Sex-Specific Quantitative Trait Loci Govern Susceptibility to Theiler's Murine Encephalomyelitis Virus-Induced Demyelination

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ABSTRACT

Susceptibility to Theiler's murine encephalomyelitis virus-induced demyelination (TMEVD), a mouse model for multiple sclerosis (MS), is genetically controlled. Through a mouse-human comparative mapping approach, identification of candidate susceptibility loci for MS based on the location of TMEVD susceptibility loci may be possible. Composite interval mapping (CIM) identified quantitative trait loci (QTL) controlling TMEVD severity in male and female backcross populations derived from susceptible DBA/2J and resistant BALBc/ByJ mice. We report QTL on chromosomes 1, 5, 15, and 16 affecting male mice. In addition, we identified two QTL in female mice located on chromosome 1. Our results support the existence of three linked sex-specific QTL on chromosome 1 with opposing effects on the severity of the clinical signs of TMEV-induced disease in male and female mice.

MULTIPLE sclerosis (MS) is the major demyelinating disease of the central nervous system (CNS) in humans, affecting 0.1% of the North American population, and involves both genetic and environmental factors (Sadovnick and Ebers 1993; Ebers and Sadovnick 1994; Ewing and Bernard 1998; Compston 1999; Kalman and Lublin 1999; Sadovnick 2002). Concordance rates among monozygotic twins are 20–30% while dizygotic twins, full siblings, and nonbiological siblings have concordance rates of ~4% (Ebers *et al.* 1995; Sadovnick *et al.* 1996). Although a genetic component to susceptibility has been demonstrated, little is known about the genes that modulate MS. Evidence of an environmental etiology for MS comes primarily from migration studies and geographic distribution data (Ebers and Sadovnick 1994). Migration studies indicate that individuals moving from high-risk areas tend to adopt the low risk of native populations (Ebers and Sadovnick 1994; Compston 1999). Susceptibility to MS is likely the result of complex interactions of environmental triggers on a susceptible genetic background.

Viruses have long been purported to play a role in the etiology of MS. Human herpes virus-6, Epstein-Barr virus, and measles virus have been detected in the brains of MS patients, but no single virus has been associated with all cases (Challoner *et al.* 1995; Dalgleish 1997). Theiler's murine encephalomyelitis virus-induced demyelination (TMEVD) is a model for virally triggered MS. TMEV is a murine picornavirus spread in natural and laboratory populations by the fecal/oral route (Miller *et al.* 1994). Following intracerebral inoculation, the virus establishes a persistent infection of CNS white matter in susceptible strains. $CD4^+$ T cells initiate the disease by infiltrating the CNS and subsequently recruit additional lymphocytes, leading to inflammation and progressive demyelination. Clinical signs become apparent 35–40 days postinoculation and show a progressive course characterized by gait abnormalities, limb spasms, and incontinence (Miller *et al.* 1994).

H2D and two loci on chromosome 10 have been associated with viral persistence (Clatch *et al.* 1985; Bureau *et al.* 1993; Lipton *et al.* 1995; Bihl *et al.* 1999). Other loci controlling susceptibility to TMEVD have been identified on chromosomes 3, 6, 11, and 14 (Melvold *et al.* 1987, 1990; Brahic and Bureau 1998; Bureau *et al.* 1998; Aubagnac *et al.* 1999). Recently, we examined the most severely affected animals in a (BALB/cByJ × DBA/2J) × BALB/cByJ backcross by qualitative assessment (Teuscher *et al.* 1997). We reported that susceptibility to TMEVD was linked to a locus on chromosome 3 between *D3Mit29* and *D3Mit10* near *eae3*, a locus associated with susceptibility to experimental allergic encephalomyelitis (EAE), suggesting that a shared susceptibility gene or a cluster of tightly linked genes control susceptibility to both of these demyelinating diseases.

