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## Report

# *HLA-DR2* Dose Effect on Susceptibility to Multiple Sclerosis and Influence on Disease Course

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**Models of disease susceptibility in multiple sclerosis (MS) often assume a dominant action for the *HLA-DRB1**1501* allele and its associated haplotype (*DRB1**1501-DQB1**0602* or *DR2*). A robust and phenotypically well-characterized MS data set was used to explore this model in more detail. A dose effect of *HLA-DR2* haplotypes on MS susceptibility was revealed. This observation suggests that, in addition to the role of *HLA-DR2* in MS, two copies of a susceptibility haplotype further increase disease risk. Second, we report that *DR2* haplotypes modify disease expression. There is a paucity of benign MS and an increase of severe MS in individuals homozygous for *DR2*. Concepts of the molecular mechanisms that underlie linkage and association of the human leukocyte antigen (HLA) region to MS need to be revised to accommodate these data.**

The association of multiple sclerosis (MS [MIM 126200]) with the human leukocyte antigen (HLA) region has been known for more than a quarter of a century (Bertrams and Kuwert 1972; Naito et al. 1972). A specific association with the *DRB1**1501 molecule and its associated haplotype (*DRB1**1501-DQB1**0602* or *DR2* [MIM 142857 and MIM 604305, respectively]) is present in most populations with MS (Haines et al. 1998; Rubio et al. 2002); the only exceptions are an apparent association with *DR3* and *DR4* in patients with MS from Sardinia (Marrosu et al. 2002) and, perhaps, no *DR2* association in some Asian populations who have a restricted form of MS, termed "neuromyelitis optica," selectively affecting optic nerve and spinal cord myelin (Kira et al. 1996). Whole-genome mapping studies provide additional support for the presence of an MS susceptibility gene located within chromosome 6p21 (Haines et al. 1996, 1998). However, fine-mapping studies have not settled whether the effect is explained by the *DRB1* gene itself; by variation at another closely linked gene within the class 2 HLA region in very high linkage disequilibrium (LD) with *DRB1**1501*, such as *DQB1**0602*; or by some other nearby gene in LD. Identification of the true predisposing gene or genes has been made more complex by extensive LD across the HLA region and by the presence of >240 genes within this superlocus, many of which have roles in immune function and are thus plausible MS candidates.

Linkage to the HLA region plus a specific association with the *DR2* haplotype suggests that *DRB1**1501*-bearing haplotypes confer an effect on MS susceptibility that is distinct from that of other major histocompatibility (MHC) haplotypes. In support of this hypothesis, no evidence for linkage to 6p21 could be discerned in families who did not carry *DR2* (Chataway et al. 1998; Haines et al. 1998; Barcellos et al. 2002). Locus heterogeneity may not be present in all populations, however, underscoring the complex genetic nature of this disease (Ligers et al. 2001).

To better delineate the role of the HLA locus in MS and, in particular, the risk and phenotype associated with the *DR2* homozygous state, we analyzed a large and well-

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**Table 1**

**Disease Course and Severity Phenotypes in Patients with MS**

| Clinical Phenotype | *N* (%) |
|---|---|
| RR | 487 (62.8) |
| SP | 235 (30.3) |
| Other[a] | 53 (6.8) |
| Mild MS: EDSS <3 after ⩾10 years[b] | 71 (14.8) |
| Mild MS: EDSS <3 after ⩾15 years[b] | 40 (11.9) |
| Severe MS: EDSS >6 in ⩽10 years[c] | 35 (10.8) |

NOTE.—A total of 808 patients with clinically definite MS was studied. Complete clinical data were available for >95% of individuals.

[a] PP and RP clinical subtypes.

[b] Classifications of benign or mild MS. Patients in this group can walk normally or have mild gait disability only after ⩾10 or ⩾15 years from disease onset.

[c] Patients in this group require bilateral assistance to walk or are wheelchair dependent in ⩽10 years from disease onset. The percentages shown for mild and severe phenotypes reflect the proportion of individuals in patient groups restricted by disease duration; for example, for mild MS, patients with disease duration of ⩾10 or ⩾15 years only are included ($n = 480$ and $n = 335$, respectively), and, for severe MS, only patients with disease duration of ⩽10 years are included ($n = 323$).

characterized family-based cohort of 549 families prone to MS (187 multicase and 362 single affected, or "singleton") comprising 2,382 individuals, including 808 affected individuals and 1,574 unaffected family members. Diagnostic criteria, ascertainment protocols, and clinical and demographic characteristics of the multicase families are summarized elsewhere (Goodkin et al. 1991; Multiple Sclerosis Genetics Group [MSG Group] 1998; Haines et al. 1998; Barcellos et al. 2002). Singleton families were collected using identical ascertainment criteria; these families were required to have an affected proband with either two living parents or at least one unaffected sibling. The singleton family ascertainment was coordinated at the University of California San Francisco in collaboration with a network of specialized clinical sites throughout the United States.

