Association between the Phe206Leu polymorphism of L-selectin and brucellosis

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Brucellosis remains a major zoonosis worldwide; therefore, better understanding of its immunology is a priority for the development of new therapeutic and vaccination strategies. Genetic factors appear to have an important role in the pathogenesis of infectious diseases such as brucellosis. Adhesion molecules, such as members of the selectin family, participate in the interaction between leukocytes and the endothelium, as well as in inflammatory cell recruitment. The impact of L-selectin polymorphisms on brucellosis has not so far been investigated. The aim of this study was to assess an L-selectin Phe206Leu (F206L) polymorphism in patients with active brucellosis, and to analyse its possible relationship with disease progression. A case-control association study was carried out on 619 subjects, including 374 patients with brucellosis and 245 age- and sex-matched healthy controls. Genomic DNA was isolated, and amplification of L-selectin genomic regions was performed by PCR incorporating sequence-specific primers (PCR-SSP) to distinguish the genotypes. The frequencies of the F206L polymorphism were studied. A significant difference in F206L polymorphism was found between patients with brucellosis and controls. The 206Leu allele was more frequent in patients than in healthy individuals (36.6 versus 28 %, $P=0.003$). In addition, there was an association between the presence of the 206Leu allele and a relapse of brucellosis (odds ratio 6.53, 95 % confidence interval 1.5–28.8, $P=0.005$). The higher frequency of L-selectin genotypes in patients with brucellosis than in control individuals, as well as the association between the 206Leu allele and the occurrence of brucellosis relapse, suggest that the F206L polymorphism could make individuals more vulnerable to brucellosis.

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INTRODUCTION

Brucellosis, which is caused by *Brucella* spp., is a zoonosis that is easily transmitted to humans. The disease occurs worldwide, particularly in the Mediterranean region, the Middle East, India, and Central and South America (Amato-Gauci, 1995). The disease tends to be chronic, and there are numerous cases of therapeutic failure, relapses and complications. Furthermore, it can present with heterogeneous responses among members of the same species, supporting the idea that individuals respond differently to *Brucella*

infection. In addition, despite environmental factors and the varying potency of different strains, it is generally accepted that host genetic factors are the major determinants of susceptibility and outcome of infectious diseases in humans (Murphy *et al.*, 2001). Candidate gene studies have implicated several immunogenetic polymorphisms in infectious diseases (Caballero *et al.*, 2000). There have been limited studies of the role of genetic background in mediating susceptibility or resistance to brucellosis (Caballero *et al.*, 2000; Bravo *et al.*, 2003).

Immune surveillance of the human body is predominantly carried out by leukocytes, which undergo transendothelial migration (TEM) to exit the bloodstream and enter inflammatory sites. The primary stages of TEM involve a group of cell adhesion molecules known as selectins. L-selectin, a

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Abbreviations: CI, confidence interval; EGF, epidermal growth factor; OR, odds ratio; PCR-SSP, PCR incorporating the sequence-specific primer.

member of the selectin family, is a cell adhesion molecule constitutively expressed on peripheral lymphocytes, monocytes and neutrophils (Carlos & Harlan, 1994). It plays a key role in the initiation of leukocyte migration from vessels into secondary lymphoid tissues or different areas of local inflammation (Ley & Kansas, 2004; Butcher & Picker, 1996; Nicholson *et al.*, 1998). Also, the binding of L-selectin to its ligand on the surfaces of other leukocytes mediates leukocyte attachment to already adherent cells, hence increasing the overall leukocyte recruitment to inflammatory sites (Walcheck *et al.*, 1996; Eriksson *et al.*, 2001; Guyer *et al.*, 1996).

L-selectin has an extracellular calcium-dependent lectin domain on the amino-terminus, followed by an epidermal growth factor (EGF)-like domain, a short consensus repeat (SCR) domain consisting of two SCRs, a membrane-spanning region, and a cytoplasmic tail (Kansas, 1996; Vestweber & Blanks, 1999). The selectins mediate cell binding through interactions between their lectin-like domain and cell surface carbohydrate ligands (McEver *et al.*, 1995; Springer & Lasky, 1991). There are two known single-base polymorphisms in the L-selectin gene in humans, resulting in amino acid exchange from threonine to serine in the lectin domain at position 49 (Tre49Ser), and from phenylalanine to leucine in the EGF domain at position 206 (PheF206Leu, F206L) (Tedder *et al.*, 1989). F206L has been reported to be associated with chronic renal allograft failure (McLaren *et al.*, 1999) and atherosclerosis (Blankenberg *et al.*, 2003). A study of F206L polymorphisms in the L-selectin gene was of particular interest, as the presence of large numbers of leukocytes is important in inflammation, L-selectin is the first protein to adhere to the endothelial cells, and the occurrence of polymorphism in the EGF domain can alter ligand interactions (Jutila *et al.*, 1992). Therefore, it was hypothesized that these associations reflect genetic variability in L-selectin expression, which influences the clinical outcome of infectious diseases. Since no study has yet been undertaken on the possible association between the genotypes of this adhesion molecule and human brucellosis, we assessed the contribution of L-selectin polymorphisms in susceptibility to brucellosis. The results clearly showed that there are differences in allele and genotype frequencies between patients with active brucellosis and healthy individuals.

