Multiple sclerosis (MS) is a common neurological disease caused by genetic and environmental factors. Previous genetic analyses have suggested that the MHC/HLA region on chromosome 6p21 contains an MS-predisposing component. Which of the many genes present in this region is primarily responsible for disease susceptibility is still an open issue. In this study, we evaluated, in a large cohort of MS families from the Mediterranean island of Sardinia, the role of allelic variation at the HLA-DRB1, DQA1 and DQB1 candidate loci in MS predisposition. Using the transmission disequilibrium test (TDT), we found significant evidence of association with MS in both the Sardinian-specific DRB1*0405/DR4–DQA1*0501–DQB1*0301 haplotype and the DRB1*0301/DR3–DQA1*0501–DQB1*0201 haplotype. Detailed comparative analysis of the DRB1–DQA1–DQB1 haplotypes present in this data set did not identify an individual locus that could explain MS susceptibility. The predisposing effect is haplotype specific, in that it is confined to specific combinations of alleles at the DRB1, DQA1 and DQB1 loci. Cross-ethnic comparison between the two HLA haplotypes associated with MS in Sardinians and the DRB1*1501 (DR2)–DQA1*0102–DQB1*0602 haplotype, associated with MS in other Caucasians, failed to identify any shared epitopes in the DR and DQ molecules that segregated with disease susceptibility. These results suggest that another MHC gene(s), in linkage disequilibrium with specific HLA-DRB1, DQA1, DQB1 haplotypes, might be primarily responsible for genetic susceptibility to MS. Alternatively, the presence of complex interactions between different HLA haplotypes, other non-HLA predisposing genes and environmental factors may explain different associations in different populations.

INTRODUCTION

Multiple sclerosis (MS), the most common cause of neurological disability in young people of Caucasian descent, is strongly clustered in families, but the aetiopathological factors responsible for its occurrence are still largely unknown. Recent studies on adopted children have indicated the preponderance of genetic versus environmental factors in determining the familial clustering of the disease (1). Three whole genome scans were not able to locate major predisposing genes (2–4). However, these linkage studies did show a modest, but definite effect of the MHC region. This is in agreement with previous reports on Caucasians (5,6), generally based on case–control studies, which demonstrated an association of MS with the HLA class II DRB1*1501 (DR2)–DQA1*0102–DQB1*0602 haplotype.

The Mediterranean island of Sardinia is a genetic isolate (7), having a very high disease incidence and prevalence (8) despite the fact that the predisposing DRB1*1501–DQA1*0102–DQB1*0602 haplotype is extremely rare in this population (haplotype frequency in 700 newborns = 0.016; F. Cucca, unpublished data). We recently have found in Sardinians that MS is associated with the HLA-DRB1*03 (DR3) and -DRB1*04 (DR4) alleles (9). The fact that MS is associated with different HLA-DR alleles in Sardinians and in other Caucasians gives rise to the question of whether HLA class II alleles themselves are primarily responsible for disease predisposition or whether they are in linkage disequilibrium with the true aetiological determinants.

To explore this issue further, we have performed a transmission disequilibrium test (TDT) for the whole DRB1–DQA1–DQB1 haplotype in a large collection of Sardinian MS families with at least one affected offspring, exploring: (i) the existence of predisposing HLA-DRB1–DQA1–DQB1 haplotypes in Sardinian patients; (ii) the position of the primarily associated allele(s) and (iii) the presence of DRB1, DQA1 and DQB1 shared sequences between Caucasian and Sardinian predisposing haplotypes.

RESULTS AND DISCUSSION

In total, we found 55 different DRB1–DQA1–DQB1 haplotypes in MS patients and 46 in healthy siblings. For TDT analysis, we considered the 10 most frequent DRB1–DQA1–DQB1 haplotypes, each one being detected in at least 25 heterozygous parents.

