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TNFA promoter polymorphism and susceptibility to brucellosis

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SUMMARY

The aim of this study was to investigate the possible influence of the tumor necrosis factor alpha (TNFA) gene promoter polymorphisms and HLA class II genes on the susceptibility to or development of human brucellosis. TNFA genotypes (at positions −308 and −238) were determined in 59 patients with brucellosis and 160 healthy controls by polymerase chain reaction-restriction fragment length polymorphism. There were no significant differences between the patients and the controls for the TNFA-238 genotypes. However, when the overall TNFA-308 genotype distribution of the brucella patients was compared with that of the control subjects, a significant skewing was observed ($P = 0{\cdot}02$). The TNFA-308.1/2 genotype was present at significantly higher frequency in the total patient as a whole compared with control subjects (30% *versus* 15%; $P = 0{\cdot}01$, odds ratio (OR) 2·49, 95% confidence interval (CI) 1·16–5·33). No statistically significant differences in the distribution of HLA-DRB1 or DQB1 alleles were observed between brucella patients and control subjects. Stratification to correct for interdependence of TNFA-308.2 and HLA-DR3 alleles confirmed that, in spite of their strong linkage disequilibrium, the association of TNFA-308.2 with brucellosis was independent of HLA-DR3.

Keywords HLA class II human brucellosis polymorphism TNF

INTRODUCTION

Brucellosis is a zoonosis transmittable to humans, caused by *Brucella* spp. infection. The disease exists worldwide, especially in the Mediterranean basin, the Middle East, India and Central and South America. In the USA, where the disease is far less frequent, changes in the predominant species have been observed throughout the past decades, the most frequently isolated species being *B. melitensis* [1]. Besides environmental factors and pathogen strain differences, it is generally accepted that host genetic factors are major determinants of susceptibility to or outcome of infectious diseases in humans. Candidate gene studies have implicated several immunogenetic polymorphisms in human infectious diseases, HLA and cytokine genes being the more relevant ones [2].

Macrophages play a fundamental role in the control of *Brucella* spp. infection, mainly through the secretion of cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) [3,4]. TNF-α production seems to be necessary for full expression for macrophage anti-brucella activity [5]. In addition to being important in resistance to brucella, TNF-α might be associated with immunopathology, depending on the timing, levels and persistence of its production by the host in response to *Brucella* infection.

Variation in the TNFA promoter region has been associated with severe forms of malaria, leishmaniasis, meningococcal infection and leprosy [6–9], diseases with high serum TNF-α levels. As a result, it has been proposed that these associations reflect genetic variability in TNF-α production which influences the clinical outcome of infectious diseases. We therefore decided to investigate the contribution of the TNFA gene promoter region polymorphisms and HLA class II genes to susceptibility to or development of clinical forms of *B. melitensis* infection, in a population from the South of Spain, where the disease is endemic.

MATERIALS AND METHODS

The present study included 59 patients with brucellosis from the Infectious Diseases Unit at the hospital Carlos Haya in Málaga, Spain. Their mean age was 45·5 years (range 17–74 years), 31 (52%) were women and 28 (40%) men. A control group was composed of 160 healthy volunteers matched for age and sex, living in the same area as the patients. The diagnosis of brucellosis was established by isolation of *B. melitensis* in blood culture (70%), and in the other cases (30%) diagnosis was based on a compatible clinical picture together with the presence of high titres of specific antibodies or a four-fold increase or greater of the initial titres in two paired serum samples drawn 2–4 weeks apart.

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Table 1. Distribution of the TNFA −308 and −238 genotypes in control subjects, brucella patients overall and brucella patients stratified by the presence of focal forms of the disease

		Controls		Brucella patients		Non-focal forms		Focal forms	
		$n = 160$	(%)	$n = 59$	(%)	$n = 36$	(%)	$n = 23$	(%)
TNFA −308	1/1	134	(84)	41	(70)	26	(72)	15	(65)
	1/2	24	(15)	18	(30)	10	(28)	8	(35)
	2/2	2	(1)	–	–	–	–	–	–
TNFA −238	G/G	132	(82.5)	47	(79)	28	(78)	19	(82)
	G/A	28	(17.5)	12	(21)	8	(22)	4	(18)
	A/A	–	–	–	–	–	–	–	–

High titres were considered to be $>1/160$ for Wright's seroagglutination test or $>1/320$ for Coomb's anti-brucella test. Those patients with focal forms of the disease comprised a subgroup of 23 (39%) patients, the most common complication being osteoarticular (67%), followed by genitourinary (23%), and to a lesser extent hepatobiliary (7%) and pulmonary (3%).