METHODS

Patients and controls. A total 374 patients with brucellosis, 202 (54%) males and 172 (46%) females, were recruited for the study. A control group of 245 healthy volunteers, matched for age, sex and geographical area, was chosen. The control group had the same epidemiological conditions as patients, but were negative in *Brucella*-specific tests. All subjects were of Iranian Caucasian origin. Diagnosis of brucellosis was established by isolation of *Brucella* in blood or bone-marrow cultures. In negative cultures, diagnosis was based on compatible epidemiological and clinical manifestations, together with the presence of high titres of specific antibodies or a fourfold or greater increase of the initial titres in two paired samples taken 2–4 weeks apart. High titres were considered to be a standard tube agglutination (STA) of $\geq 1/160$ or a Coombs anti-brucella test of

$\geq 1/320$ confirmed by a 2-mercaptoethanol (2-ME) test titre of $\geq 1/160$. Upon diagnosis, all the patients were placed on the same treatment regimen, consisting of doxycycline for a period of 6 weeks, in combination with streptomycin for 2 weeks followed by rifampicin for 4 weeks. The study was approved by the Medical Ethics Committee of Mazandaran University of Medical Sciences and informed consent was obtained from all subjects.

Determination of L-selectin genotypes. Venous blood from each subject was collected in tubes containing 50 mmol EDTA l^{-1} . Genomic DNA was isolated from anti-coagulated peripheral blood buffy coat samples using Miller's salting-out method (Miller *et al.*, 1988), and was stored at $-70^{\circ}C$ until analysis. A PCR incorporating the sequence-specific primer (PCR-SSP) method was used to detect substitutions responsible for L-selectin polymorphism. The primer pairs for delineating the polymorphism at nucleotide +668 were 5'-ATGGGCCCCAGTGTCTAGT-3' (T allele specific), 5'-ATGGGCCCCAGTGTCTAGC-3' (C allele specific) and 5'-CAAGCTCATTATGTCGTGAGC-3' (generic primer). Internal control primer 1 (HGH forward), 5'-GCCTTCCCAACCATTCCCTT-3', and internal control primer 2 (HGH reverse), 5'-TCACGGATTTCTGTTGTGTTC-3', were used. The internal control primers were used in all reactions to amplify a 927 bp segment of the human growth hormone gene to check for successful PCR amplification. Amplification was carried out using a PCR Techne Flexigene apparatus (Roche) in a total volume of 50 μl containing 0.2 ng genomic DNA, 10 pmol allele-specific primers and 10 pmol common primers, 200 mM each dNTP, 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase. PCR performed without DNA template represented the negative controls. The reaction was carried out as follows: initial denaturation at $94^{\circ}C$ for 2 min, followed by 30 cycles of amplification at $94^{\circ}C$ for 15 s, annealing at $60^{\circ}C$ for 45 s, and extension at $72^{\circ}C$ for 45 s; with final extension for 2 min at $72^{\circ}C$. The PCR reactions were performed in triplicate for every subject. In addition, the PCR reaction was repeated on 30% of the subjects to verify the accuracy of the procedure. The amplified PCR products were analysed by 2% agarose gel electrophoresis followed by ethidium bromide staining and ultraviolet visualization.

Statistical analysis. Statistical analyses were performed using SPSS statistical software (version 11.0, 2003; SPSS). Allelic and genotypic frequencies were obtained by direct counting. The Hardy-Weinberg equilibrium was tested by using a chi square goodness of fit test. The frequencies of alleles and genotypes were compared by the Fisher exact and chi square tests, respectively. The correlated P (P_c) value was calculated as P multiplied by the number of comparisons. The odds ratio (OR) and 95% confidence interval (CI) for the occurrence of relapse were computed from a logistic regression model. All two-tailed P values of less than 0.05 were considered to be statistically significant.

