute virus titrations, and were killed in parallel with the bm14D2F1 mice to provide a parental CNS virus growth curve.

Experiment 1, chronic phase The remaining 12 B6D2F1 and 26 bm13D2F1 mice (all inoculated with virus) were examined regularly for clinical disease until days 91–97 PI, when they were taken for either virus titrations (30 animals) or assayed for virus-specific DTH and then killed for histopathology (8 animals).

Experiment 2 All infected bm13D2F1, bm14D2F1 and B6D2F1 mice were taken at day 21 PI for virus titers.

Statistics

Two by two tests for independence were used to analyze the differences in disease frequency between groups. Differences between groups in $\log_{10}$ transformed values of virus titers were analyzed with the Mann-Whitney U test (unpaired samples).

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