The results are shown in Table 1. Evidence of association with MS was found with both the Sardinian-specific DRB1*0405/DR4–DQA1*0501–DQB1*0301 and the DRB1*0301/DR3–DQA1*0501–DQB1*0201 haplotypes, which were transmitted preferentially to affected patients (67.9 and 58%; P = 0.005 and $\chi^2$ test for heterogeneity = 7.4 and 9.7, respectively). In contrast, the DRB1*1101–DQA1*0501–DQB1*0301 haplotype was decreased...
in transmission to affected patients (32.8%; \( P = 0.008 \)), but increased in transmission to healthy siblings (54.2%; \( \chi^2 \) test for heterogeneity = 4.9). These results provide a clearcut demonstration of the utility of including unaffected siblings in association studies on complex diseases.

Positive associations of genetic markers with disease can arise for two different reasons: (i) the allelic marker is the variant directly responsible for the disease or (ii) the association is due to linkage disequilibrium with the primary associated mutation.

Detailed comparative analysis of associated and non-associated DRB1–DQA1–DQB1 haplotypes in this data set may help to locate the primarily associated gene (Fig. 1). An individual role for the DQA1 locus in MS predisposition could be excluded by the fact that the DQA1*0501 is present not only in the DRB1*0405–DQA1*0501–DQB1*0201 predisposing haplotypes, but also in the non-predisposing DRB1*1101–DQA1*0501–DQB1*0301 haplotype. Furthermore, the DRB1*1501(DR2)–DQA1*0102–DQB1*0602 haplotype associated with MS in Caucasians and the DRB1*1601(DR2)–DQA1*0102–DQB1*0502 haplotype which is not associated with MS in Sardinians share the same DQA1 allele.

Further analysis excludes an individual role also for the DRB1 locus and for the DQA1–DQB1 haplotype (Fig. 1). In fact, on the one hand, the DRB1*0405 allele, present in the predisposing DRB1*0405–DQA1*0501–DQB1*0301 haplotype, is also shared with the DRB1*0405–DQA1*0301–DQB1*0302 haplotype, which is not associated with MS. On the other hand, focusing on the DQA1*0501–DQB1*0301 portion of the DRB1*0405 haplotype, this is shared also by the non-predisposing DRB1*1101–DQA1*0501–DQB1*0301 haplotype.

Two mutually exclusive hypotheses can be suggested by these data: (i) in analogy with type I diabetes mellitus, the predisposing region of HLA is constituted by the whole DRB1–DQA1–DQB1 haplotype and requires a combination of alleles at these three loci (14) or (ii) none of the DRB1, DQA1 or DQB1 genes is directly responsible for predisposition to MS, and the primarily associated allele is in linkage disequilibrium with the different DRB1, DQA1 and DQB1 haplotypes. Several lines of evidence are in favour of the second hypothesis.

First, although a minor increase has been noted in Swedish MS patients (6), the association of the HLA-DR3 haplotype at this extent seems to be peculiar for Sardinian MS. Interestingly, whereas in Caucasians of Northern European descent the DR3 allele is more often included in the common HLA-A1, -B8, -DRB1*0301, -DQB1*0201 extended haplotype, in Sardinians it is more often associated with HLA-A30, -B8, resulting in the HLA-A30, -B18, -DRB1*0301, -DQB1*0201 ancestral haplotype (15). The two HLA-DR3 haplotypes share the same HLA-DRB1, DQA1 and DQB1 genes, but differ distally in the class I and III regions starting from the HLA-DRB3 locus (16). Therefore, the fact that DR3 is associated with the Sardinian, but not with the large majority of the Northern European DR3 haplotype could indicate that the predisposing gene is located in the non-shared part of these extended haplotypes.

Second, we failed to find any shared DR/DQ epitopes between the DR3 and DR4 haplotypes associated with MS in Sardinians and the DRB1*1501–DQA1*0102–DQB1*0602 haplotype associated with MS in other Caucasian populations (unpublished data).

