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Identification of a Locus on Mouse Chromosome 3 Involved in Differential Susceptibility to Theiler's Murine Encephalomyelitis Virus-Induced Demyelinating Disease

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Theiler's virus-induced demyelinating disease results from a chronic infection in the white matter of the central nervous system and provides an excellent model for human multiple sclerosis. Like multiple sclerosis, there are genetic risk factors in disease development, including genes associated with the major histocompatibility complex and with those encoding the β chain of the T-cell receptor. Comparisons of the susceptible DBA/2 and resistant C57BL/6 strains have indicated an important role for the *H-2D* locus and for a non-*H-2* gene (not involving the β chain of the T-cell receptor) in differential susceptibility. In the present report, analysis of recombinant-inbred strains (BXD) between the DBA/2 and C57BL/6 strains indicated that this non-*H-2* locus is located at the centromeric end of chromosome 3 near (4 ± 4 centimorgans) the carbonic anhydrase-2 (*Car-2*) enzyme locus.

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus which exists normally as a enteric pathogen of both wild and laboratory mice. Viral replication in the gut causes no clinical symptoms, but upon gaining access to the central nervous system (CNS) (either spontaneously or experimentally), TMEV establishes a chronic low-grade infection (41). In susceptible strains of mice, this infection triggers a destructive immune response which leads to CNS demyelination and results in both clinical and histopathological symptoms. This infection serves as an important model for multiple sclerosis (MS) (10, 18, 19, 25, 42).

Both resistant and susceptible mice can generate antibody and proliferative T-cell responses against the virus after intracerebral inoculation. In contrast, it has been repeatedly demonstrated that susceptible mice develop significant levels of TMEV-specific delayed-type hypersensitivity (DTH), while resistant mice do not. The absence of this DTH response in resistant animals after intracerebral inoculation is specific and does not represent a generalized immunological deficiency. In fact, resistant animals can generate DTH against UV-inactivated TMEV when the virus is injected peripherally with complete Freund adjuvant (6). It is believed that this DTH response against the infected cells of the CNS is, in large part, responsible for the demyelination (5–7). In addition, some investigators have postulated a role for cytotoxic T lymphocytes in the demyelinating process (36), but there is no definitive evidence in this regard.

There is a clear genetic basis for differences in susceptibility to development of the demyelinating disease (7, 21, 22, 27, 33, 34). Multiple loci are involved, and comparisons employing different combinations of susceptible and resistant genotypes have revealed different sets of loci as having primary effects (22, 27). Thus far, effects have been demonstrated for the major histocompatibility complex (MHC) class I locus, *H-2D* (7, 34) as well as for a non-MHC gene located in or near those encoding the β chain of the T-cell receptor (27).

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In comparisons of susceptible DBA/2 and resistant C57BL/6 mice, both MHC and non-MHC genes are involved. The relevant MHC gene has been shown to be *H-2D*, and single gene mutations at this locus in appropriate hybrids can convert the animals from resistant to susceptible (28). However, a non-*H-2* gene from the susceptible DBA/2 genome is also essential for disease development. In this study, we localized that gene to chromosome 3 near the *Car-2* locus.

MATERIALS AND METHODS

Animals and inoculations. All the mice used were bred in our facility at Northwestern University Medical School. BXD recombinant-inbred breeders were received from the Jackson Laboratory (Bar Harbor, Maine) through the courtesy of Ben Taylor. At 4 to 6 weeks of age, mice were anesthetized with methoxyfluorane and inoculated in the right cerebral hemisphere with 1.3×10^6 PFU of BeAn 8386 virus. All animals were examined daily for the first 3 weeks and weekly thereafter up to 90 to 120 days postinoculation for development of clinical neurologic symptoms, particularly the chronic gait abnormality caused by demyelination (19). Control mice were uninjected or injected with phosphate-buffered saline alone, but kept in the same environment as the experimental animals.

Virus. BeAn 8386 was isolated from a feral mouse in Belem, Brazil, in 1957 and was later classified as a TMEV by complement fixation serology. After plaque purification and titer amplification by serial passage in BHK-21 cells, two working stocks, A20 and 173J, were prepared which had titers of 1.3×10^8 and 2.6×10^8 PFU/ml, respectively.

