

Tumour necrosis factor microsatellites and HLA-DRB1*, HLA-DQA1*, and HLA-DQB1* alleles in Peruvian patients with rheumatoid arthritis

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Abstract

Objective—To study the association between rheumatoid arthritis (RA) and HLA and tumour necrosis factor (TNF) polymorphism in Peruvian mestizo patients in comparison with ethnically similar controls.

Methods—Seventy nine patients with RA and 65 ethnically matched healthy controls were genotyped for HLA-DRB1, HLA-DQA1, HLA-DQB1, and TNF α and TNF β alleles using PCR amplification. Clinical severity was assessed as mild, moderate, or severe in 35 of the patients.

Results—TNF α 6 showed the strongest association with disease susceptibility. The TNF α 6 allele was more common in patients than in controls ($p < 0.0076$) and the proportion of patients with at least one copy of this allele was greater ($p < 0.015$, relative risk 2.35). Among the HLA-DRB1* alleles with the shared epitope sequence, only the DRB1*1402 allele was significantly increased in patients compared with controls ($p < 0.0311$), as was the proportion of patients with at least one copy of this allele ($p < 0.0232$, relative risk 2.74). In contrast, the overall frequency of alleles with the shared epitope was not different in patients and controls. The haplotype HLA-DRB1*1402-DQB1*0301-DQA1*0401 was significantly more common in patients. TNF α 6 was more common in patients whether or not they had this haplotype. None of the 11 patients lacking the TNF α 6 allele had severe disease.

Conclusions—This study shows for the first time that TNF gene polymorphism is associated with susceptibility to RA in a non-white population. TNF α 6 and HLA-DRB1*1402 independently conferred significantly increased risk in Peruvian mestizo patients.

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Rheumatoid arthritis (RA) is a chronic inflammatory disease with worldwide distribution. Its manifestations and progression rate show pronounced individual variation both within and between cohorts of different geographical origin.^{1,2} Concordance in monozygotic twins is 10–30%, indicating that both genetic and environmental factors are important pathogenetically.³ Several studies in different populations

have shown that only a few of the 15 or more DRB1* alleles are associated with susceptibility to RA and these contain the amino acid sequence QRRAA, QKRAA, or RRRRAA in position 70–74 on the third hypervariable region of the DRB1* β chain, the so called shared epitope.⁴ The presence of a shared epitope was found in some studies to be correlated with a more destructive form of RA, particularly if it was present on both alleles.^{5,14} More recently, polymorphism has been described in the genes for tumour necrosis factors (TNF) located on the short arm of chromosome 6 within the major histocompatibility complex.⁶ Disease correlations have been described with systemic lupus erythematosus (SLE), but these have been secondary mainly to linkage disequilibrium due to the presence of alleles with previously established disease associations.⁷ The best studied polymorphisms relate to the TNF α and TNF β microsatellites located 3.5 kB upstream of the TNF gene, and 13 and eight alleles, respectively, have been identified.⁸ Interestingly, TNF polymorphism has been linked to the in vitro response of macrophages to lipopolysaccharide^{9,10} and therefore it could be of potential importance in the regulation of the host reaction in inflammation.

RA is an important cause of chronic and disabling polyarthritis in Peru, but very little is known about HLA and TNF polymorphisms in this population. We report such data in unselected Hispano-Indian (mestizo) patients with RA seen in two hospitals in Lima and in ethnically matched local controls.

Subjects and methods

PATIENTS AND CONTROLS

Seventy nine patients with RA and 65 ethnically matched healthy controls were studied (table 1). All patients fulfilled the ACR criteria for RA. All patient samples were available for DQA1* typing but, for technical reasons, accurate DQB1* typing was possible in only 76

Table 1 Number of patients with rheumatoid arthritis and controls typed for HLA and tumour necrosis factor (TNF)

Locus	Patients		Controls	
	Chromosomes	Individuals	Chromosomes	Individuals
DRB1	156	78	130	65
DQB1	152	76	130	65
DQA1	158	79	130	65
TNF α	158	79	130	65
TNF β	156	78	130	65

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patients and DRB1* typing in 78 patients. All 79 samples were tested for TNF α but only 78 for TNF β alleles. In 35 patients the disease was graded as mild, moderate, or severe based on functional status, extent and severity of joint involvement, and presence or absence of extra-articular manifestations. Rheumatoid factor was determined in the routine laboratory by the sheep cell agglutination test.