All known ancestors were non-Hispanic whites of European descent. Complete clinical data were available for 96.0% of patients. For affected individuals, the female-to-male ratio was 3.0:1. The overall mean age at onset was 30.1 ± 8.8 years, and mean disease duration was 14.6 ± 10.2 years. Age at onset was defined as the first episode of neurological dysfunction suggestive of demyelinating disease (Doolittle et al. 1990; Barcellos et al. 2000). Disease course was recorded for patients at entry into study as either relapsing remitting (RR), secondary progressive (SP), primary progressive (PP), or relapsing progressive (RP). Disability was also assessed at entry with the Expanded Disability Status Scale (EDSS) (Kurtzke 1983). "Mild" (benign) and "severe" disease patient classifications were based on EDSS scores that were maintained over or achieved within designated time intervals were also used in the present study (see table 1 for details). The appropriate institutional review boards approved all studies, and informed consent was obtained from all participants.

HLA typing for *DRB1* and *DQB1* loci was performed using a nonradioactive PCR-based sequence-specific oligonucleotide probe reverse line-blot assay (PCR-SSOP) (Dynal). Generation of all genotypes was performed blind to pedigree structure and clinical status of the individual. PEDCHECK (O'Connell and Weeks 1998) was used to check for Mendelian consistency within all families with MS. Family-based association testing for alleles at the *HLA-DR* locus was performed for the combined family data set, using the "sum" option of the pedigree disequilibrium test (PDT v.3.11 [available at the Duke University Center for Human Genetics Web site]; Martin et al. 2000, 2001). The PDT can utilize genetic data from related nuclear families and discordant sibships within extended pedigrees. A strong association with *HLA-DR* overall ($P < 1.0 \times 10^{-10}$) and with the *DR2* haplotype specifically ($P < 1.0 \times 10^{-11}$) was observed, as reported elsewhere, in a subset of this population (Barcellos et al. 2002). No evidence for excess transmission of other *DR* alleles to affected individuals was present.

All patients and unaffected family members were stratified by *HLA-DR* genotype (*DR2/DR2*, *DR2/DRX*, and *DRX/DRX*, where X denotes other *DR* alleles) to test for effects of *HLA-DR2* dose on disease susceptibility. An effect of *HLA-DR2* copy number on disease risk was suggested by the observed difference in proportion of affected individuals within each *HLA-DR* genotypic category (fig. 1). A total of 52.8% of *DR2/DR2* individuals

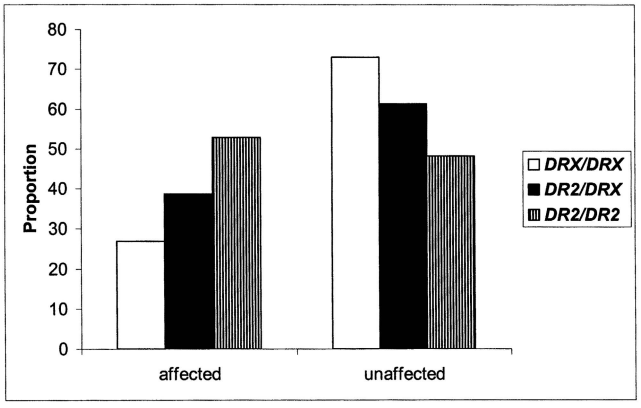


**Figure 1** Proportion of affected and unaffected individuals within 549 families with MS grouped by *HLA-DR2* genotypes. The data set contained 2,382 individuals comprising 808 patients with MS and 1,574 unaffected family members. A total of 26.9% ($n = 319$) *DRX/DRX* and 38.7% ($n = 395$) *DR2/DRX* individuals were affected, in contrast to 52.8% ($n = 94$) *DR2/DR2* individuals, demonstrating a dose effect of *HLA-DR2* on susceptibility.