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The shared gene hypothesis for non-MHC-linked genes underlying immunopathologically based phenotypes, first proposed by this laboratory (Teuscher 1985; Sudweeks *et al.* 1993; Meeker *et al.* 1995), was recently validated with the identification of *Bphs*, an autoimmune disease susceptibility gene linked to EAE and autoimmune orchitis as histamine receptor H_1 (Ma *et al.* 2002). Interestingly, CD2, a known polymorphic cell surface protein important in T-cell activation, colocalizes with *Tmevd2* and *eae3* on chromosome 3 (Altevogt *et al.* 1989; Moseley and Seldin 1989).

Males and females of the same strain can differ in susceptibility to TMEVD (Kappel *et al.* 1990; Hill *et al.* 1998). In addition, a sex effect has been associated with viral persistence in the CNS. *Tmevp2* and *Tmevp3* were identified on chromosome 10, with males exhibiting a greater viral load than females (Bihl *et al.* 1999). In this work we used composite interval mapping (CIM; Zeng 1993, 1994) to identify sex-dependent quantitative trait loci (QTL) on chromosomes 1, 5, 15, and 16 in males and two QTL on chromosome 1 in females controlling severity of TMEVD.

MATERIALS AND METHODS

Animals: Male and female BALB/cByJ and DBA/2J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). (BALB/cByJ × DBA/2J) × BALB/cByJ backcross mice (BC1) were bred at Northwestern University School of Medicine (Chicago) and the University of North Dakota (Grand Forks, ND). All mice were maintained in polycarbonate cages and received standard mouse chow and water *ad libitum*. Mice used in these studies were maintained according to the guidelines of the Animal Care and Use Committees of the University of North Dakota and Northwestern University, fully accredited by the American Association of Animal Laboratory Care. Of the 170 BC1 animals included in this study, 71 were male and 99 were female.

Induction of disease: The BeAn 8386 strain of TMEV was used for disease induction in this study. After plaque purification and titer amplification by serial passage in BHK-21 cells, a working stock was prepared with a titer of 9.7×10^8 PFU/ml. At 7 weeks of age, the mice were anesthetized with methoxyflurane and inoculated in the right cerebral hemisphere with 2.9×10^6 PFU of virus. Control mice were injected with media or were mock infected with BHK lysate in the same manner. Both the control and the experimental animals were housed in the same environment.

Evaluation of phenotype: Following inoculation, the animals were examined independently by two investigators for a period of 13 weeks. Severity of clinical signs was scored on the following basis: 0 for asymptomatic, 1 for moderate (swaying) gait abnormality, and 2 for severe (waddling) gait abnormality. Clinical signs have been previously shown to provide a good correlation with demyelination when compared with histological examination or testing of TMEV-specific delayed-type hypersensitivity responsiveness (McGavern *et al.* 2000). A quantitative trait value for estimating the overall severity of the disease as a function of time postviral challenge, similar to that used in studies on murine EAE (Butterfield *et al.* 1998), was calculated by averaging the scores for each animal over the course of the experiment.

