Influence of HLA-DRB1 and TNF microsatellite polymorphisms on the expression of extraarticular manifestations in rheumatoid arthritis patients from northwest Spain

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Abstract

Objective

To determine whether extraarticular manifestations (EAM) in rheumatoid arthritis (RA) patients from northwest (NW) Spain are associated with particular HLA-DRB1 alleles and/or TNF microsatellite polymorphisms.

Methods

The frequencies of HLA-DRB1 alleles and TNF microsatellite polymorphisms were compared between RA patients with and without extraarticular disease in a population from Lugo, NW Spain. HLA-DRB1 and TNF typing were carried out using molecular based methods on 181 clinic-based RA patients and 145 healthy controls. Associations were investigated using Chi-square analyses or Fisher's exact test. Multivariate logistic regression analyses were used to investigate independent and interactive effects of HLA and TNF alleles.

Results

*The frequencies of HLA-DRB1 and TNF microsatellite polymorphisms in patients with EAM were not significantly different from those without extraarticular disease, although an association between HLA-DRB1*0101 and nodular disease approached significance (p = 0.054). There was no evidence for an increased frequency of homozygous or heterozygous combinations of disease associated DRB1 alleles in RA patients with EAM. The TNF a8 microsatellite allele was found at a higher frequency (6.9%) in patients with EAM compared to those without EAM (1.8%), and controls (1.5%) (p = 0.03 and 0.02, respectively). However, significance was lost after correction for multiple testing. No evidence was found for an interaction between HLA-DRB1 and TNF alleles being associated with the expression of EAM.*

Conclusion

*In an RA population from NW Spain the frequencies of HLA-DRB1 and TNF microsatellite alleles in patients with extra-articular manifestations were not significantly different to those without extraarticular disease, although there was a trend towards increased frequency of HLA-DRB1*0101 in patients with nodular disease. There was no evidence for an interaction between HLA-DRB1 and TNF alleles in relation to the expression of EAM.*

Key words
Rheumatoid arthritis, extraarticular disease, HLA, TNF, polymorphisms.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by the destruction of synovial joints and various systemic extraarticular manifestations. Genetic and environmental factors are believed to contribute to the disease, with the genetic component accounting for up to 60% of disease susceptibility (1). The major genetic contribution is provided by genes of the HLA region, particularly the MHC class II HLA-DRB1 alleles. Those DRB1 alleles associated with RA all encode a conserved amino acid sequence (QKRAA, QRRAA or RRRAA) in the third hypervariable region (HVR3) of the DR 1 molecule, which is commonly called the shared epitope (SE) (2). There is still considerable debate as to whether the HLA-DRB1 alleles influence susceptiblity, disease severity or both.

Extraarticular manifestations in RA (e.g. nodules, vasculitis, fibrosis, amyloidosis) are usually associated with more severe disease. A number of studies have shown an association between SE alleles and extraarticular disease in RA. An early study by Ollier *et al.* (3) suggested that the frequency of HLA-DR4 was increased in patients with nodular disease, and that an HLA haplotype carrying DR4 (i.e. Cw3-Bw62-Dw4-DR4-DRw53) was associated with patient subsets displaying features of extraarticular disease. HLA-DR4 was also found to be associated with extraarticular manifestations of RA in a population from Northern Italy (4). Further studies in US and French populations supported a role for the HLA-DRB1*0401 allele in susceptibility to the extraarticular manifestations of RA (5, 6). Both homozygosity for the HLA-DRB1*0401 allele and the presence of two different alleles carrying the SE accounted for an increased risk of severe EAM (5, 6). Homozygosity for the HLA-DRB1*401 allele was associated with major organ involvement (5), while heterozygosity for two SE alleles was associated with rheumatoid nodules (5) and vasculitis (6). In contrast, in Dutch patients with RA the occurrence of rheumatoid vasculitis with major organ involvement was not increased in patients carrying HLA-DRB1*04 alleles (7).