DNA was isolated from anti-coagulated peripheral blood mononuclear cells (PBMC) using standard methods. TNFA −308 and −238 genotyping was carried out by double polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with amplification-created restriction sites, as described [10]. HLA-DRB1 and DQB1 alleles were typed using a commercially available kit (Inno-Lipa HLA-DRB and DQB; Innogenetics, Zwijndrecht, Belgium) based on reverse hybridization with sequence-specific oligonucleotides following PCR amplification (reverse PCR-SSO). Data were analysed by χ^2 test with Yates' correction or Fisher's exact test using the Stalcalc program (Epi Info, version 6.0; USD, Stone Mountain, GA). $P < 0{\cdot}05$ was considered significant. The magnitude of associations was estimated as odds ratios with 95% confidence intervals (OR, 95% CI).

RESULTS

Comparison of TNFA genotype frequencies between brucella patients and controls

The distribution of the TNFA promoter genotypes in the patient groups and in the controls is shown in Table 1. In the control population, the TNFA allele and genotype frequencies were consistent with the Hardy–Weinberg equilibrium. No differences in the distribution of the TNFA −238 genotypes were detected between the groups under study. However, when the overall TNFA −308 genotype distribution of the brucella patients was compared with that of the control subjects, a significant skewing was observed ($P = 0{\cdot}02$, by χ^2 test from 3×2 contingency table) (Table 1). The TNFA-308.1/2 genotype was present at significantly higher frequency in the total patient as a whole compared with control subjects (30% *versus* 15%; $P = 0{\cdot}01$, OR 2·49, 95% CI 1·16–5·33). No differences in TNFA −308 genotype distribution was observed between patients with or without focal forms of the disease.

Comparison of HLA frequencies between brucella patients and controls

Table 2 shows phenotype frequencies for DRB1 and DQB1 in brucella patients and controls subjects. No statistically significant differences in the distribution of HLA-DRB1 or DQB1 alleles were observed between brucella patients overall and control subjects, or between patients with or without focal forms of the disease (data not shown).

Interaction between TNFA and HLA alleles

The existence of linkage disequilibrium between alleles at TNF and HLA loci is well known [11], therefore we examined the possibility that the TNF–brucellosis observed associations were primary, or secondary to HLA class II antigen associations. TNFA-308.2 allele was in linkage disequilibrium with HLA-DRB1*03 (OR = 7·03; $P = 0{\cdot}0007$) and, because of this association, stratification was necessary to rule out an eventual confounder effect of HLA-DR3 to the susceptibility to brucella infection. This method allows the division of the study population into HLA-DR3-negative and -positive individuals. Then, to estimate the total contribution of a risk factor in a stratified analysis, the Mantel–Haenszel procedure was used as described [12]. It resulted in a weighted Mantel–Haenszel odds ratio for TNFA-308.2 of 2·54 (95% CI 1·13–5·60; $P = 0{\cdot}02$) (Table 3). Likewise, the study population was divided into TNFA-308.2-negative and -positive individuals. This stratification indicated that HLA-DR3 was not individually associated with brucellosis (combined OR = 0·80; 95% CI 0·33–1·90) and that the observed disease associations with TNFA −308 genotypes appear to be independent of HLA class II antigens (Table 3).

DISCUSSION

We have found that TNFA −308 genotypes influence the susceptibility to brucellosis. The fact that after correction for the presence of HLA-DR3 the OR of the TNFA-308.2 allele was 2·54 ($P = 0{\cdot}01$) indicates that the TNFA-308.2 allele contributes independently to the susceptibility to brucellosis (Table 3). This study adds to a growing body of evidence showing that this polymorphism, directly or by linkage with other polymorphisms, is important in infectious diseases. Previous studies have linked TNFA-308.2 allele with cerebral malaria, mucocutaneous leishmaniasis, fatal meningococcal disease and lepromatous leprosy [6–9]. The fact that all these conditions are associated with high serum TNF-α levels, along with the evidence from transfection studies indicating that the TNFA-308.2 allele could act to increase constitutive and inducible levels of TNF-α [13], prompted the idea that this variation predisposes to overproduce TNF-α which,

Table 2. HLA-DRB1 and DQB1 phenotype frequencies in brucella patients and controls