Genotyping and linkage analysis: Genomic DNA was isolated from liver tissue. PCR-based genotyping using 199 polymorphic microsatellite markers was performed as previously described (Sudweeks *et al.* 1993; Meeker *et al.* 1995; Wardell *et al.* 1995). Microsatellite primers were either purchased from Research Genetics (Huntsville, AL) or synthesized according to sequences obtained through the Whitehead Institute/MIT genome database (http://www.genome.wi.mit.edu/). Microsatellite size variants were resolved by electrophoresis either on agarose gels stained by ethidium bromide or on large denaturing polyacrylamide gels visualized with autoradiography. A linkage map was estimated using MAPMAKER/EXP computer package (Lander *et al.* 1987; Lincoln *et al.* 1992) with the Kosambi map function. CIM was used for localization of QTL governing severity of TMEVD using model 6 of the Zmapqtl program in QTL Cartographer software, version 1.13g (http://statgen.ncsu.edu/qtlcart/cartographer.html; Basten *et al.* 1997). By combining classical interval mapping with multiple regression, CIM allows for more precise QTL localization than does classical interval mapping. Additionally, CIM controls for spurious ghost loci (Zeng 1993, 1994; Doerge *et al.* 1997). Significant markers are first chosen using a linear regression model with a forward/backward selection procedure in the SRmapqtl program of QTL Cartographer. Markers flanking the test interval are added to the regression model to control for the presence of linked QTL. Additional markers, unlinked to the test interval, but with significant effects on the trait, are added to the model to control for the genetic background. In this study, background markers for CIM were chosen using the SRmapqtl module of QTL Cartographer in forward/backward selection with an accepting/rejecting significance level of 0.10 using 198 markers (all markers except *D15Mit209*, which was subsequently added to the analysis). Composite interval mapping was performed using 2-cM increments with a window size of 10 cM, and the 10 most significant background markers selected via SRmapqtl as described above were used in our CIM analyses. Tests of significant linkage for QTL are reported as likelihood-ratio test (LRT) statistics. Significance of the linkage between marker loci and putative QTL was assessed by permutation-based threshold analysis (Churchill and Doerge 1994; Doerge *et al.* 1997). Significant ($\alpha = 0.05$) and suggestive ($\alpha = 0.10$) experimentwise critical values were determined using the distribution of maximum LRT statistics from 1000 permutations of our data.

RESULTS AND DISCUSSION

Gait abnormalities were seen in 120 of the 170 BC1 animals in the study. Significant differences in sex-specific susceptibility were not seen in parental BALB/cByJ, DBA/2J, or F_1 hybrid mice using cohort sizes of 9 male and female mice (see Table 1). In our BC1 population, however, a greater number of males (56 of 71) than females were susceptible to TEMVD (64 of 99, $\chi^2 = 4.03$, $P = 0.045$). Kappel *et al.* (1990) have also shown an increase in susceptibility to TMEVD in male mice. Sex-specific effects have also been observed in overall susceptibility to MS (Duquette *et al.* 1992) as well as in disease subtypes (Runmarker and Anderson 1993). As such, identification of the genes uniquely controlling susceptibility to TMEVD in males and females may lead to a better understanding of the role of sex-specific QTL in inflammatory diseases of the CNS.

Loci involved in TMEVD severity were identified using CIM on subpopulations consisting of males and

TABLE 1

Incidence of symptoms of TMEVD in DBA/2J, BALB/cByJ, (BALB/cByJ × DBA/2J) F_1 hybrid, and (BALB/cByJ × DBA/2J) × BALB/cByJ backcross mice by sex

			Symptoms of TMEVD	
Strain	Sex	Total	Unaffected (clinical score = 0)	Affected[a] (clinical score = 1 or 2)
DBA/2J	M	9	0	9
DBA/2J	F	9	0	9
BALB/cByJ	M	9	7	2
BALB/cByJ	F	9	8	1
F1	M	9	7	2
F1	F	9	9	0
BC1	M	71	15	56
BC1	F	99	35	64

[a] Animals were considered affected if they displayed either mild (score = 1) or severe (score = 2) abnormalities in gait for three consecutive weekly evaluations or for four out of five consecutive weekly evaluations.