In addition to class II alleles, other genes within the MHC regions such as tumor necrosis factor (TNF) alpha seem to be implicated in RA susceptibility. The TNF locus is polymorphic and located within the HLA class III region. A number of biallelic polymorphisms have been identified within the TNF locus, as well as 5 microsatellite markers (a-e) (8). Several conserved HLA-DRB1/TNF microsatellite haplotypes have been found at higher frequencies in RA patients (9), and an additional effect of TNF microsatellite polymorphism on susceptibility to RA has been described in patients carrying the rheumatoid SE (10). Recently, Martinez *et al.* have observed that polymorphism in the TNF region is associated with RA, even in those patients without the HLA-DRB1 SE (11). These authors have observed that TNF a/b microsatellites not only confer susceptibility, but also increase the susceptibility conferred by HLA-DRB1 SE alleles (11). In patients with RA from NW Spain, TNF and HLA-DRB1 gene regions were also found to be independently associated with RA susceptibility (12). Moreover, TNF microsatellite polymorphism was associated with the susceptibility to erosive and seropositive disease, which was independent of HLA-DRB1 and SE status.

To further investigate the role of TNF and HLA-DRB1 alleles in the severity of RA we have examined the influence of different gene loci within the TNF and HLA-DRB1 regions on the susceptibility to extraarticular manifestations of RA.

Patients and methods

Patients

All patients and ethnically matched controls were from the area surrounding Lugo in Galicia (Northwest Spain). The patients (n = 181) satisfied the 1987 American College of Rheumatology (ACR) classification criteria for RA (13), and were all recruited from the Hospital Xeral-Calde in Lugo. The rheumatology unit is the only one available for a population of approximately 250,000 people. All patients

were treated by the same group of rheumatologists (principally MAGG and CGP), either as in-patients or in hospital out-patient clinics. The cohort consisted of an unselected series of RA patients seen over a period of approximately one year. 94% of the patients had been treated with one or more DMARDs, including chloroquine, sulphasalazine, gold, cyclosporine and methotrexate. More than 50% of the patients were currently being treated with methotrexate, either alone or in combination with chloroquine or cyclosporine. Controls (n = 145) were ethnically matched healthy volunteers from the same region. Partial information on this series of RA patients has been described in previous studies (12,14). The main characteristics of the population of Lugo have previously been described (15,16). It is a mixed rural and urban population with a Celtic background which has seen little population movement from or into the area.

Inclusion criteria. Patients with RA were considered to have severe EAM and included in this study if they had at least one of the following clinical features.

1) Rheumatoid subcutaneous nodules (usually found on periarticular structures, extensor surfaces or other areas subjected to mechanical pressure).
2) Pleuropulmonary manifestations: If there was evidence of pleuritis with typical fluid containing low glucose levels in the absence of infection or interstitial pulmonary fibrosis confirmed by computed tomography (CT) scan and lung function tests or pulmonary nodules confirmed by CT scan.
3) Clinically apparent pericarditis confirmed by echocardiographic evidence of pericardial effusion.
4) Rheumatoid vasculitis: A diagnosis of vasculitis related to rheumatoid arthritis was considered if the patient fulfilled the ACR criteria for RA and had a biopsy showing vasculitis in any involved tissue (14).
5) Felty's syndrome: A diagnosis of Felty's syndrome was considered if the patient fulfilled the ACR criteria for RA and had splenomegaly and leukopenia with selective neutropenia, with or without anemia and thrombocytopenia.
6) Amyloidosis confirmed by abdominal subcutaneous fat pad aspirate or rectal biopsy.
7) Glomerulonephritis if the patient fulfilled the ACR criteria for RA, had renal manifestations, and a renal biopsy confirmed the presence of glomerulonephritis.
8) Autoimmune thrombocytopenia: If a patient who fullfilled the ACR criteria for RA had thrombocytopenia with normal or high megakaryocyte count in an otherwise normal bone aspirate and no disease other than RA was known to be associated with the thrombocytopenia (17).

