# HLA class III region and susceptibility to rheumatoid arthritis

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## Abstract

### Objective

*We examined the contribution of the HLA class III region in susceptibility to rheumatoid arthritis (RA).*

### Methods

*Patients with RA, healthy subjects and homozygous typing cell (HTC) lines were typed for HLA class I (A, B, C), class II (DR, DQ) and class III (D6S273, Bat 2, and TNFa microsatellites, and HSP70 promoter region) alleles by molecular techniques.*

### Results

*Based on the distribution of microsatellites D6S273, Bat2 and TNFa, and HSP70 promoter region alleles in HTCs and homozygous unrelated individuals, a class III region haplotype, D6S273 138 - HSP70c - Bat2 138 - TNFa2 was identified. This haplotype showed a significant primary association with susceptibility to RA in DRB 1 QKRAA/QRRAA epitope-negative patients.*

### Conclusion

*Since the QKRAA/QRRAA epitope does not provide any risk for disease susceptibility in RA-susceptibility DRB1 epitope-negative patients, the present data suggest that the class III region haplotype D6S273 138 - HSP70c - Bat2 138 - TNFa2 provides an additional risk for the development of RA. These results show that two regions in MHC, class II (DRB1) and class III (D6S273 138 - HSP70c - Bat 2 138 - TNFa2), contribute to susceptibility to RA and more completely define the risk for development of the disease.*

**Key words**

Bat2, D6S273, HSP70, TNFa, RA.

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## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease caused by tissue-destructive autoimmune response(s). Although the etiology of RA is not yet known, a genetic predisposition conferred by HLA class II (HLA-DR) is well established (1, 2). It has been demonstrated that the susceptibility to develop RA and the severity of the disease are associated with DR alleles that share a five amino acid sequence motif, QKRAA/QRRAA, from positions 70 to 74 in the third hypervariable region of the DRβ1 molocule. These associations between DRB1 genes and susceptibility to RA are, however, incomplete in that about one-fourth of patients do not carry the disease susceptibility DRB1 epitope and not all disease epitope-positive individuals develop the disease. We therefore hypothesized that additional susceptibility and/or modifying gene(s) contribute to the development of RA.

In this regard, it has been suggested that the HLA class III region contains genes that are good candidates for involvement in susceptibility to RA (3). This region contains genes and new polymorphic markers (microsatellites), such as Bat2, D6S273, TNFa, HSP70, which are in linkage disequilibrium with each other and with HLA class II and class I markers of previously reported extended haplotypes (4-13). For example, the extended haplotype HLA-B8,DR3 is associated with specific alleles of microsatellites TNFa, D6S273 and Bat2, and of the promoter region of HSP70. We have recently reported that the microsatellite D6S273 138 and Bat2 138 alleles provide an additional markers for susceptibility to RA (14, 15).

In the present study, we extend these data to gene (i.e., microsatellites TNFa and Bat2, and HSP70 promoter region) polymorphisms to obtain a mapping of RA-susceptibility genes in the HLA class III region and to identify a class III region haplotype that causes susceptibility to RA.

## Materials and methods

Ninety-nine adult Caucasian patients with RA who attended the rheumatology clinics at the Hamilton Health Sciences Corporation (McMaster University Medical Centre Division) and the St. Joseph's Hospital in Hamilton were studied. Since the number of RA susceptibility DRB 1 epitope-negative patients in this series was small, we included an additional 19 DRB1 QKRAA/QRRAA-negative patients. All of the patients had definite classical seropositive RA according to the American College of Rheumatology 1987 revised criteria (16). Secondary Sjögren's syndrome (sSS) associated with RA was diagnosed in 18 patients. Of these, 4 sSS patients were positive for antinuclear antibody (ANA).

One hundred unrelated healthy Caucasian subjects from the same geographical area served as controls. In addition, homozygous typing cell (HTC) lines and unrelated homozygous individuals were used for the identification of HLA class I, II and III extended haplotypes. All of the patients, healthy controls and HTCs were previously typed for the HLA-A, B, C, DR, and D6S273 alleles by molecular techniques (14, 17, 18, unpublished).