	Controls		Brucella patients	
	$n = 160$	(%)	$n = 59$	(%)
HLA-DRB1				
01	37	(23)	14	(13)
15	35	(16)	14	(20)
16	8	(5)	4	(7)
03	32	(20)	12	(20)
04	42	(26)	10	(17)
11	38	(24)	8	(13)
12	2	(1)	1	(2)
13	35	(22)	18	(30)
14	6	(4)	1	(2)
07	47	(29)	19	(32)
08	6	(4)	7	(12)
09	4	(2)	4	(7)
10	11	(7)	2	(3)
HLA-DQB1				
02	75	(47)	26	(44)
0301	52	(32)	15	(25)
0302	33	(21)	6	(10)
0303	7	(4)	6	(10)
0304	1	(1)	1	(2)
0401	–	–	1	(2)
0402	7	(4)	7	(12)
0501	43	(27)	16	(27)
0502	7	(4)	5	(8)
0503	7	(4)	1	(2)
0601	7	(4)	2	(3)
0602	29	(18)	11	(19)
0603	20	(12)	11	(19)
0604	5	(3)	4	(7)

in turn, would favour the development of the above-mentioned conditions. However, other reporter gene studies have not been able to reproduce the effect of this polymorphism on up-regulation of TNF-α production [14,15]. In addition, Conway *et al*. [16] found that scarring trachoma was also associated both with TNFA-308.2 polymorphism and with elevated TNF-α levels in tear fluid; however, detection of TNF-α in tear fluid samples was not associated with particular TNFA promoter genotypes. Furthermore, it has been reported that the levels of TNF-α induced through stimulation of PBMC with *Mycobacterium leprae* were higher in patients with the paucibacillary tuberculoid form of the disease than in those patients with the multibacillary lepromatous form [17], in spite of the latter condition being associated with high circulating levels of TNF-α. In the same way, a lower capacity to secrete TNF-α upon *ex vivo* stimulation of whole blood with meningococcal endotoxin has been demonstrated among relatives of non-survivors from severe meningococcal disease than in relatives of survivors [18].

In spite of the known immunopathological aspects of TNF-α, high levels of this cytokine may reflect an advanced stage of disease, and the data suggest that other mechanisms in relation with TNF-α physiology may be acting on the pathogenesis of at least some complicated forms of infectious disease. In a murine model of *Brucella* infection it has been demonstrated that TNF-α plays an important role in enhancing IL-12 production *in vitro* and *in vivo*, which is the key cytokine controlling the induction of acquired cellular immunity against *Brucella* and other intracellular pathogens [4]. Interestingly, direct evidence has been provided that a secreted *Brucella* factor specifically inhibits TNF-α expression at the mRNA level on human, but not murine, macrophage-like cells [19]. Therefore, it is possible that individuals bearing TNFA-308.2 allele are more sensitive to down-regulation by such a factor, making them more susceptible to develop disease upon *Brucella* infection. Thus, it is tempting to speculate that the pathogenic mechanism underlying the development of complicated forms of infectious diseases may be more related to an inability to secrete suitable levels of TNF-α at some crucial points in the natural history of the infective process, allowing intracellular survival, multiplication and spreading of pathogens, than to an increased capacity to secrete high levels of TNF-α.

Individuals with different types of HLA might differ in their susceptibility or resistance to infectious pathogens [20]. Very little information is available on the importance of the HLA genes in brucellosis. Although increased frequencies of the HLA-DR4 allele have been reported in patients with brucellosis [21], we found no differences between patients and controls for this allele. This discordance may be due to the different typing techniques employed, since we used DNA typing in contrast to serological typing. Studies have been carried out to try to associate HLA antigens with focal forms of the disease, however, and in agreement with the results of the present study, no clear associations have been found [22,23].

Table 3. Odds ratios (OR) of the TNFA −308 and HLA-DR3 genotypes for susceptibility to human brucellosis

OR between genotypes	Subgroup	OR	95% CI
TNFA-308.1/2 and 2/2 *versus* -308.1/1	HLA-DR3-negative	1·96	0·71–5·34
	HLA-DR3-positive	4·20	0·85–23·1
	Combined (Mantel–Haenszel)	2·49	1·09–5·60
HLA-DR3/x *versus* -DRx/x	TNFA-308.2-negative	0·60	0·16–1·97
	TNFA-308.2-positive	1·20	0·30–4·89
	Combined (Mantel–Haenszel)	0·80	0·33–1·90

The Mantel–Haenszel odds ratio is the weighted mean of the OR of the risk factor in two or more strata. It is used to calculate the Mantel–Haenszel OR if no statistical interaction between the risk factors is present. The weighted Mantel–Haenszel OR for TNF-308.2 was of 2·49 (95% CI 1·09–5·60; $P = 0·02$) and for HLA-DR3 was 0·80 (0·33–1·90; $P =$ not significant).

In conclusion, our study suggests that the TNFA −308 genotypes may be involved in the susceptibility to brucellosis, although we realise that larger studies in different populations will be needed to confirm these observations.

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