Patients with secondary Sjögren's syndrome without any of the severe EAM described above have recently been described (14) and they were not included among the group of patients with severe EAM.

Patients with RA were considered as seropositive if the rheumatoid factor (by nephelometry) was found to be positive in at least two determinations during the course of the disease.

HLA typing

DNA was obtained from EDTA anticoagulated blood samples using a phenol-chloroform extraction procedure. HLA-DRB1 phenotypes were determined using a semi-automated commercially available reverse dot blot method (INNO-LiPA, Abbott Laboratories UK). Reaction patterns were interpreted using Inno-LiPA software. HLA-DR4 subtypes were identified using either single strand conformational polymorphism (SSCP) following amplification with DR4 specific primers (18) or the INNO-LiPA technology.

TNF microsatellite typing

TNF microsatellite markers (a, b, c and d) were typed as previously reported (9). Fluorescently-labelled forward primers and unlabelled reverse primers were used to amplify TNF a-d microsatellite markers. Following PCR amplification, products were pooled and mixed with an internal standard (TAMRA-350). Alleles were separated on 6% acrylamide gels using an Applied Biosystems 373A automated DNA sequencer. Allele calling was performed using fragment analysis software (672 Genescan analysis) and Genotyper software (Perkin Elmer). DNA with known TNF genotypes were included on each gel as positive controls; TUBO (a3/13, b1/4, c1/2, d4/5); VAVY (a2/2, b3/3, c1/1, d1/1), OMW (a7/7, b1/1, c2/2, d2/2) and IBW9 (a4/4, b7/7, c2/2, d5/5).

Statistical analysis

The frequency distribution of all the HLA-DRB1 alleles and TNF microsatellite markers in the disease groups was initially compared to that of controls by Chi-square tests for homogeneity. P values were corrected for multiple comparisons by the Bonferroni (versus controls) method. The cells containing alleles which contributed most to the Chi-square were determined by examining the adjusted residuals (deviations from expected values). Each allele in the disease groups which demonstrated significant differences to controls was then examined further by either Chi-square or Fisher exact analysis of 2 x 2 contingency tables. The strength of the association between severe EAM and alleles of DRB1 and TNF was estimated using odds ratios (OR) and 95% confidence intervals (CI). To examine whether TNF and DRB1 allelles were independently associated with EAM in RA, we undertook bivariate and multivariate logistic regression analyses with different combinations of TNF and DRB1 alleles as independent variables, and presence or absence of EAM as the dependent (group) variable. In some analyses we also examined the effect of gene interactions using logistic regression models which contained the interaction term as well as the corresponding main effects.

Results

Thirty-five of the 181 patients (19.3%) had EAM. Twenty-five of them developed subcutaneous nodules. In 7 patients the rheumatoid subcutaneous nodules coexisted with other unequivo-

cal severe EAM. Ten patients presented severe EAM without subcutaneous nodules (Table I). The main epidemiological and clinical differences between patients with severe EAM and the rest of the RA patients are shown in Table II. The mean disease duration of patients with EAM was longer than that of patients without EAM ($p = 0.006$). All patients (100%) with EAM were rheumatoid factor positive, compared with 71.2% of patients without EAM (OR 28.9, $p < 0.0001$). There was also a significantly higher number of patients with erosions in the group with EAM compared to those without extraarticular disease (91.4% v 71.9%, OR 4.2, $p = 0.016$).

Table I. Severe extra-articular manifestations (EAM) in a series of 181 unselected rheumatoid arthritis patients from northwest Spain.