Genomic DNA was prepared from peripheral blood lymphocytes and from HTCs. Samples of DNA (50 ng) were amplified in a total volume of 10 µl of 10 mM Tris-HCl, 50 mM KCl and 1.5 mM $MgC1_2$, pH 8.3. The sequences of TNFa and Bat2 microsatellites, and HSP70 promoter region primers used for amplification were as follows (Table I):

TNFa-1 = 5'-GCCTCTAGATTTCATCC AGCCACA-3',

TNFa-2 = 5'CCTCTCTCCCCTGCAA CACACA-3';

Bat2-1 = 5'-CTCCAGCCTGGATAA CAG-3',

Bat2-2 = 5'-ACAAGGGCTTTAGGAG GTCT-3';

HSP70-1 = 5'-CGCCATGGAGACCAA CACCC-3',

HSP70-2 =5'-GCGGATCCGCGGTTC CCTGCTCTC-3'.

Primers TNFa-1 and Bat2-1 were 5' end labeled with [$r^{32}$p]d = ATP (4500 ci/mmol) (ICN Pharmaceuticals, Costa Mesa, CA). The amplification was performed with 35 cycles using following conditions: 95°C for 60 sec, 62°C for 60 sec, and 72°C for 60 sec, followed by extension at 72°C for 5 min. The HSP70 promoter region was amplified as described previously (8).

**Table I.** Nucleotide sequences of primers for TNFa and Bat2 microsatellite alleles, and of primers and sequence-specific oligonucleotide probes for the HSP70 promoter region alleles.

| | | |
|---|---|---|
| Primers | | |
| TNFa | 5' end primer: 5'-GCCTCTAGATTTCATCCAGCCACA-3'<br>3' end primer: 5'-CCTCTCTCCCCTGCAACACACA-3' | |
| Bat2 | 5' end primer: 5'-CTCCAGCCTGGATAACAG-3'<br>3' end primer: 5'-ACAAGGGCTTTAGGAGGTCT-3' | |
| | | Position |
| HSP70 | 5' end primer: 5'-CGCCATGGAGACCAACACCC-3' | -273 to -254 |
| | 3'end primer: 5'-GCGGATCCGCGGTTCCCTGCTCTC-3' | 200 to 215 |
| Oligonucleotide probes | | |
| HSP70 | Sequence | Position / Specificity |
| | GGCGAAACCCCTGGA | -117 to -103 / B + C |
| | GGCGAAAACCCTGGA | -117 to -103 / A |
| | TCGAGTTTCCGGCGT | 113 to 127 / A + C |
| | TCGAGTTCCCGCCGT | 113 to 127 / B |

**Table II.** Prevalence of the Bat2 138 microsatellite allele in normal healthy subjects and RA patients.

| | All | | | | RA-susceptibility epitope-positive | | | | RA-susceptibility epitope-negative | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normals n = 100 | Patients n = 99 | p | pc | Normals n = 52 | Patients n = 78 | p | pc | Normals n = 48 | Patients n = 21 | p | pc |
| Bat2 allele 138 | 21.0 | 22.2 | n.s | - | 26.9 | 14.1 | n.s. | - | 14.6 | 52.4 | 0.001 | 0.013[a] |

[a]RR = 6.44, 95% CI limit = 1.99 - 20.82

For the identification of TNFa and Bat2 microsatellite alleles, PCR-amplified DNA was electrophoresed on a 6% denaturing polyacrylamide sequencing gel. The gels were dried and autoradiographed overnight at -70°C using Quanta Blue (Eastman Kodak, Rochester, NY) intensifying screens as described earlier (14). TNFa and Bat2 microsatellite alleles were identified by the size of the amplified fragments, which was determined using a sequencing ladder. HSP70 promoter region alleles were identified by dot blot analysis of PCR-amplified DNA with sequence-specific oligonucleotide probes (Table I), as described previously (8).