females. In the male population ($n = 71$), CIM revealed significant loci ($\alpha = 0.05$) on chromosomes 1, 5, and 15. A QTL on chromosome 1 (*Tmevd6*) near *D1Mit170* at 19.5 cM accounted for 9.5% of the variation in the severity of the clinical signs associated with TMEVD severity (Figure 1, Table 2). (Mouse chromosomes are acrocentric; thus all centimorgan distances are relative to the centromere.) Interestingly, the negative additive effect (−0.26; see Table 2) indicated that the susceptibility allele was derived from the TMEVD-resistant BALB/cByJ. A QTL on chromosome 5 (*Tmevd7*) at 72 cM, near *D5Mit30*, accounted for 16.6% of the variation (Figure 1, Table 2) and increased severity at this locus was associated with the DBA/2J allele. On chromosome 15 at 4.7 cM near *D15Mit12*, a QTL (*Tmevd8*) accounted for 14.1% of the trait variation. Additionally, in male mice, suggestive linkage ($\alpha = 0.10$) was found on chromosomes 15 and 16. A QTL on chromosome 15, at 22.2 cM near *D15Mit5*, accounted for 8.1% of the variation in the trait, while a locus on chromosome 16 near *D16Mit50* at 53.5 cM accounted for 8.1% of the variation (Figure 1, Table 2). Interestingly, the QTL identified on chromosome 16 in males colocalizes with *eae11*, a locus controlling lesion severity and susceptibility to EAE in males (Butterfield *et al.* 1999). Linkage to this region of chromosome 16 may reflect a hormonally regulated gene or gene complex controlling immunologically mediated demyelination. Independent verification of the suggestive loci on chromosomes 15 and 16 will be required before these QTL will be given TMEVD designations.

Analysis of the female population ($n = 99$) revealed two QTL influencing the severity of disease symptoms. A significant QTL, *Tmevd9*, was found on chromosome 1 at 32.8 cM, near *D1Mit76*, and accounted for 7.6% of the variation (Table 2, Figure 2). This QTL colocalizes

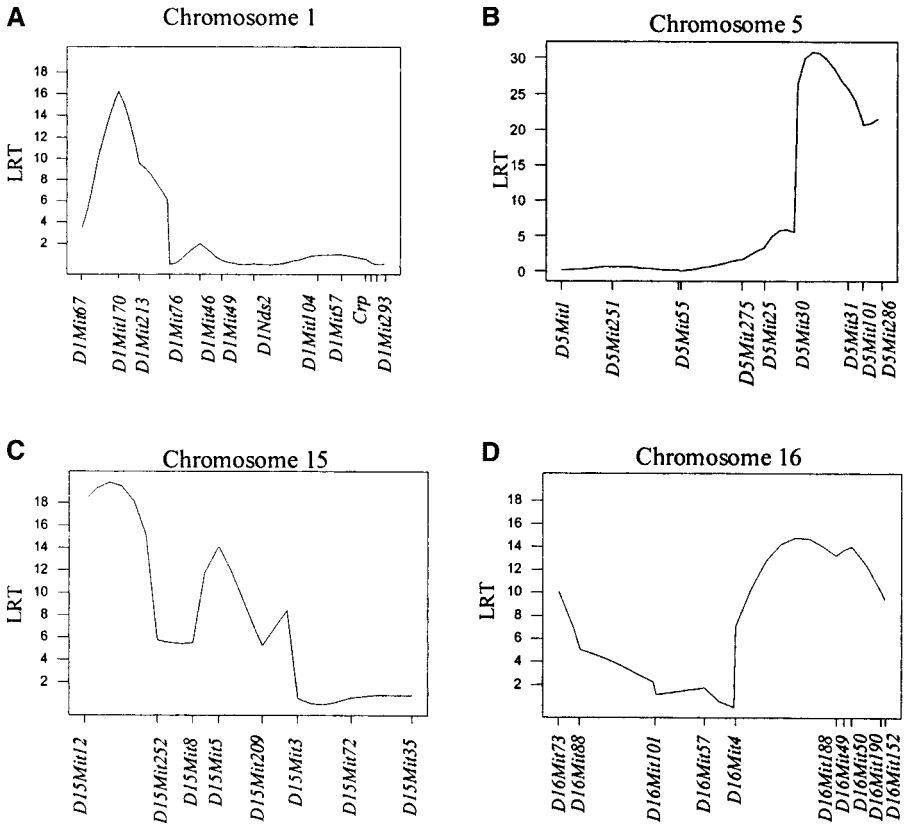

FIGURE 1.—Composite interval mapping results for the male population. Tick marks on the *x*-axis represent the positions of microsatellite markers on the genetic map. Permutation-derived significance cutoffs were based on 1000 permutations. Significance cutoffs for males are based on LRTs: LRTs = 15.6 ($\alpha = 0.05$) and 13.7 ($\alpha = 0.10$).