EAM	No. of patients	%
No. of patients with severe EAM	35	19.3
Subcutaneous nodules	25	14.0
Alone	18	10.1
Associated with other severe EAM	7	3.9
Other severe EAM	10	5.6
Pleuropulmonary manifestations	10	5.6
Biopsy-proven vasculitis	3	1.7
Small and middle-sized vasculitis	2	1.1
Small-sized vasculitis	1	0.6
Felty's syndrome	2	1.1
Pericarditis	2	1.1
Focal proliferative glomerulonephritis	1	0.6
Autoimmune thrombocytopenia	1	0.6

Table II. Clinical features of RA patients with and without severe EAM.

Group	F: M ratio	Age at onset onset (SD)	Disease duration (SD)	Rheumatoid factor (+)	Erosions[§]
With severe EAM (n = 35)	27: 8	49.6 (11.8)	13.2 (7.9)*	100%**	91.4%†
Without severe EAM (n = 146)	103: 43	49.2 (14.8)	9.9 (9.3)	71.2%	71.9%

[§]Observed on plain radiographs of hands and/or feet. Erosive status was based on radiological assessment following a minimum of 2 years disease duration.
*$p = 0.006$; **OR 28.9, 95% CI 3.4 - , $p < 0.0001$; † OR 4.2, 95% CI 1.1 - 18.1, $p = 0.016$.

HLA-DRB1 phenotype and genotype frequencies

The HLA-DRB1*0101 and HLA-DRB1*04 phenotype frequencies were significantly increased in the RA patient group with severe EAM compared with controls. In the case of DRB1*04 this was largely accounted for by an increase in DRB1*0401 (Table III). There were no significant differences between RA patients with and without severe EAM, although in patients with nodular disease the increased frequency of HLA-DRB1* 0101 approached significance (41.7% vs 23.2%, $p = 0.054$) compared to patients without nodules. Correction for age, sex and disease duration in a logistic regression model made little difference to the significance ($p= 0.058$) of this result. This possible weak association also appeared to be independent of HLA-DRB1* 0401, since inclusion of DRB1*0401 together with DRB1*0101 as independent variables in the regression model made virtually no difference to the significance level ($p = 0.057$).

A greater number of patients with EAM carried a single SE allele (60.9%) compared with those without EAM (46.6%), although this was not statistically significant. There was no evidence for an increased frequency of homozygous or heterozygous combinations of SE alleles in RA patients with EAM. Only 6/33 (18.2%) patients with EAM carried two SE alleles compared with 26/146 (17.8%) patients without EAM. Of 24 patients with nodules, only 3 (12.5%) were heterozygous for two SE alleles compared with 26/155 (16.8%) of patients without nodules ($p = 0.6$).

TNF microsatellite allele frequencies

Allele frequencies of TNF microsatellite markers in RA patients with and without severe EAM and controls are presented in Table IV. Global Chi-square analyses revealed no differences in the overall frequencies of TNF alleles in patients with and without EAM. However, examination of adjusted residuals indicated that the frequency of the TNF a8 allele in patients with EAM were significantly different to patients without EAM as well as controls, and made the largest contribution to the Chi-square value. Using 2 x 2 contingency tables, TNF a8 was found to be significantly increased in patients with EAM compared with patients without EAM (6.9 vs 1.8%, OR 4.01, 95% CI 0.9 – 18.0, $p = 0.03$), and controls (6.9 vs 1.5%. OR 4.8, 95% CI 1.0–24.6, $p=0.02$). However, these results should be treated with caution since significance was lost when correction for multiple testing was carried out.

Investigation of TNF and HLA-DRB1 interactions in extraarticular disease

We have shown previously in a UK population that the radiographic severity of RA is associated with an interaction between the SE and the TNF a6 microsatellite polymorphism (19). Mu *et al*. also found an interaction between the SE and TNF a11 in relation to RA severity (20). We therefore investigated whether any interactions between HLA and TNF alleles were associated with extraarticular manifestations in RA.