Statistical analyses of the distribution of the TNFa and Bat2 microsatellites and HSP70 promoter region alleles in patients with RA and in normal healthy controls was done by the $\chi^2$ test. P values (pc) were corrected by multiplication by the number of comparisons made. Relative risk (RR) values were calculated by the method of Woolf (19). The relative (primary versus secondary) importance of the class III (D6S273, HSP-70, Bat2, TNFa), class I (A, B) and class II (DR) alleles in RA susceptibility was determined using the methods described by Svejgaard and colleagues (20, 21). Haplotype frequencies and linkage disequilibrium values (LD, delta) were calculated as described by Imanishi *et al.* (22).

## Results

The prevalence of RA susceptibility DRB 1 epitope QKRAA or QRRAA in patients with RA (78.8%) was significantly ($\chi^2 = 15.76$, $p < 0.00008$; RR = 3.43, 95% CI = 1.84-6.38) higher as compared to normal healthy control subjects (52.0%). No differences were observed in the prevalence of sSS in DRB1 epitope-positive patients as compared to DRB1 epitope-negative patients (19.2% versus 14.3%, $p > 0.75$).

In the present study, we were able to identify 13 TNFa and 13 Bat2 microsatellite alleles, and three HSP70 promoter region alleles. We then compared the prevalence of these alleles in patients with RA to those in normal healthy controls. For these comparisons, patients with RA and normal controls were considered in three groups: (i) all; (ii) RA-susceptibility DRB1 epitope-positive; and (iii) RA susceptibility DRB1 epitope-negative. The distribution of TNFa microsatellite and HSP70 alleles in patients with RA was essentially the same as in normal healthy subjects in all 3 groups (data not shown). Among the Bat2 alleles, the distribution of Bat2 138 was significantly ($\chi^2 = 10.82$, $p = 0.001$, $pc = 0.013$; RR = 6.44, 95% CI = 1.99 - 20.82) higher in RA susceptibility epitope-negative patients (52.4%) as compared to the respective group of normal controls (14.6%) (Table II). No differences were observed in the distribution of the remaining Bat2 alleles in patients with RA and normal controls.

We then analyzed the association of the TNFa microsatellite, Bat2 microsatellite and HSP70 promoter region alleles with HLA-DR specificities. TNFa/HLA-DR, Bat2/HLA-DR and HSP70/HLA-DR haplotype frequencies (HF) and linkage disequilibrium (LD, delta) values in normal healthy controls are shown in Table III. It is evident that significant LD values were observed between DR alleles and specific TNFa microsatellite, Bat2 microsatellite and HSP70 promoter region alleles. For example, TNFa2, Bat2 138 and HSP70c showed a significant association with DR3; TNFa4 with DR6; TNFa6 with DR1; TNFa7 with DR7; Bat2 150 with DR4; and HSP70a with DR2 and DR5. Similar associations of TNFa, Bat2 and HSP70 alleles with DR specificities were observed in patients with RA, e.g. DR3 showed a significant positive association with TNFa2 ($p < 0.013$), Bat2 138 ($p < 10^{-6}$) and HSP70c ($p < 0.003$).

Next, we examined the associations between the TNFa microsatellite, Bat2 microsatellite, and HSP70 promoter region alleles, and between these alleles and other (D6S273) microsatellite alleles in the HLA class III region. These analyses showed that the DR3-associated HLA class III region alleles, i.e. TNFa2, HSP70c, Bat2 138 and D6S273 138, are significantly associated among them-

**Table III.** Association of microsatellites TNFa and Bat2, and HSP70 promoter region alleles with HLA-DR specificities in normal subjects.