Histology. At 105 to 120 days postinoculation, two mice from each strain were anesthetized and sacrificed by perfusion through the left ventricle with 10 ml of phosphate-buffered saline (pH 7.3) followed by 100 ml of chilled 3% glutaraldehyde in phosphate buffer (pH 7.3). Spinal cords were dissected from the vertebral canal, sectioned at approximately 1-mm intervals, postfixed in 1% osmic acid for 1 h, and processed for Epon embedding. Fifteen 1-μm-thick

TABLE 1. Susceptibility of BXD recombinant-inbred mice to TMEV-induced demyelination: role of *H-2*

BXD strain	Affected/ total (%)	Day of onset[a]	Susceptibility type[b]	*H-2* type[b]
1	5/9 (56)	63, 63, 67, 67, 71	D	D
2	1/9 (11)	80	B	B
5	8/11 (73)	28, 35, 38, 49, 52, 60, 60, 62	D	D
8	1/9 (11)	77	B	B
9	5/8 (63)	41, 48, 55, 85, 105	D	D
11	6/7 (84)	43, 44, 51, 51, 51, 85	D	D
12	3/3 (100)	45, 62, 71	D	D
13	0/3 (0)		B	B
14	0/9 (0)		B	B
15	0/4 (0)		B	B
18	8/15 (53)	44, 44, 55, 75, 85, 88, 88, 88	D	D
19	1/10 (10)	71	B	B
21	0/3 (0)		B	D
22	1/10 (10)	73	B	D
23	0/6 (0)		B	B
24	14/15 (93)	35, 35, 35, 35, 43, 43, 51, 51, 51, 51, 56, 62, 76, 89	D	D
25	1/10 (10)	121	B	D
27	7/8 (88)	36, 54, 54, 58, 69, 90, 90	D	D
28	0/7 (0)		B	D
29	0/9 (0)		B	B
30	4/6 (67)	45, 54, 54, 69	D	D
31	8/9 (89)	38, 46, 52, 105, 105, 149, 149, 149	D	D
32	2/10 (20)	71, 81	B	D

[a] Day postinoculation for appearance of symptoms in each affected animal is given.

[b] D, Susceptible DBA/2 phenotype and genotype; B, resistant C57BL/6 phenotype and genotype. Strains with 50 to 100% affected mice were considered susceptible. Those with 0 to 20% affected were considered resistant.

sections from each spinal cord were stained with toluidine blue and examined by light microscopy (10).

Measurement of DTH. At appropriate times (90 to 120 days) postinfection, the ears of infected and uninfected (negative control) mice were measured with a Mitutoya model 7326 micrometer. Immediately thereafter, the dorsal side of each ear received a dermal injection of purified BeAn 8386 virus (5 μg in 10 μl of phosphate-buffered saline, pH 7.2) to elicit virus-specific DTH responses (7). At 24 h after challenge, each ear was again measured to determine the increase in thickness (swelling due to mononuclear cell infiltration). The increases in thickness were expressed in units of 10^{-4} inches (2.54×10^{-4} cm).

Statistics. Statistical comparisons employed the Student *t* test and one-way analysis of variance.

RESULTS

Susceptibility among BXD recombinant-inbred strains. We obtained 23 of the BXD strains at the appropriate age for induction of TMEV-induced demyelinating disease. When inoculated intracerebrally with 1.3×10^6 PFU of BeAn 8386 virus, 10 of the 23 strains were susceptible (Table 1). All of the 8 strains carrying the *H-2* haplotype of the resistant B6 strain (*H-2*^{b}) were resistant, while 10 of 15 strains carrying *H-2*^{d} (from the susceptible D2 strain) showed clinical symptoms of CNS demyelination. Because the *H-2*^{b} haplotype has been regularly associated with resistance to TMEV-induced demyelination (7, 28), it was necessary to remove the *H-2*^{b} strains from the analysis to determine the strain distribution pattern for relevant non-*H-2* genes.

TABLE 2. Correlation of susceptibility with *Car-2* genotype in BXD strains carrying the *H-2*^{d} haplotype

BXD strain	Affected/total (%)	Susceptibility type[a]	*Car-2*
1	5/9 (56)	D	D
5	8/11 (73)	D	D
9	5/8 (63)	D	B
11	6/7 (84)	D	D
12	3/3 (100)	D	D
18	8/15 (53)	D	D
21	0/3 (0)	B	B
22	1/10 (10)	B	B
24	14/15 (93)	D	B
25	1/10 (10)	B	B
27	7/8 (89)	D	D
28	0/7 (0)	B	B
30	4/6 (67)	D	D
31	8/9 (89)	D	D
32	2/10 (20)	B	B

[a] D, Susceptible DBA/2 phenotype and genotype; B, resistant C57BL/6 phenotype and genotype. Strains with 50 to 100% affected mice were considered susceptible. Those with 0 to 20% affected were considered resistant.