HLA TYPING

Genomic DNA was extracted from whole blood using a standard phenol chloroform extraction procedure. One μ g DNA was amplified by the polymerase chain reaction (PCR) using specific primers for DRB1* and DQB1* loci. The sequences of the primers and oligonucleotide probes used for the genetic typing have been described elsewhere, as well as the conditions for amplification and hybridisation.¹¹ Five μ l of the PCR product was denatured, spotted onto nylon membranes (Pall, CA, USA) using a dot blot apparatus (Biorad, CA, USA), and hybridised with either horseradish peroxidase (HRP) or biotin labelled oligonucleotide probes. Negative controls containing no DNA and positive controls with DNA from homozygous cell lines were included on each membrane. The DQA1* typing was performed with a commercially available reverse dot blot assay (Amplitype, Cetus, CA, USA).

TNF TYPING

A non-radioactive method was used for typing TNF α and TNF β microsatellites.¹² The method included two consecutive PCR amplifications, in the second of which biotinylated primers were used. In the first PCR we obtained a fragment of 200 bp. In the second amplification 1 μ l of 1:100 dilution of the product obtained from the first PCR was used for the TNF α and TNF β specific amplification. The PCR mixture contained 1 μ M of each primer, 200 μ M of each dNTP, 1.5 mM of MgCl₂, and 2.5 units of Taq polymerase. The total volume was 50 μ l. This mixture was subjected to 30 cycles of amplification (94°C for 1 minute, 68°C for 1 minute, and 72°C for 1 minute). To the final specific PCR product, one volume of loading buffer (98% formamide) was added. The samples were heated to 80°C for 2 minutes and loaded on a routine sequencing gel at 90 W for 3 hours in 1 \times TBE (0.1 M Tris base, 0.009 M boric acid, 1 mM EDTA). After electrophoresis the PCR products were blotted onto a positively charged nylon membrane for 3 hours (Amersham-Pharmacia, Sweden). The membrane was air dried and saturated with 5% SDS and 0.9% NaCl and then left for 20 minutes in a solution containing 0.5% SDS, 0.9% NaCl, and 1 μ g/ml streptavidin-HRP. The membrane was washed twice in 0.5% SDS, 0.9% NaCl for 5 minutes. Detection of TNF α and TNF β microsatellites was performed by chemiluminescence (ECL, Amersham-Pharmacia, Sweden) according to the manufacturer's protocol.

Table 2 Results of HLA-DRB1* typing in patients with rheumatoid arthritis and controls

Allele	Patients (n=78)		Controls (n=65)	
	Alleles No (%)	Individuals No	Alleles No (%)	Individuals No
0101*	5 (3.2)	5	3 (2.3)	3
0102*	2 (1.3)	2	2 (1.5)	2
0301	7 (4.5)	5	3 (2.3)	3
0302	0	0	1 (0.8)	1
0401*	3 (1.9)	3	1 (0.8)	1
0402	2 (1.3)	1	0	0
0403	1 (0.6)	1	0	0
0404*	12 (7.7)	12	15 (11.5)	13
0405*	2 (1.3)	2	2 (1.5)	2
0406	1 (0.6)	1	2 (1.5)	2
0407	10 (6.4)	10	7 (5.4)	7
0408*	10 (6.4)	8	13 (10.0)	12
0701	6 (3.8)	6	3 (2.3)	3
0801	4 (2.6)	4	0	0
0802	14 (9.0)	14	12 (9.2)	12
0803	0	0	2 (1.5)	2
0804	1 (0.6)	1	4 (3.1)	3
0901	27 (17.3)	23	18 (13.8)	16
1001*	2 (1.3)	2	0	0
1101	1 (0.6)	1	6 (4.6)	6
1103	0	0	2 (1.5)	2
1104	0	0	2 (1.5)	2
1301	3 (1.9)	3	0	0
1302	2 (1.3)	2	7 (5.4)	7
1303	1 (0.6)	1	1 (0.8)	1
1304	0	0	3 (2.3)	3
1305	2 (1.3)	2	1 (0.8)	1
1402*	28 (17.9)	26	11 (8.5)	10
DR2†	10 (6.4)	9	9 (6.9)	9

*Alleles with the shared epitope.