| | HLA-DR | HF[a] | LD[b] | $\chi^2$ | P |
|---|---|---|---|---|---|
| Microsatellite TNFa | | | | | |
| a2 | DR3 | 0.2402 | 0.0564 | 6.82 | < 0.009 |
| a4 | DR6 | 0.0419 | 0.0203 | 6.98 | < 0.008 |
| a6 | DR1 | 0.0824 | 0.0320 | 7.57 | < 0.006 |
| a7 | DR7 | 0.0593 | 0.0290 | 10.24 | < 0.001 |
| Microsatellite Bat2 | | | | | |
| 138 | DR3 | 0.1527 | 0.0852 | 47.23 | $< 10^{-6}$ |
| 150 | DR4 | 0.1731 | 0.0415 | 5.17 | < 0.024 |
| HSP70 promoter region | | | | | |
| a | DR2 | 0.2995 | 0.0606 | 5.54 | < 0.019 |
| a | DR5 | 0.2311 | 0.0545 | 5.84 | < 0.016 |
| c | DR3 | 0.3825 | 0.0920 | 12.26 | < 0.0005 |

[a]HF = haplotype frequency; [b]LD = linkage disequilibrium (delta) value.

**Table IV.** Association of microsatellites TNFa2 and Bat2 138, and HSP70c promoter region alleles with the microsatellite D6S273 138 allele in normal subjects.

| Alleles | | HF[a] | LD[b] | $\chi^2$ | P |
|---|---|---|---|---|---|
| TNFa2 | Bat2 138 | 0.1625 | 0.0585 | 14.46 | < 0.0002 |
| TNFa2 | HSP70c | 0.5629 | 0.0909 | 7.00 | < 0.008 |
| TNFa2 | D6S273 138 | 0.1907 | 0.0594 | 10.59 | < 0.001 |
| Bat2 138 | HSP70c | 0.2373 | 0.0573 | 6.52 | < 0.011 |
| Bat2 138 | D6S273 138 | 0.1466 | 0.0977 | 83.85 | $< 10^{-6}$ |
| HSP70c | D6S273 138 | 0.2647 | 0.0642 | 7.50 | < 0.006 |

[a]HF = haplotype frequency; [b]LD = linkage disequilibrium (delta) value

selves. The haplotype frequencies and LD values of TNFa2/Bat2 138, TNFa2/HSP70c, TNFa2/D6S273 138, Bat2 138/HSP70c, Bat2 138/D6S273 138, and HSP70c/D6S273 138 haplotypes in normal healthy controls are shown in Table IV. It is evident that these alleles showed significant associations among themselves, with significant LD values. Similar associations between microsatellite D6S273 138, Bat2 138 and TNFa2, and HSP70c promoter region alleles were observed in patients with RA (data not shown).

We then determined the distribution of microsatellite D6S273, Bat2 and TNFa, and HSP70 promoter region alleles in DR3-bearing extended haplotypes in HLA-A, B, DR homozygous HTCs (n = 5) and unrelated individuals (n=10). The results demonstrated that the A1, B8, DR3 haplotypes carried D6S273 138, HSP70c, Bat2 138 and TNFa2 alleles; the B18, DR3 haplotypes carried D6S-273 128, HSP70c, Bat2 140 and TNFa1/a2 alleles; and the non-B8, non-B 18, DR3 haplotypes carried a combination of different D6S273, Bat2 and TNFa alleles. The same patterns of DR3-bearing extended haplotypes was observed in normal healthy control subjects (A1, B8, DR3 n = 15; B18, DR3 n = 5; and non-B8, non-B18, DR3 n = 7) and in patients with RA (A1, B8, DR3 n = 13; B18, DR3 n = 3; and non-B8, non-B18, DR3 n = 2). However, seven DR3-negative individuals carried the D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype; normal subjects = two RA susceptibility epitope-positive, RA patients = three RA-susceptibility epitope-positive and two RA susceptibility epitope-negative. In addition, five normal subjects (three RA epitope-positive and two RA epitope-negative) and one patient (RA epitope-positive), who were Al, B8, DR3-positive, did not carry the D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype.

We examined the prevalence of haplotypes A1-B8-DR3, A1-B8-DR3-D6S273 138-HSP70c Bat2 138-TNFa2, and D6S273 138-HSP70c-Bat2 138-TNFa2 in three groups of normal healthy subjects and in patients with RA (Table V). It is evident that the prevalence of these three haplotypes was significantly lower in RA patients as compared to normal controls in the RA-susceptibility DRB 1 epitope-positive group.