RESULTS

Characteristics of patients

The mean age was 38.17 ± 19.97 and 42.67 ± 22.38 years in the patients and healthy individuals, respectively. *Brucella* species were isolated from blood cultures in 127 cases (34%). All *Brucella* species were identified as *Brucella melitensis*. Most of the patients described a gradual onset of the disease. The time of onset of symptoms until the patients were admitted ranged from 2 weeks to 3 months. Fever, sweats, myalgia, arthralgia, loss of appetite and back pain were common in most of the patients. Other symptoms at presentation are listed in Table 1.

Table 1. Frequency distribution of the main complaints of the 374 patients with brucellosis

Complaint	No. of patients	
	<i>n</i>	Percentage of total
Fever	258	69.0
Sweating	254	67.9
Arthralgia	242	64.7
Headache	197	52.7
Myalgia	200	53.5
Anorexia	256	69.4
Back pain	227	60.7
Weight loss	139	37.2
Relapse	18	4.8
Depression	100	26.7
Delirium	29	7.8
Hepatomegaly	11	2.9
Osteoarthritis	11	2.9
Haematology*	10	2.7

*Anaemia and monocytosis were the most common haematological manifestations.

A presumed source of the *Brucella* infections was identified in 258 (69 %) patients, including ingestion of unpasteurized dairy products in 151 (58.5 %), animal husbandry in 87 (33.7 %), and the laboratory and veterinary professions in 20 (7.8 %) patients. In the remaining 116 cases (31 %), no epidemiological source could be traced. Eighteen patients experienced a relapse of the disease 3–6 months after treatment.

Genotype distribution in brucellosis patients and healthy subjects

The F206L mutation of L-selectin was detected using a PCR-SSP analysis that revealed a substitution of thymine to cytosine at position 668 (T668C) within the EGF domain in

the L-selectin gene. In the patients and control subjects, the L-selectin allele and genotype frequencies were consistent with the Hardy–Weinberg equilibrium (data not shown).

The allelic and genotypic frequencies in brucellosis patients were shown to be significantly different from those of control subjects. The allele frequencies and genotype distribution of the L-selectin F206L polymorphism in patients with brucellosis and healthy subjects are given in Table 2. An increased frequency of the L-selectin 206Leu allele was observed in brucellosis (36.6 versus 28 %, $P=0.003$). In addition, patients with brucellosis were categorized into two groups of ≤ 50 and > 50 years old. When the overall L-selectin 206Leu allele distribution of the brucellosis patients ≤ 50 years old was compared with that of the patients > 50 years old, a significant skewing was found (47 versus 21 %; OR 2.63, 95 % CI 1.72–4.03, $P<0.0001$) (Table 3). Interestingly, homozygous 206Leu/Leu was found only in patients of ≤ 50 years old.

L-selectin 206Leu allele influences brucellosis severity

An effort was made to determine the clinical relevance of the L-selectin F206L polymorphism with respect to disease severity in patients with brucellosis. In order to investigate the influence of the L-selectin F206L polymorphism on the development of brucellosis, allele frequencies between patients with and without relapse were compared. Of the 374 patients, 18 (4.8 %) experienced a relapse of brucellosis between 3 and 9 months after treatment. Since the majority of the patients with relapse were homozygous or heterozygous for leucine, further analysis was undertaken. At first, both groups were compared based on minor alleles. As shown in Table 4, the Phe/Leu+Leu/Leu genotype was more frequent in the patients with brucellosis than in healthy individuals (56.7 versus 43.7 %, $P=0.002$). Subsequently, comparison between genotypes of the patients with and without relapse showed that carriers of the 206Leu allele were more frequently found in the relapsing group

Table 2. L-selectin F206L polymorphism in brucellosis patients and healthy subjects

L-selectin alleles and genotypes were determined in patients and controls.

L-selectin F206L polymorphism	<i>n</i> /total (%) for:		<i>P</i> value
	Brucellosis, <i>n</i> = 374	Controls, <i>n</i> = 245	
Allele			
Phenylalanine (F)	474/748 (63·4)	353/490 (72)	<i>P</i> = 0·202*
Leucine (L)	274/748 (36·6)	137/490 (28)	<i>P</i> = 0·003†
Genotype			
Major homozygotes (F/F)	162/374 (43·3)	138/245 (56·3)	<i>P</i> = 0·006
Heterozygotes (F/L)	150/374 (40·1)	77/245 (31·4)	
Minor homozygotes (L/L)	62/374 (16·6)	30/245 (12·2)	

*Versus controls, $P_c=0.404$ ($P=0.202$).