†DR2 is used instead of DRB1*15 and *16 families, the alleles of which were not distinguishable with our primers and probes.

For unambiguous typing, control DNA—that is, previously sequenced DNA—was included in all typing experiments.

STATISTICS

Statistical analysis was performed using the χ^2 test. The level of significance was set at 0.05. Relative risk (RR) was estimated according to Wolf with Haldane's correction.¹³

Results

Table 2 shows the results of DRB1* typing. DRB1*1402 was the only allele with the shared epitope sequence which was significantly more common in patients than in controls (17.9% v 8.5%, $p < 0.0311$). The proportion of patients with at least one copy of this allele was similarly significantly increased compared with controls (33.3% v 15.4%, $p < 0.0232$, relative risk 2.74). In contrast, the overall frequency of alleles with the shared epitope was not different between patients and controls. The proportion of

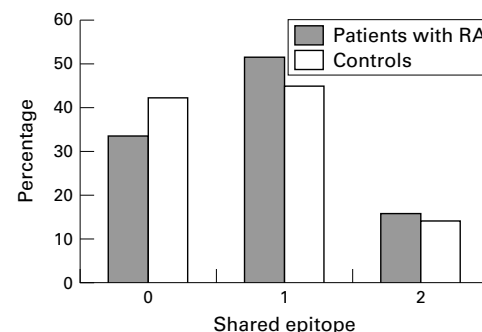


Figure 1 Proportion of patients and controls carrying none, one, or two alleles with the shared epitope sequences. The distribution is not significantly increased ($\chi^2=1.03$; $p > 0.5$).

Table 3 Results of HLA-DQA1* typing in patients with rheumatoid arthritis and controls

Allele	Patients (n=79)		Controls (n=65)	
	Alleles No (%)	Individuals No	Alleles No (%)	Individuals No
0101	8 (5.1)	8	6 (4.6)	6
0102	9 (5.7)	9	9 (6.9)	9
0103	3 (1.9)	3	6 (4.6)	6
0201	9 (5.7)	9	4 (3.1)	4
0301	65 (41.1)	52	57 (43.8)	45
0401	64 (40.5)	49	48 (36.9)	40

Table 4 Results of HLA-DQB1* typing in patients with rheumatoid arthritis and controls

Allele	Patients (n=76)		Controls (n=65)	
	Alleles No (%)	Individuals No	Alleles No (%)	Individuals No
0201	12 (7.9)	11	9 (6.9)	8
0301	47 (30.9)	36	36 (27.7)	30
0302	23 (15.1)	21	28 (21.5)	26
0303	31 (20.4)	25	23 (17.7)	20
0401	9 (5.9)	9	11 (8.5)	11
0402	8 (5.3)	8	6 (4.6)	6
0501	10 (6.6)	9	5 (3.8)	5
0502	2 (1.3)	2	0	0
0503	0	0	1 (0.8)	1
0601	0	0	2 (1.5)	2
0602	4 (2.6)	4	2 (1.5)	2
0603	3 (2.0)	3	1 (0.8)	1
0604	3 (2.0)	3	6 (4.6)	6

subjects with none, one, or two alleles with the shared epitope was similar in patients and controls (fig 1). The DRB1*11 containing alleles were more common in controls than in patients (7.7% v 0.6%, p<0.0045).

Data on clinical severity (assessed as mild, moderate, or severe) were available in 35 patients. Those with none, one, or two alleles with the shared epitope were equally represented in all three categories of disease severity (data not shown).

DQA1* and DQB1* allele frequencies are shown in tables 3 and 4, respectively. No significant differences were detected between patients and controls.