### Table 1. Transmission disequilibrium test of the DRB1–DQA1–DQB1 haplotype in affected patients and unaffected siblings from 264 MS Sardinian families

| DRB1 | DQA1 | DQB1 | Affected | | Unaffected | | \( \chi^2 \) for heterogeneity |
|------|------|------|----------|-------|-------------|----------------------|
| 0301 | 0501 | 0201 | 120      | 87    | 58.0        | 0.013                | 80       | 109    | 42.3    | 9.7       |
| 1101 | 0501 | 0301 | 19       | 39    | 32.8        | 0.008                | 26       | 22     | 54.2    | 4.9       |
| 0405 | 0501 | 0301 | 38       | 18    | 67.9        | 0.005                | 16       | 24     | 40.0    | 7.4       |
| 0405 | 0301 | 0302 | 23       | 25    | 47.9        |                      | 26       | 19     | 57.8    |           |
| 1601 | 0102 | 0502 | 30       | 71    | 41.3        |                      | 45       | 38     | 54.2    |           |
| 1104 | 0501 | 0301 | 41       | 29    | 58.6        |                      | 37       | 30     | 55.2    |           |
| 0101 | 0101 | 0501 | 20       | 27    | 42.6        |                      | 19       | 14     | 57.6    |           |
| 0701 | 0201 | 0201 | 16       | 18    | 47.1        |                      | 14       | 7      | 66.7    |           |
| 0102 | 0101 | 0501 | 10       | 16    | 38.5        |                      | 13       | 9      | 59.1    |           |
| 0403 | 0301 | 0302 | 14       | 11    | 56.0        |                      | 12       | 14     | 46.2    |           |
Taken together, our results give rise to doubts about a direct role of the HLA class II loci in the genetic predisposition to MS, and suggest that the HLA-DRB1–DQA1–DQB1 haplotypes associated with MS in different populations could be markers in linkage disequilibrium with another MHC gene(s) primarily associated with MS. Further fine mapping work within the MHC region is necessary to identify the region(s) of the MHC most commonly shared by identical-by-descent patients with MS. Nevertheless, considering also the well-known clinical heterogeneity of MS, recently supported by neuropathological (17) and “in vivo” MR spectroscopy (18) data, we cannot exclude that genetic predisposition to the disease differs in several ethnic backgrounds, as the result of a complex relationship between the various HLA class II genes, other MS predisposing genes and environmental triggers.

MATERIALS AND METHODS

Patients

The study has been carried out on 264 MS families, all Sardinians and of Sardinian descent, born and living on the island. Two hundred and forty one families were comprised of one patient, both parents and of Sardinian descent, born and living on the island. Two hundred and forty one families were comprised of one patient, both parents and of Sardinian descent, born and living on the island. Twenty one and forty one families were comprised of one patient, both parents and of Sardinian descent, born and living on the island. Two hundred and forty one families were comprised of one patient, both parents and of Sardinian descent, born and living on the island. Twenty one and forty one families were comprised of one patient, both parents and of Sardinian descent, born and living on the island. All patients had clinically defined MS according to the criteria of Poser et al. (10). Moreover, magnetic resonance imaging demonstrated that all patients had white matter lesions consistent with MS, according to the criteria of Paty (11). Among the MS patients, 96 were males (mean age 33.5, range 16–57 years) and 192 were females (mean age 27.9, range 15–56 years). The course of the disease was relapsing–remitting in 195, secondary progressive in 76 and primary progressive in 17 patients. Among healthy siblings, there were 103 males (mean age 38.9, range 19–67 years) and 161 females (mean age 34.6, range 20–65 years).

HLA-DRB1, DQA1, DQB1 analysis

DNA of patients and controls was extracted from peripheral blood using standard methods. Amplification of the polymorphic second exon of the DRB1, DQA1 and DQB1 genes and dot-blot analysis of amplified DNAs with sequence-specific oligonucleotide probes (SSO) were carried out using the procedures described elsewhere (12).

Transmission disequilibrium test (TDT) analysis

TDT analysis was performed according to Spielman et al. (13). The test counts the number of transmissions and non-transmissions of each allele from heterozygous parents and tests the statistical deviation from the expected 50:50 rate of transmission using a χ² test with one degree of freedom. The χ² level of significance was set at 0.05 and the P-values were generated by analysis of the TDT results in MS patients.

In order to exclude any spurious association with MS owing to segregation distortion, we also compared the TDT results in MS patients and healthy siblings using a χ² test of heterogeneity.

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