TABLE 2

Location and effects of QTL controlling severity of symptoms in (BALB/cByJ × DBA/2J) × BALB/cByJ backcross mice

	QTL designation	Chr.	cM[a]	Marker[b]	LRT[c]	Additive % Effect[d]	Additive % Variance[e]
Males	*Tmevd6*	1	19.5	*D1Mit170*	16.21	−0.26	9.5
	Tmevd7	5	72	*D5Mit30*	26.45	0.37	16.6
	Tmevd8	15	4.7	*D15Mit12*	18.34	−0.36	14.1
		15	22.2	*D15Mit5*	14.10	0.29	8.1
		16	53.5	*D16Mit50*	14.06	0.24	8.1
Females	*Tmevd6*	1	19.5	*D1Mit170*	12.15	0.25	6.8
	Tmevd9	1	32.8	*D1Mit76*	14.61	−0.33	7.6

[a] Location according to Mouse Genome Informatics (MGI; http://www.informatics.jax.org/).
[b] Marker at the peak linkage, determined by permutation threshold (Figures 1 and 2).
[c] Likelihood-ratio test statistic.
[d] Additive effect of the QTL relative to the BALB/cByJ homozygote. A positive value indicates that the mean trait value for the BALB/cByJ homozygotes is greater than the mean trait value for the heterozygous animals.
[e] Percentage variance accounted for by a QTL at the specified location.

with *Cd28* and *Cd152* (*Ctla4*), important cell surface molecules in the control of T-cell activation. In contrast to *Tmevd6*, the DBA2/J allele at this locus decreased disease severity in females.

Additionally, suggestive linkage in females was seen on chromosome 1 at 19.5 cM (*D1Mit170*), accounting for 6.8% of the experimental variation. This QTL is at the same location as *Tmevd6* identified in males. In contrast to males (additive effect = −0.26), the additive effect of *Tmevd6* in females was 0.25, indicating that the DBA/2J allele increased severity in females while a BALB/cByJ allele increased severity in males. The presence of a QTL in the same interval of chromosome 1 in male and female populations with opposite additive

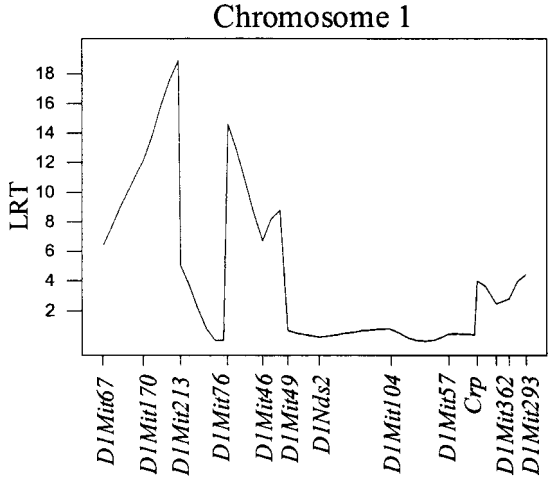

FIGURE 2.—Composite interval mapping results for the female population. Tick marks on the *x*-axis represent the positions of microsatellite markers. Permutation-derived significance cutoffs were based on 1000 permutations. Significance cutoffs for females are based on LRTs: LRTs = 14.1 (α = 0.05) and 12.8 (α = 0.10).

effects suggests that *Tmevd6* may contain two closely linked QTL with opposite effects in males and females. Alternatively, sex hormones may differentially regulate the same QTL in males and females. Interference of the sex-specific QTL at *Tmevd6* on chromosome 1 most likely prevented their identification by classical interval mapping since they had effects in opposite directions (ZENG 1993, 1994). Further studies will be necessary to elucidate the position and effects of the *Tmevd6* locus in males and females.