Tables 5 and 6 show the results of TNFα and TNFβ typing, respectively. The TNFα6 allele occurred more frequently in patients than in controls (36.7% v 21.5%, p<0.0076), and the proportion of patients with at least one copy of this allele was greater (59.5% v 38.5%, p<0.015, relative risk 2.35). There were more homozygous and heterozygous TNFα6 individuals in the patient group than in the control

Table 5 Results of TNFα typing in patients with rheumatoid arthritis and controls

Allele	Patients (n=79)		Controls (n=65)	
	Alleles No (%)	Individuals No	Alleles No (%)	Individuals No
α1	14 (8.9)	13	13 (10.0)	13
α2	16 (10.1)	15	15 (11.5)	15
α3	0	0	2 (1.5)	2
α4	2 (1.3)	2	3 (2.3)	3
α5	0	0	5 (3.8)	5
α6	58 (36.7)	47	28 (21.5)	25
α7	11 (7.0)	11	4 (3.1)	4
α8	0	0	0	0
α9	3 (1.9)	3	0	0
α10	43 (27.2)	35	49 (37.7)	43
α11	10 (6.3)	9	10 (7.7)	10
α12	0	0	0	0
α13	1 (0.6)	1	1 (0.8)	1

Table 6 Results of TNFβ typing in patients with rheumatoid arthritis and controls

Allele	Patients (n=78)		Controls (n=65)	
	Alleles No (%)	Individuals No	Alleles No (%)	Individuals No
β1	9 (5.8)	8	16 (12.3)	16
β2	0	0	0	0
β3	3 (1.9)	3	0	0
β4	58 (37.2)	46	63 (48.5)	51
β5	84 (53.8)	59	49 (37.7)	40
β6	0	0	0	0
β7	2 (1.3)	2	2 (1.5)	2

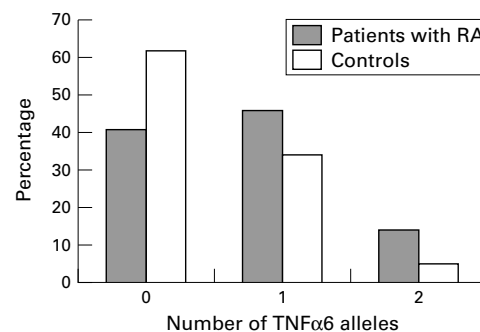


Figure 2 Proportion of patients and controls carrying none, one, or two TNFα6 alleles. The distribution differs significantly ($\chi^2=7.54$; $p<0.023$).

group (fig 2). TNFα6 seems to be more closely related to RA than DRB1*1402. The number of patients with the TNFα10 allele was decreased compared with the controls (44.3% v 66.2%, p<0.014).

The overall TNFβ5 allele frequency was significantly increased among the patients (53.8% v 37.7%, p<0.009) and a similar trend was found when comparing phenotypic frequencies (75.6% v 61.5%, p<0.10). All but one of the TNFα6 positive patients were also positive for TNFβ5. Both TNFβ1 and TNFβ4 phenotype frequencies (10.3% v 24.6%, p<0.019, and 59.0% v 78.5%, p<0.021) were decreased in patients.

There was no correlation between the occurrence of rheumatoid factor and TNF status. Furthermore, there was no significant correlation between disease severity and the presence of TNFα6. However, five of 24 (20.8%) patients with TNFα6 were classified as severe compared with none of 11 TNFα6 negative patients (NS).

The extended haplotype DRB1*1402-DQB1*0301-DQA1*0401 (based on known linkage disequilibrium) was found in 26 of 78 patients (33.3%) compared with 10 of 65 controls (15.4%; p<0.023). TNFα6 was more common among patients, whether or not they had this haplotype (16 of 26 (61.5%) v 30 of 52 (57.7%)). An association between DRB1*1402 and TNFα6 in this population is likely since about half of DRB1*1402 individuals also have TNFα6β5, with no differences between RA patients and controls. Moreover, an association between these two alleles is described in the cell line ASHI no EK 9054. However, since we did not perform a family study, we cannot formally confirm the linkage. To test for independent associations, TNFα6 frequencies were also analysed in DRB1*1402

negative subjects and a statistically significant increase was detected in patients with RA compared with controls (57.7% *v* 36.4%; $\chi^2=4.065$; $p<0.044$).