Using logistic regression analysis with EAM as the dependent variable we initially looked to see whether interactions between the SE and TNF a6 or TNF a11 were associated with EAM. No significant associations were found with EAM in general, or with particular manifestations such as nodular dis-

Table III. HLA-DRB1 phenotype frequencies (%) in controls and RA patients with and without extraarticular manifestations (EAM).

DRB1*	Controls N = 145	RA with EAM N = 33	RA without EAM N = 146
0101	15.8	36.4[1]	23.3
0103	1.4	3.0	0.7
15 or *16	26.2	30.3	24.7
03	20.0	6.1	15.1
04	26.2	54.5[2]	52.1[3]
0401	10.3	30.3[4]	29.5[5]
0402	2.0	3.0	2.1
0403	1.4	3.0	3.4
0404	4.1	12.1	8.2
0405	3.4	9.1	7.5
0406	1.4	0.0	0.7
0407	0.7	0.0	2.1
0408	2.8	0.0	4.1
0409	0.0	0.0	0.7
11 or 12	22.8	18.2	3.7
13 or 14	35.2	12.1[6]	19.2
07	26.9	21.2	15.8
08	7.6	0.0	2.1
09	3.4	0.0	2.1
10	4.1	3.0	4.1
SE-/-	62.1	21.2[7]	35.6[8]
SE+/-	33.1	60.6[9]	46.6[10]
SE+/+	4.8	18.2[11]	17.8[12]

All comparisons versus controls
[1]OR = 3.0 (95% CI 1.2-7.6, p = 0.007)
[2]OR = 3.4 (95% CI 1.5-7.9, p = 0.002)
[3]OR = 3.1 (95% CI 1.8-5.2, p = 6.3×10^{-6})
[4]OR = 3.8 (95% CI 1.4-10.3, p = 0.003)
[5]OR = 3.6 (95% CI 1.8-7.2, p = 4.5×10^{-5})
[6]OR = 0.25 (95% CI 0.07-0.8, p = 0.01)
[7]OR = 0.2 (95% CI 0.1-0.4, p = 2.1×10^{-5})
[8]OR = 0.3 (95% CI 0.2-0.6, p = 6.4×10^{-6})
[9]OR = 3.1 (95% CI 1.3-7.3, p = 0.003)
[10]OR = 1.8 (95% CI 1.1-2.9, p = 0.02)
[11] OR = 4.4 (95% CI 1.2-16.2, p = 0.007)
[12]OR = 4.3 (95% CI 1.7-11.3, p = 0.0004)

ease or fibrosis. Since the TNF a8 allele appeared to show an increased frequency in EAM patients, we also examined whether this was associated with any particular DRB1 allele(s). No associations were found. Likewise, the close to significant association of HLA-DRB1*0101 alleles with nodular disease was independent of any TNF alleles. Overall, we found no evidence of any interaction between HLA-DRB1 and TNF microsatellite alleles that might influence the expression of EAM (data not shown).

Discussion

We have examined the frequency of HLA-DRB1 alleles and TNF microsatellite polymorphisms in clinic-based group of RA patients from NW Spain. In contrast to other studies we found no significant difference in the frequencies of HLA-DRB1 alleles in patients with and without extra-articular manifestations of RA. The only association that approached significance was between the HLA-DRB1*0101 phenotype and the presence of rheumatoid nodules, where there was a trend towards increased frequency of DRB1*0101. One report on patients from the USA indicated that nodular disease was associated with heterozygosity for two SE alleles while homozygosity for HLA-DRB1*0401 was characteristic of patients with major organ development (5). In our patients from NW Spain the frequency of two different SE alleles in patients with rheumatoid nodules was no different to that in patients without nodules. Furthermore, there was no evidence that homozygosity for HLA-DRB1*0401 was associated with major organ involvement in this population. This may be partly explained by the low frequency (1.7%) of DRB1*0401 homozygotes in this Spanish population.