On the other hand, the occurrence of these three haplotypes was significantly higher in patients than control subjects in the RA-susceptibility DRB1 epitope-negative group. These data suggest that these three haplotypes, A1-B8-DR3, A1-B8-DR3-D6S273 138-HSP70c-Bat2 138-TNFa2, and D6S273 138-HSP70c - Bat2 138-TNFa2, cause susceptibility to RA in RA-susceptibility DRB1 epitope-negative subjects. However, the highest level of significance and RR values were obtained with the D6S273 138-HSP70c - Bat2 138-TNFa2 haplotype (normals = 8.33%, patients 52.38%; $p < 0.0002$; RR= 12.10, 95% CI = 3.19-45.96).

Similar results were obtained when additional (n = 19) RA susceptibility DRB1 epitope-negative patients were included in the analyses. For example, the data show that these haplotypes, A1-B8-DR3 (normals = 12.50%, patients = 40.0%; $p < 0.003$; RR = 4.67, 95% CI = 1.61 - 13.52), A1-B8-DR3 - D6S273 138 - HSP70c-Bat2 138- TNFa2 (normals = 8.33%, patients = 40.0%; $p < 0.0004$; RR = 7.33, 95% CI = 2.21 - 24.35), and D6S-273 138 - HSP70c-Bat2 138 - TNFa2 (normals = 8.33%, patients = 45.0%; $p < 0.0001$; RR = 9.00, 95% CI = 2.72 - 29.83) were associated with susceptibility to RA in DRB1 epitope negative subjects, and the highest level of significance and RR values were obtained with class III D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype.

Since the HLA class III region haplotype D6S273 138-HSP70c-Bat2 138-TNFa2 showed a significant positive association with class I and class II region haplotype A1 -B8-DR3 in both normal controls ($p < 7 \times 10^{-12}$) and in patients with RA ($p < 2 \times 10^{-12}$) and since the A1-B8-DR3 haplotype also showed an association with RA in disease susceptibility epitope-negative subjects, we analyzed our data to elucidate the relative roles of class III region versus class I and class

**Table V.** Prevalence of haplotypes A1-B8-DR3, Al-B8-DR3-D6S273 138-HSP70c-Bat2 138-TNFa2, and D6S273 138-HSP70c-Bat2 138-TNFa2 in normal healthy subjects and RA patients.

| Haplotype | All | | | | RA-susceptibility epitope-positive | | | | RA-susceptibility epitope-negative | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normals n=100 % | Patients n=99 % | p | RR | Normals n=52 % | Patients n=78 % | p < | RR | Normals n=48 % | Patients n=21 % | p < | RR |
| A1-B8-DR3 | 20.00 | 14.14 | n.s. | - | 26.92 | 6.41 | 0.002 | 0.19 | 12.50 | 42.86 | 0.005 | 5.25 1.56-17.71* |
| A1-B8-DR3-D6S273 138 HSP70c-Bat2 138-TNFa2 | 15.00 | 13.13 | n.s. | - | 21.15 | 5.13 | 0.006 | 0.20 | 8.33 | 42.86 | 0.001 | 8.25 2.16-31.49* |
| D6S273 138-HSP70c Bat2 138-TNFa2 | 17.00 | 18.18 | n.s. | - | 25.00 | 8.97 | 0.014 | 0.30 | 8.33 | 52.38 | 0.0002 | 12.10 3.19-45.96* |

* 95% CI limit

II region haplotypes in susceptibility to RA in DRB 1 QKRAA/QRRAA epitope-negative patients and normal healthy subjects. There analyses were carried out in a total of 40 RA susceptibility DRB 1 epitope-negative patients (Table VI). Analysis of the relative roles of these haplotypes showed that the D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype is significantly ($p<4x10^{-5}$) associated with RA in the presence or absence of the A1-B8-DR3 haplotype (Table VIb). In contrast, no association (p = 0.23) between A1-B8-DR3 haplotype and RA was observed when the data were analyzed in the presence or absence of the class III region haplotype D6S273 138-HSP70c-Bat2 138-TNFa2 (Table VIa). These data suggest that the class III region haplotype shows a primary association with disease in RA-susceptibility DRB1 epitope-negative individuals.