Discussion

HLA-DR genetics has been studied in patients with RA for two decades, establishing susceptibility associated with a structure in the third hypervariable region of the DRB1 chain in position 70–74. Susceptibility is conferred by the sequence QRRAA, QKRAA, or RRRRAA, called the shared epitope.⁴ Of particular interest is the fact that the correlation with DRB1* genes varies between ethnic groups.¹⁴ The most common association found in white populations and Eskimos is with DRB1*0401 and 0404, in Asians with DRB1*0405, in Mediterranean populations with DRB1*0101 and 1001, and in North American Indians with DRB1*1402.^{14–17} Our study shows that DRB1*1402 is also over-represented in Peruvian mestizos, a population of descendants of Hispanics and American Indians. Its presence conferred susceptibility with a relative risk of 2.74. This significant association was shown, despite the limited number of individuals studied. The modest level of the association could be explained in part by the relatively high prevalence of DRB1*1402 in the general mestizo population. Likewise, the overall prevalence of alleles with the shared epitope in the Peruvian population was as high as 58.5% compared with 66.7% in patients with RA. The presence of either a single or two shared epitopes is therefore not significantly predictive of RA in this population (fig 1). Some, but not all, studies have found a positive correlation between disease progression and the dose of the shared epitope.¹⁴ The limited clinical data in our study do not allow definite conclusions in this regard.

It has been proposed that only one third of the genetic predisposition for RA is related to HLA-DRB1*.^{14, 18} The remaining candidate genes are still largely unknown. DQB1* has been suggested in some studies, but this was later found to be secondary to linkage disequilibrium with HLA-DRB1*.¹⁹ Our findings support the previous observations. Other gene polymorphisms that did not vary between patients with RA and controls independently of HLA-DRB1* include TAP1 and TAP2.^{20, 21}

The Gm system has been studied in white populations and, although anti-Gm antibodies showed strong correlation with destructive disease in one study,²² no association between the genes of the Gm system and RA was detected. Conflicting results have been reported regarding HLA-DM.^{23, 24} The class III C4 null genes have been found to be associated with susceptibility to RA in Asians and with radiographic progression in a Finnish study.^{25, 26}

In our study the strongest association was with the TNF α 6 allele, which was present in almost 60% of the patients compared with less than 40% of the controls. All but one of the TNF α 6 positive patients were also TNF β 5 positive. Figure 2 shows that there is a significant difference in the distribution of

TNF α 6 alleles between patients and controls. TNF polymorphism has recently attracted increasing interest in relation to RA. Early work suggested no correlation with gene polymorphisms in TNF β or TNF α ,^{27, 28} but an Irish study reported results from multicase families where a TNF α 6 β 5 containing haplotype was present in 35.3% of affected individuals but in only 20.5% of non-affected individuals.²⁹ In a British study the TNF α 6 allele was found to be increased with an odds ratio of 2.5.¹⁹ Importantly, as in our study, these studies indicate an influence that is independent of HLA-DRB1* status. It has been reported that the association is more pronounced in female patients.^{30, 31} A Spanish study found that the presence of TNF α 6 β 5 increased susceptibility to RA, regardless of the presence or absence of the shared epitope.³² One recent study described an association with disease progression and guanine to adenine transition at position –238 of the promoter region of the TNF α gene.^{33, 34} Robust evidence, gathered in several populations and looking at different regions, is therefore emerging which links TNF gene polymorphism with susceptibility to RA, and perhaps with disease progression. Our study shows for the first time an association in a Hispano-Indian population with an HLA polymorphism, HLA-DRB1*1402, that differs from that in white patients with RA. Our results also show that the TNF α 6 allele is associated with RA independently of HLA-DRB1*1402.

As a result of the strong linkage disequilibrium between TNF α and TNF β alleles, an increase in TNF β 5 (which is in linkage with TNF α 6) and a decrease in TNF β 4 (which is in linkage with TNF α 10) was an expected result in our study.

An intriguing finding was the low prevalence of the TNF α 10 allele. Whereas no information is available regarding altered TNF production in individuals with TNF α 6, high TNF production has been connected with TNF α 10 haplotypes.¹⁰ Complex interactions between HLA and TNF gene polymorphism have been suggested in a recent report.³⁵ Future studies will address the question if and how this polymorphism affects disease manifestations, and explore the correlation between TNF α 6 and disease severity.

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