Another study from France (6) showed that a higher risk of vasculitic disease was found in patients with 2 SE alleles, one characterised by the QKRAA sequence (DRB1*0401) and the other by the QRRAA sequence (e.g. DRB1*0101, *0102, *0404, *0405 or *0408). In our Spanish population the number of patients presenting with vasculitis was too small to carry out any meaningful comparisons, although 2/3 of the patients carried the HLA-DRB1*0401 allele.

We found no convincing evidence for an association between the EAM of RA and TNF microsatellite alleles. There may a very weak association between EAM and TNF a8, but this was not significant after correction for multiple testing. Furthermore, the frequency of TNF a8 was very low in this population so the influence of this allele (if any) is unlikely to have much impact on the numbers of patients expressing EAM. In a previous study on this group of patients we found a weak association between the TNF a2 polymorphism and erosive disease (12). However, TNF a2 appears to have no association with EAM since the frequency of TNF a2 alleles was no different between patients with and without EAM in the present study.

Investigation of possible interactions between HLA-DRB1 and TNF alleles failed to reveal any associations with EAM. We initially concentrated on interactions between the SE and TNF a6 or TNF a11, since these had been shown to be associated with more severe disease in previous studies in the UK and USA (19,20). However, we found no evidence for such interactions being associated with EAM in this Spanish population. We also looked for other interactions between HLA-DRB1*0101 and TNF alleles since DRB1*0101 appeared to have some possible association with nodular disease. Again, we found no interactions with any TNF alleles. Similarly we found no interactions between TNF a8 and DRB1 alleles.

Overall, our data suggests that the distribution of HLA-DRB1 and TNF microsatellite alleles in RA patients expressing EAM is not significantly different to that in patients who do not express EAM. This does not appear to be due to differences in age, sex or disease duration since these were corrected for in the analyses. Obviously, there could be other genetic factors that are important, but we do not have information on other genetic polymorphisms in these patients at present. Our study suggests that in RA patients from NW Spain the expression of EAM cannot be explained by the influence of particular HLA-DRB1 and TNF alleles. The difference between our results and those from other studies could possibly be explained by differences in the distribution of HLA-DRB1 alleles in different RA populations. However, we have shown previously that the primary association with RA in NW Spain is with DRB1*0401 (12), and the associations in general appear similar to those observed in caucasian populations in the USA and

Table IV. Allele frequencies of TNF microsatellite markers in controls and RA patients with or without EAM.

		Allele frequencies	
	Control	RA with EAM	RA without EAM
TNF a	(2N = 266)	(2N = 58)	(2N = 276)
1	0.041	0.017	0.018
2	0.192	0.207	0.210
3	0.011	0.0	0.014
4	0.086	0.052	0.062
5	0.056	0.121	0.069
6	0.158	0.103	0.174
7	0.128	0.034	0.098
8	0.015	0.069	0.018
10	0.173	0.224	0.181
11	0.090	0.121	0.101
12	0.008	0.017	0.0
13	0.023	0.017	0.033
14	0.000	0.0	0.007
TNF b	(2N = 294)	(2N = 60)	(2N = 278)
1	0.125	0.133	0.111
2	0.014	0.0	0.007
3	0.047	0.183	0.101
4	0.412	0.433	0.410
5	0.361	0.183	0.345
6	0.024	0.011	0.011
7	0.017	0.014	0.014
TNF c	(2N = 266)	(2N = 66)	(2N = 286)
1	0.658	0.788	0.752
2	0.342	0.212	0.248
TNF d	(2N = 272)	(2N = 62)	(2N = 288)
1	0.004	0.0	0.003
2	0.077	0.081	0.101
3	0.018	0.016	0.021
4	0.529	0.500	0.479
5	0.301	0.339	0.299
6	0.063	0.065	0.069
7	0.007	0.0	0.028

UK (21). Further studies are clearly needed on other RA populations to determine the importance of HLA-DRB1 and/or TNF polymorphisms in susceptibility to developing extraarticular disease.

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