Finally, we analyzed our data to elucidate the role of the DRB 1 QKRAA/QRRAA epitope and/or class III region haplotype D6S273 138 - HSP70c - Bat2 138-TNFa2 in susceptibility to RA. The results demonstrate that the presence of the QKRAA/QRRAA epitope and/or the D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype shows a stronger association with disease susceptibility (patients = 89.9%, controls = 56.0%; $\chi^2 = 28.91$, $p < 5.8 \times 10^{-8}$; RR = 6.99, 95% CI = 3.26-15.01) than that observed with the DRB1 QKRAA/QRRAA epitope (patients = 78.8%, controls = 52.0%; $\chi^2 = 15.76$, $p < 0.00008$; RR = 3.43, 95% CI = 1.84-6.38).

**Table VI.** Primary versus secondary associations between haplotypes HLA-A1-B8-DR3 and D6S273 138-HSP70c-Bat2 138-TNFa2 with RA susceptibility in DRB1 QKRAA/QRRAA epitope-negative patients and normal healthy subjects.

**A.** Association between RA and A1-B8-DR3 haplotype in the presence or absence of the D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype*.

| | D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype | | | |
|---|---|---|---|---|
| | Positive | | Negative | |
| A1-B8-DR3 haplotype | + | - | + | - |
| Patients | 16 | 2 | 0 | 22 |
| Normals | 4 | 0 | 2 | 42 |

* p value for the significance of association of A1-B8-DR3 haplotype = 0.23.

**B**. Association between RA and D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype in the presence or absence of the A1-B8-DR3 haplotype*.

| | A1 -B8-DR3 haplotype | | | |
|---|---|---|---|---|
| | Positive | | Negative | |
| D6S273 138-HSP70c- | + | - | + | - |
| Bat2 138-TNFa2 | | | | |
| Patients | 16 | 0 | 2 | 22 |
| Normals | 4 | 2 | 0 | 42 |

*p value for the significance of association of the D6S273 138-HSP70c-Bat2 138-TNFa2 haplotype < $4 \times 10^{-5}$.

## Discussion

The results in the present study demonstrate that the HLA class III region haplotype D6S273 - 138HSP70c - Bat2 138-TNFa2 is associated with susceptibility to RA in DRB1 QKRAA/QRRAA epitope-negative patients. Since this haplotype has a primary association with RA susceptibility and since the QKRAA/QRRAA epitope does not provide any risk for disease susceptibility in this group of individuals, the present data suggest that this class III region haplotype provides an additional risk in susceptibility to RA. In addition, the present data suggest that the DRB1 QKRAA/QRRAA epitope and class III region hap-

lotype D6S273 138-HSP70c-Bat2 138-TNFa2 define more completely the risk for the development of RA.

The human MHC region, located on chromosome 6p21.3, includes genes in the class I and class II regions that encode for class I (HLA-A, B, C) and class II (HLA-DR, DQ, DP) antigens. The class III region is located between the class I and class II regions and contains genes that encode for proteins involved in the immune response (e.g. TNF-A, B, and three members of the HSP70 family). The MHC region is characterized both by a high degree of polymorphism and by strong linkage disequilibrium between different class I, class II and class III region alleles (5-15). For example, these studies have demonstrated strong linkage disequilibrium between class I, class II and certain class III region gene and microsatellite alleles (e.g., HSP70, Bat2, D6S273 and TNFa).