Susceptibility among *H-2*^{d} BXD recombinant-inbred strains. Among 15 BXD strains bearing *H-2*^{d}, a strain distribution pattern was determined in which 10 strains (1, 5, 9, 11, 12, 18, 24, 27, 30, and 31) were like the susceptible D2 parental strain, while 5 strains (21, 22, 25, 28, and 32) were, like the B6 parental strain, resistant (Table 2). In a survey of the strain distribution patterns of other loci among these 15 strains, the only significant match was that for carbonic anhydrase-2 (*Car-2*) on chromosome 3. The strain distribution patterns for *Car-2* and for susceptibility to TMEV-induced demyelinating disease were concordant in 13 of 15 strains, differing only in strains 9 and 24. Concordance in 13 of 15 cases is statistically significant at the 0.02 level by the statistical methodology of Green (14), suggesting linkage of at least one locus important in determining susceptibility and *Car-2* on chromosome 3. The linkage distance between the two (susceptibility to TMEV-induced demyelinating disease and *Car-2*) is estimated at 4 ± 4 centimorgans, according to the methodology of Taylor and Meier (40).

Histopathology. Examination of spinal cords from infected animals of the various BXD strains indicated that clinical diagnoses were consistent with the histopathological data. Strains designated as susceptible by clinical symptoms typically exhibited demyelination and inflammatory cell infiltrates, while animals from resistant strains typically exhibited few or no such lesions (data not shown).

Role of non-*H-2* loci in segregating populations. The influence of non-*H-2* loci is evident in examining the proportion of susceptible progeny in backcross and F_2 progeny (Table 3). Unlike completely resistant (B6D2)F_1 animals, which are *H-2*^{b}/*H-2*^{d} and have B6 and D2 non-*H-2* genes in a 1:1 ratio, diseased animals are often seen among *H-2*^{b}/*H-2*^{d} heterozygotes in which the ratio of B6 to D2 alleles is 1:3 for the entire genome (Table 3, top) or for only some loci (Table 3, bottom). Thus, an excess of non-*H-2* genes from the susceptible D2 parent can overcome the protective effect of a single copy of the *H-2*^{b} haplotype routinely seen in comparisons among inbred and congenic strains involving this same strain combination (28).

Association of susceptibility with TMEV-specific DTH re-

TABLE 3. Influence of non-*H-2* genes on susceptibility in segregating populations

Progeny	*H-2*	Resistant	Susceptible	Total (% susceptible)
(B6D2)F_1 × D2 backcross	*b/d*	25	10	35 (29)
	d/d	6	33	39 (85)
Total		31	43	74 (58)
(B6D2)F_2	*b/b*	9	0	9 (0)
	b/d	21	4	25 (16)
	d/d	6	7	13 (54)
Total		36	11	47 (23)

sponsiveness. Figure 1 illustrates the concordance of TMEV-induced demyelinating disease with the ability to mount TMEV-specific DTH responses after intracerebral inoculation. As in previous reports on susceptibility-resistance differences in a variety of strain comparisons, including *H-2*-related differences between the B6 and D2 strains (28), such DTH responsiveness is strongly correlated with disease incidence. Other antiviral parameters examined (TMEV-specific T-cell proliferation and anti-TMEV antibody titers) did not correlate with disease incidence (data not shown), in accordance with results reported for other strain combinations (5–7, 22, 28).

DISCUSSION

Previous reports from this and other laboratories have identified multiple loci influencing differential resistance to TMEV-induced demyelinating disease. These include the *H-2D* locus on chromosome 17 (7, 34) and a locus very near the constant gene for the β chain of the T-cell receptor on chromosome 6 (27), possibly one (or more) of the variable genes contributing to the T-cell receptor β-chain repertoire. In both of these cases, a logical connection can be drawn between the gene functions and an immunological basis for the disease process. Furthermore, the human equivalents are known for both loci. In the current report, however, we identified an additional significant influence of at least one additional locus, on chromosome 3 near the *Car-2* locus (about 4 ± 4 centimorgans from *Car-2*). Unfortunately, there are no known loci other than *Car-2* mapped to this particular area of chromosome 3 which might be specifically analyzed. Attempts to further isolate the gene affecting susceptibility using a cDNA probe for the *Car-2* gene have been initiated via restriction fragment length polymorphism analysis.