**Table 2**

**OR for *HLA-DR* Genotypes in Patients with MS Compared with Unaffected Family Members**

| | | OR (95% CI) | | |
|---|---|---|---|---|
| TEST GROUP | REFERENCE[a] | All Families | Multicase Only | Singleton Only |
| *DR2/DR2* | *DRX/DRX* | 6.7 (4.2–10.7)[b] | 6.1 (3.4–11.0)[b] | 7.9 (3.7–17.0)[b] |
| *DR2/DRX* | *DRX/DRX* | 2.7 (2.1–3.6)[b] | 2.6 (1.8–3.7)[b] | 3.0 (2.0–4.5)[b] |
| *DR2/DR2* | *DR2/DRX* | 2.5 (1.7–3.7)[c] | 2.4 (1.4–3.9)[d] | 2.7 (1.4–5.1)[e] |

NOTE.—The analyses include all affected and unaffected individuals from families with multicase ($n$ = 187) and singleton ($n$ = 362) MS (total $n$ = 2,382 individuals; $n$ = 808 affected and 1,574 unaffected individuals).

[a] The *DRX* group refers to all other non-*DR2* (*DRB1**1501–*DQB1**0602) alleles. All analyses were performed using conditional logistic regression modeling and controlling for sex as implemented in PROC PHREG (SAS v. 8.2). All individuals were stratified by family for analyses. Unaffected family members included all parents and siblings of affected individuals.

[b] $P < 10^{-6}$.

[c] $P < 10^{-5}$.

[d] $P < 10^{-3}$.

[e] $P < .01$.

was affected, in contrast to 37.8% of *DR2/DRX* and 26.9% of *DRX/DRX* individuals. Conditional logistic regression modeling was then used to evaluate the effect of *DR* genotype on disease risk in the family data set. For these analyses, carriers of two (*DR2/DR2*) and one (*DR2/DRX*) *DR2* haplotypes were compared with *DRX/DRX* individuals. Significant effects on disease risk were observed for both *DR2/DR2* and *DR2/DRX* genotypes in all families (odds ratio [OR] = 6.7, $P < 1.0 \times 10^{-6}$ and OR = 2.7, $P < 1.0 \times 10^{-6}$), as well as multicase and singleton families considered separately (table 2). Significant results were also obtained when *DR2/DR2* individuals were compared with individuals with *DR2/DRX* genotypes (table 2), indicating that two copies of *DR2* confer a greater risk compared with one copy (OR = 2.5, $P < 1.0 \times 10^{-5}$, OR = 2.4, $P < 1.0 \times 10^{-3}$, and OR = 2.7, $P < .01$ for all families, multicase, and singleton families, respectively). When analyses were restricted to sibships only, or just one single discordant sib pair selected from each family, the results were very similar (data not shown).

A second approach, a modification of the PDT, was also used to examine *HLA-DR* genotypic associations in the combined family data set. This test, the geno-PDT, is also applicable to nuclear family or extended pedigree data and can be used to test any specific genotype, or a global statistic can be computed to test all genotypes simultaneously. The PDT and geno-PDT both test the same null hypotheses but can have different powers, depending on the genetic model. Significant evidence for excess transmission of both *DR2/DR2* and *DR2/DRX* genotypes was observed in the families with MS ($P < .01$ and $P < 1.0 \times 10^{-5}$, respectively; data not shown), providing further support for both genotypes as disease risk factors.

**Table 3**

**Relative Penetrance Values for *HLA-DR* Genotypes**

| Index Cases from | *DR2/DR2* | *DR2/DRX* |
|---|---|---|
| All families[a] | 11.8 | 3.8 |
| Singleton families only[b] | 9.1 | 2.9 |
| Multicase families only[c] | 20.0 | 6.7 |

NOTE.—Control genotype frequencies for P/C ratios were derived using nontransmitted alleles from singleton families assuming Hardy Weinberg proportions (Thomson 1995) (*DR2* or *DRB1**1501-*DQB1**0602: $f$ = 0.122). Nontransmitted allele frequencies derived using additional trios from multicase families (one per family) were similar (data not shown). All relative penetrance values were normalized to a value of 1 for the reference *DRX/DRX* genotype.

[a] $n$ = 549.

[b] $n$ = 362.

[c] $n$ = 187.