In the present study, we examined the patterns of microsatellite (D6S273, Bat2, TNFa) and HSP70 promoter region alleles in HTCs, homozygous normal subjects, unrelated normal controls and patients with RA. Our results confirm and extend data published earlier, in that different class I-bearing DR3 haplotypes (A1 -B8-DR3, B 18-DR3, and non-B8-nonB 18-DR3) carry different class III region D6S273, Bat2, TNFa microsatellite alleles. Of these, the A1 -B8 DR3 haplotype carries the D6S273 138, Bat2 138, TNFa2 and HSP70c alleles. In addition, no association between the TNFa alleles and DR4 was observed in the present study, which is in agreement with earlier extensive investigations of HTCs, unrelated individuals and family members (6, 7).

Since the MHC class III region contains genes that are involved in the immune response, it has been suggested that class III region genes are good candidates for involvement in autoimmune diseases (3). In fact, a number of studies have provided suggestive evidence that the class III region genes and microsatellite markers are associated with susceptibility to insulin-dependent diabetes mellitus (IDDM) (13, 23-27), celiac disease (28, 29), multiple sclerosis (30, 31), systemic lupus erythematosus (12, 32-35), sarcoidosis (36), Grave's disease (37), and ankylosing spondylitis (38). Investigation of multiplex families demonstrated that the class III region containing the TNF-lymphotoxin region influences susceptibility to RA, distinct from HLA-DR (39, 40). In addition, Hajeer and colleagues demonstrated an association between the TNFa6 allele and susceptibility to RA (41, 42). In addition, these investigators observed a strong association between the TNFa6 allele and DR4; consequently their results showed that the association of the TNF microsatellite allele with RA was secondary to that of the class II (DR) association. In the present study, we did not observe an association between the TNFa microsatellite alleles and susceptibiity to RA. The differences between the present data and those reported earlier by Hajeer *et al.* (41, 42) could be due to the lack of an association between the TNFa6 allele and DR4 in the present study. In fact, extensive investigations of HTCs, unrelated subjects and family members by others also showed no association between the TNFa6 allele and DR4 (6, 7). The lack of an association of the TNFa6 allele with RA in the present study could therefore be due to: (a) the lack of an association of the TNFa6 allele with DR4; and (b) the nature of the association of the TNFa6 allele with RA, i.e. secondary to that of DR4.

HSP70 has been suggested to play a role in self surveillance and susceptibility to autoimmune diseases, including RA (42-45). Furthermore, it has been shown that HSP70 proteins specifically bind to the QKRAA motif in DRB1*0401 and RRR AA in DRB 1*1001, suggesting that this property may influence antigen processing pathways relevant for the development of RA (47). Polymorphisms in the HSP70 gene, including that in the HSP-70 promoter region, however, showed no association with susceptibility to RA (34, 48). The results in the present study are in agreement with those published earlier in that the HSP70 alleles were not associated with susceptibility to RA.

Christiansen *et al.* (49) reported that the prevalence of DR3 was higher in DR4-negative RA patients as compared to a respective group of normal controls, suggesting that DR3 may be associated with disease susceptibility in DRB1 RA-susceptibility epitope-negative subjects. In the present study, we also observed a higher prevalence of DR3 in RA epitope-negative patients; however, analysis of the relative importance of the A1, B8, DR3 haplotype versus class III haplotype D6S273 138-HSP70c-Bat2 138-TNFa2 showed that the class III haplotype has a primary association with RA susceptibility in DRB1 epitope QKRAA/QRRAA-negative subjects.

Moghaddam and colleagues (27) examined the role of HLA class II and microsatellite markers located in the class III region in susceptibility to IDDM. These investigators provided strong evidence that two regions, DQ (class II) and D6S-273 (class III), in MHC are involved in susceptibility to IDDM.

Similarly, results in the present study show that two regions in MHC, class II (DRB1) and class III (D6S 273 138-HSP70C-Bat2 138-TNFa2) contribute to susceptibility to RA. It is likely therefore that class III genes are additional to susceptibility genes that cause the development of RA in DRB 1 QKRAA or QRRAA epitope-negative individuals. It is not yet known, however, if alternate pathogenetic mechanisms are involved in the development of disease.

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