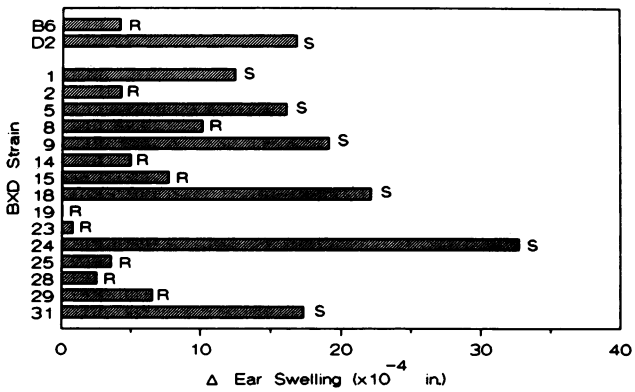

FIG. 1. Correlation of susceptibility to TMEV-induced demyelinating disease with TMEV-specific DTH responsiveness. Resistant (R) or susceptible (S) phenotypes (from Table 1) are given for each tested strain. Data are given only for those BXD strains in which susceptibility-resistance and DTH data were obtained from at least three animals each.

Among the tested BXD recombinant-inbred strains, a strong correlation was found between susceptibility and the presence of a significant DTH response against TMEV. This observation is consistent with numerous previous studies utilizing several inbred, *H-2* congenic, *H-2* recombinant, and R-I strains (5–7, 28), further supporting a critical role for DTH in the disease process. Presumably, the relevant locus on chromosome 3 has some role in permitting the DTH, and the disease process, to proceed.

Because the analysis does not identify a known genetic locus, we have no specific basis at this point for proposing a particular function for the locus, although numerous hypothetical functions could be postulated for this genetic locus, e.g., (i) regulation of viral replication rates within the infected cells, (ii) encoding cell surface molecules used by the virus as specific receptors for entry into particular cell types, (iii) encoding surface molecules which (in concert with viral components) provide suppressive-enhancing signals to the DTH effector arm of the immune system, or (iv) encoding surface molecules whose expression and subsequent function might be modulated by TMEV infection (such that the susceptible and resistant allelic forms may be quantitative, rather than qualitative, variants). Other functions may surely be postulated as well, but there is currently no evidence for or against any of these. The role of this locus in differential susceptibility remains purely speculative for the moment, awaiting further analysis.

MS, a disease documented as far back as the 14th century (26), results from inflammatory demyelination of axons in the CNS white matter. Studies indicate that the immune system has a major role in the pathogenesis of MS (4, 42, 48), and there is strong evidence to suggest that MS is triggered by viral infection (1, 25), although no specific virus has yet been consistently found in MS patients.

There is clearly a genetic component among the risk factors for MS (reviewed in reference 38). Both family studies (12, 32, 37) and twin studies (9, 11, 47) have indicated a heritable risk for MS. Numerous studies also indicate an association between MS and the human leukocyte antigen (HLA) complex on chromosome 6 (3, 13, 16, 17, 24, 39, 46), especially for the A3, B7, and DRw2 alleles at their respective loci within the HLA complex. However, the situation is complex and no single gene has been identified as having a substantial effect, with even the cited HLA genes having low relative risks for MS.

With the belief that there is a viral etiology for MS (1, 25), animal models for MS provide an opportunity to correlate viral infections and disease, especially when resistance to the virus can be shown to have a genetic basis (2). Several such animal models for virally induced CNS demyelination exist: caprine encephalitis virus infection in goats (8), canine distemper virus infection in dogs (49), murine hepatitis virus type in mice and rats (43–45), visna virus infection in Icelandic sheep (15, 29), and TMEV infection in mice, among others.

TMEV, a picornavirus which is a natural pathogen of mice (30), provides a particularly strong model for human MS (23) because of its viral etiology (18–20, 35, 41), its immunological involvement in the disease process (7, 20, 31, 36), and its

occurrence in a species in which genetics can be exquisitely controlled and manipulated. Identification of genes involved in risk from this animal model may be applicable in humans, especially when the human equivalent is known, perhaps in both predictive terms and providing clues as to mechanisms.

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