Maximum-likelihood estimates of the relative penetrance values for MS-associated *DR2/DR2* and *DR2/DRX* genotypes were determined using the ratio of the observed genotype frequency in patients over the frequency in controls (or P/C ratio), as described elsewhere (McWeeney and Thomson 2000). Control genotype frequency estimates were derived under the expectation of Hardy-Weinberg equilibrium, using nontransmitted (or non-MS) *HLA-DR* allele frequencies in the singleton families with MS (Thomson 1995). The penetrance values shown in table 3 have been normalized using a value of 1 for the reference *DRX/DRX* genotype. The results suggest that the relative penetrance for the *DR2* homozygous genotype is at least twice as large as that for the *DR2/DRX* genotype in all families and in both multicase and singleton families considered separately.

The effect of *HLA-DR2* genotype on clinical phenotypes, including age at onset and disease course and severity, was tested in the patient data set, using linear and logistic models estimated by generalized estimating equations (Liang and Zeger 1986; Zeger and Liang 1992), which take into account any correlation between family members. The proportions shown in table 1 are derived from the total number of individuals with a particular clinical phenotype present within the data set. Although

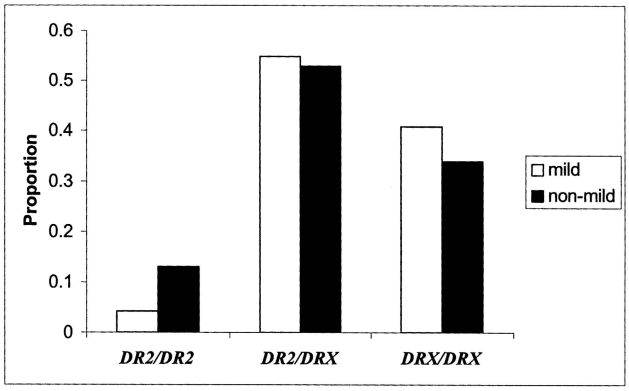


**Figure 2** A comparison of *HLA-DR* genotypes for patient subgroups of mild and nonmild MS. Analyses were restricted to patients with disease duration of at least 10 years ($n = 480$). *P* values are from PROC GENMOD (SAS version 8.2), using logistic regression with correction for familial correlations and adjustment for age at onset and sex. OR of mild MS for *DR2* homozygotes (*DR2/DR2*) = 0.3, 95% CI = 0.1–0.9, $P < .05$. Reference group = *DRX/DRX* genotype. When *DR2/DR2* individuals were compared with individuals with *DR2/DRX* genotype, OR = 0.3, 95% CI = 0.1–1.1, $P < .10$. There was no evidence for increased risk of mild MS phenotype in individuals carrying just one copy of *HLA-DR2* haplotype, OR = 0.8, 95% CI = 0.5–1.4, $P > .10$. Because no *DR2/DR2* individuals were present in analyses of mild MS ⩾15 years from onset, the statistical methods used above were not appropriate. Therefore, to determine statistical significance, unrelated mild MS cases were compared to other randomly selected unrelated cases using Fisher's exact test, as implemented in PROC FREQ (version 8.2, SAS; $P < .01$; data not shown). Logistic regression modeling with correction for familial correlations and adjustments for age at onset and sex was also used for comparisons of *HLA-DR* genotypes in patient subgroups of severe and nonsevere MS (data not shown). See text for results.

phenotypic categorization results in smaller sample sizes and a potential loss in statistical power, the increased clinical homogeneity in these subgroups may also increase the likelihood of detecting specific disease-modulating genetic effects. In the patient group with mild, or benign, MS, *DR2* homozygotes were significantly less frequent compared with patients classified as having nonmild MS (5.4% vs. 13.0%), using the *DRX/DRX* genotype as a reference group (OR = 0.3, 95% CI = 0.1–0.9, $P < .05$; see fig. 2). Here, "mild MS" was defined as maintaining an EDSS score of <3 for at least 10 years. When a more stringent definition of "mild MS" was applied, in which disease duration of at least 15 years was imposed, no *DR2* homozygotes were present in this subgroup. This result was also significant (Fisher's exact test, $P < .01$; see fig. 2 legend for details). Both observations provide strong support for *HLA-DR2* as a disease modifier. Furthermore, *DR2* homozygotes were observed more frequently in patients with a severe disease course (those defined as reaching an EDSS score >6 in ⩽10 years) in contrast to patients classified as "nonsevere" (17.1% vs. 10.1%, OR = 1.8, 95% CI = 0.7–5.0; data not shown), though this observation did not reach statistical significance ($P > .10$).