In this study, we have shown that sex-specific QTL play a role in susceptibility to TMEVD with QTL on chromosomes 1, 5, 15, and 16 controlling disease severity in males, while two QTL on chromosome 1 influence severity in females. These sex-specific QTL were identified only when the experimental population was stratified by sex and analyzed using CIM. Similar sex-specific QTL have been identified in the genetic control of both clinical and histopathologic EAE, the other major animal model for MS (BUTTERFIELD *et al.* 1999, 2000; BLANKENHORN *et al.* 2000). Additionally, this study demonstrates, in a practical sense, the utility of CIM in detecting multiple linked, sex-specific QTL and that resistant strains of mice may harbor TMEVD susceptibility loci that become relevant only as they interact with susceptibility loci from different strains. This may explain why significantly greater numbers of BC1 males were affected with TMEVD while parental DBA/2 and BALB/cByJ mice did not show significant differences in sex-biased susceptibility. A summary of both the sex-specific and non-sex-specific TMEVD-modifying loci identified to date is found in Table 3.

The mechanisms underlying sex-specific QTL are unknown but may arise as a result of sex hormone regulation of the polymorphic genes underlying these QTL or interactions between mitochondrially or Y-chromo-

TABLE 3

Summary of TMEVD-modifying loci identified in mice to date

Locus[a]	Chr.	Location (cM)[b]	Reference
Tmevd1	6	22	Melvold *et al.* (1987)
Tmevd2	3	46	Melvold *et al.* (1990)
Tmevd3	14	12.5	Bureau *et al.* (1998)
Tmevd4	14	39.5	Bureau *et al.* (1998)
Tmevd5	11	60	Aubagnac *et al.* (1999)
Tmevd6	1	19.5	This report
Tmevd7	5	72	This report
Tmevd8	15	4.7	This report
Tmevd9	1	32.8	This report
Tmevp1	17	19.1 (*H2D*)	Clatch *et al.* (1985)
Tmevp2	10	51.5–62	Bihl *et al.* (1999)
Tmevp3	10	62–70	Bihl *et al.* (1999)

[a] *Tmevd* loci are defined as susceptibility loci for TMEV-induced demyelination. *Tmevp* loci are defined as loci controlling viral persistence.

[b] Location according to MGI (http://www.informatics.jax.org/).

some-linked genes. The role of sex hormones in the sexual dimorphism observed in immune responsiveness as well as in immunopathologically based diseases has been well documented (Grossman *et al.* 1991; Da Silva 1999) as has the regulation of immunologically relevant genes such as cytokines (Cutolo 2002). With respect to potential interactions with mitochondrially linked genes, evidence suggests that mutations in mitochondrially encoded genes contribute to an MS-like syndrome, Leber's hereditary optic neuropathology, which occurs primarily in women (Wissinger *et al.* 1997). Such mutations, however, do not appear to be a risk factor for MS *per se* (Reynier *et al.* 1999). Additionally, a number of immunologically relevant genes are known to be on the X chromosome (http://www.informatics.jax.org/searches/linkmap.cgi). The precedence for Y-chromosome-linked genes that influence immune responses is best exemplified by *Yaa*, a Y chromosome-encoded gene that interacts with autosomal susceptibility loci to accelerate the development of spontaneous lupus and lymphoproliferation in male mice (Murphy and Roths 1979). A similar male-specific form of hereditary lupus is seen in humans (Lahita *et al.* 1983). Although the mice used in this study do not possess the classically defined accelerator allele at the *Yaa* locus, *Yaa* nevertheless establishes a precedent for the existence of Y-chromosome-linked genes affecting autoimmune disease. Thus, the existence of sex-specific QTL may be responsible for confounding the interpretation of human MS genetic data or masking the presence of susceptibility loci. In light of our findings, MS genetic studies, as well as studies using animal models, should account for the potential sex-specific genetic differences in disease.

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