When *HLA-DR* genotypic proportions for individuals with either mild or severe disease classifications were compared with nonmild or nonsevere groups, respectively, the observed effect on disease expression was limited to those individuals carrying two copies of the *HLA-DR2* haplotype. Using *DR2/DRX* as a reference group, trends for association were observed for both mild and severe phenotypes (OR = 0.3, 95% CI = 0.1–1.1, $P < .10$ and OR = 2.3, 95% CI = 0.8–6.4, $P > .10$), respectively. There was no evidence for effects of *DR2/DRX*, as compared with *DRX/DRX*, for either clinical phenotype ($P > .40$ for both mild and severe subgroups of MS, respectively; data not shown), suggesting that one copy of the haplotype may be insufficient for expression of these disease variants. The difference in *DR2/DR2* genotype frequencies between the two most extreme clinical phenotypes, mild (disease duration of at least 15 years) and severe, was striking (Fisher's exact test, $P < .01$; see fig. 3 for details), although the results must be interpreted cautiously because of small sample sizes. Significant effects were not present for *DR2* dose on disease course (RR, SP, and PP), and no *DR2*-dependent difference in mean age at onset was detected.

The use of a well-characterized data set of white MS-prone families permitted, for the first time in a large and prospectively ascertained population, an analysis of disease risk in individuals homozygous for the MS-associated *HLA-DR2* haplotype. A dose effect on MS sus-

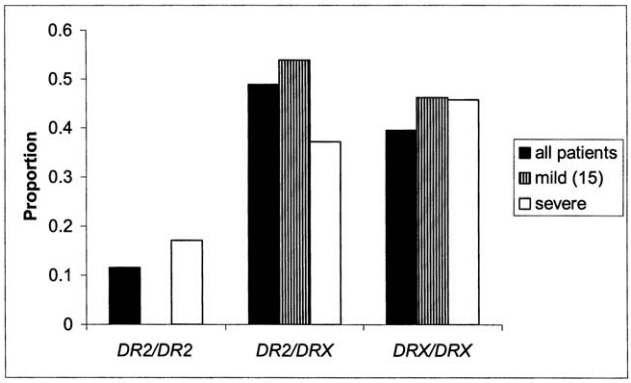


**Figure 3** *HLA-DR* genotype frequencies for all patients (index cases only, $n = 549$) and most extreme phenotypes, patients with mild (<3 for ⩾15 years) and severe MS. Because no *DR2/DR2* individuals were present in the patient group with mild MS, unrelated mild MS cases ($n = 39$) were compared with unrelated severe cases ($n = 33$), using Fisher's exact test, as implemented in PROC FREQ (version 8.2, SAS). The overall difference between genotype distributions for patients with mild and severe MS was significant ($P < .01$); 18.2% of patients with severe MS were homozygous for *HLA-DR2*, compared with 0% in patients with mild MS.

ceptibility was revealed. This finding is unexpected, although it is also supported by a recent meta-analysis of three small published data sets comprising a total of 35 *DR2* homozygous individuals (Rasmussen et al. 2001). Although relative penetrance values for *HLA-DR* genotypes derived in the present study also support a *DR2* dose effect for disease risk, the available data do not distinguish between additive and multiplicative models. In experimental models of autoimmune demyelination, a single copy of a disease-associated MHC haplotype, when present in the context of an appropriate genetic background, is generally sufficient for the induction of susceptibility. Dominantly acting MHC genes are thought to function via high-affinity binding to certain self-peptides, which are then efficiently presented to pathogenic T cells (Todd et al. 1987).

In the case of *DRB1**1501, binding and structural data support a model of the peptide-binding region that is composed in part of a large hydrophobic pocket displaying high affinity for aromatic amino acids, including phenylalanine at aa 92 of an immunodominant peptide (aa 89–96) of the autoantigen myelin basic protein (MBP) (Valli et al. 1993; Smith et al. 1998). A direct role for autoimmunity against the aa 89–96 region of MBP in the pathogenesis of MS is suggested by the findings of immunodominance of the T-cell response to this peptide in *DRB1**1501-positive individuals (Martin et al. 1990; Ota et al. 1990), specific activation of these T cells in the circulation of patients with MS (Allegretta et al. 1990; Scholz et al. 1998), and presence in MS lesions of T-cell receptors bearing antigen recognition CDR3 motifs likely to recognize this peptide (Oksenberg et al. 1993). The "tight binder" concept and immunodominant peptide model has been applied to a variety of other autoimmune diseases in which MHC alleles function as dominantly acting susceptibility genes (Svejgaard et al. 1983), including pemphigus vulgaris (Wucherpfennig et al. 1995).

One model to explain a dose effect of MHC genes on MS predicts that more than one gene within the MHC class 2 region, and perhaps beyond this region, contributes to disease risk. This model has been most fully developed for rheumatoid arthritis (RA) (Zanelli et al. 2000). Although disease-associated alleles differ between RA and MS, a dose effect of MHC genes on risk is common to the two conditions. In RA, *DRB* genes that encode a sequence containing a shared epitope—consisting of the motif Q(or R)K(or R)RRA in the third hypervariable region of the DRB protein contributing to the antigen binding properties of the molecule—influence susceptibility in a dominant manner (Gregersen et al. 1987). The adjacent DQ locus also influences risk but via a different mechanism, which may involve binding of the shared epitope itself to DQ, which results in the activation of protective regulatory T cells (van der Horst-Bruinsma et al. 1999).

In MS, the observed dose effects might be similarly explained by a dominantly acting susceptibility gene present on *DRB1**1501 haplotypes plus the absence of a protective gene required for the maintenance of peripheral tolerance present on non-*DRB1**1501 haplotypes. Loss of protection could conceivably result from a perturbation in the balance of Th1 and Th2 cytokines influenced by other class 2 MHC loci, such as *DQ,* or by other genes in the HLA region, such as tumor necrosis factor (TNF). Because of the tight LD between *DRB1**1501 and *DQB1**0602 in whites, it has not been possible to discern whether *DQB1**0602 has any independent role in MS (Rubio et al. 2002). With respect to TNF, *DRB1**1501 haplotypes are associated with a promoter polymorphism in TNF that modulates levels of expression of this proinflammatory Th1 cytokine (Garcia-Merino et al. 1996). T-cell clones triggered by antigen presented in the context of *DRB1**1501 have also been reported to display a Th1-biased pattern of cytokine secretion (Zipp et al. 1995). Thus, homozygosity for *DRB1**1501 could influence the outcome of interactions with MS autoantigenic peptides, by promoting an environment in which peripheral tolerance is not maintained. As an alternative explanation, current data cannot exclude the possibility that a dose effect of *DRB1**1501 might result simply from higher levels of surface expression of *DRB1**1501 on antigen-presenting cells in the homozygous state, increasing the likelihood that myelin autoantigens will be presented to encephalitogenic T cells.

The genotype-phenotype correlation revealed that patients with MS who were homozygous for *HLA-DR2* were unlikely to have a benign course and to be at greater risk for a more severe disease outcome. In contrast to the observed *HLA-DR2* effects on disease susceptibility, a single copy of the *HLA-DR2* haplotype did not appear to influence mild or severe disease expression. The analyses of *HLA-DR2* effects on disease behavior have been contradictory. Almost all studies have been based on phenotypic (presence or absence of *DR2*) rather than genotypic data and have shown associations with both favorable and unfavorable outcomes or no association at all (for review, see Kantarci et al. [2002]). A recent population-based case-control study showed no differences in *DR2/DR2* genotype frequencies between PP MS and "bout onset" MS (Weinshenker et al. 1998). Although the numbers of individuals within the mild and severe phenotypic categories presented here are small, our results underscore the importance of including genotypic information in analyses of clinical data.

Our observation of a *DR2* effect on disease outcome is also consistent with a model of protection mediated by *DRB1**1501-negative haplotypes and the concept that a Th1 bias drives an aggressive disease course in experimental autoimmune demyelination (Powell et al.

1990) and in human MS (Beck et al. 1988; Tejada-Simon et al. 2001). Analogous to MS, in RA, a similar dose effect of MHC genes on indices of clinical severity, including erosive manifestations and rheumatoid factor production, is clearly present (Weyand et al. 1992; El-Gabalawy et al. 1999). The proposed model has several testable implications, including an expectation that myelin autoantigen-reactive T-cell clones derived from *DRB1**1501 homozygous individuals should display a strong Th1 bias and that generation of regulatory T cells from such individuals might be impaired.

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## Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Duke University Center for Human Genetics, http://wwwchg.mc.duke.edu/software/pdt.html (for Pedigree Disequilibrium Test computer program; a beta version of the genoPDT program for genotype analysis is also available upon request: emartin@chg.mc.duke.edu)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for MS [MIM 126200], *DRB1* [MIM 142857], and *DQB1* [MIM 604305])

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