†Versus controls, $P_c=0.006$ ($P=0.003$).

Table 3. Comparison of allele and genotype frequencies in patients with brucellosis based on age

L-selectin allele and genotype frequencies were determined in patients either ≤ 50 or > 50 years old.

Allele or genotype	Patients with brucellosis, n/total (%)		P value
	≤ 50 years	> 50 years	
Allele			
Phe (F)	240/452 (53)	235/298 (79)	$P < 0.0001^*$
Leu (L)	212/452 (47)	63/298 (21)	$P < 0.0001^\dagger$
Genotype			
Phe/Phe	79/226 (33.6)	86/149 (57.7)	
Phe/Leu	88/226 (38.9)	63/149 (42.3)	
Leu/Leu	62/226 (27.4)	0	$P < 0.0001$

* ≤ 50 years versus > 50 years, $P_c < 0.0002$ ($P < 0.0001$).

$^\dagger \leq 50$ years versus > 50 years, $P_c < 0.0002$ ($P < 0.0001$).

Table 4. Allele carrier frequencies between patients with brucellosis and controls

Values are the numbers of patients or controls carrying each allele or genotype. Percentage values are shown in parentheses. The P values were calculated by the chi square test from a 2×2 contingency table.

Genotype, L-selectin F206L allele carriage	n/total (%)		P value
	Brucellosis, $n = 374$	Controls, $n = 245$	
L206L + F206L carriers	212/374 (56.7)	107/245 (43.7)	$P = 0.002$
F206F carriers	162/374 (43.3)	138/245 (56.3)	

than in the patients without relapse ($P = 0.006$) (Table 5). There was a strong association between the presence of the 206Leu allele and a relapse of brucellosis (OR 6.53, 95% CI 1.5–28.8, $P = 0.005$).

DISCUSSION

The distribution of polymorphisms in the gene encoding L-selectin was investigated in a large group of Iranian patients with brucellosis and in a control group of ethnically

matched healthy individuals. This is the first study to implicate L-selectin gene polymorphism as a genetic risk factor for brucellosis. A significantly higher frequency of L-selectin F206L polymorphism was found in patients with brucellosis in comparison to healthy controls. Further analysis revealed an association of the 206Leu allele with the occurrence of relapse in brucellosis. This suggests that the L-selectin F206L polymorphism influences susceptibility to brucellosis, and that the 206Leu allele could be a genetic risk factor for the disease.

Table 5. L-selectin F206L polymorphism in brucellosis patients with or without relapse

L-selectin allele and genotype frequencies were determined in patients with and without relapse.

L-selectin F206L polymorphism	Brucellosis patients, n/total (%)		P value
	With relapse	Without relapse	
Allele			
Phenylalanine (F)	36/748 (4.8)	712/748 (95.2)	
Leucine (L)	12/36 (33.3)	465/712 (65.3)	$P = 0.004^*$
	24/36 (66.7)	247/712 (34.7)	$P = 0.003^\dagger$
Genotype			
F206F	18/374 (4.8)	356/374 (95.2)	
F206L + L206L	2/18 (11.1)	160/356 (44.9)	
	16/18 (88.9)	196/356 (55.1)	$P = 0.006$

*With relapses versus without relapses, $P_c = 0.008$ ($P = 0.004$).

† With relapses versus without relapses, $P_c = 0.006$ ($P = 0.003$).

Based on an Iranian Caucasian population, the L-selectin 206Leu allele was more frequent in patients than in healthy controls. These findings are consistent with the role of L-selectin in the development of chronic inflammation, reflecting the potential impact of an individual's genetic make-up on the risk of developing chronic infectious diseases.

L-selectin is believed to have a role in attracting leukocytes to inflammatory sites, in the activation of leukocytes, and in interactions between leukocytes and endothelial cells (Patel *et al.*, 2002; Rosen, 1999). Although the principal ligand contact points of the selectins lie within the lectin domain (Somers *et al.*, 2000), the EGF domain can modulate the binding properties of the lectin domain (Dwir *et al.*, 2000). Therefore, substitution of a phenylalanine with a leucine at residue 206 may influence L-selectin function either directly, by binding a novel ligand, or indirectly by inducing a conformation change in the lectin-EGF domains. Therefore, it may be speculated that the pathogenic mechanisms underlying the development of complicated forms of infectious diseases may be related to an inability to express suitable ligand-binding forms of the adhesion molecules, including L-selectin. The amino acid change is significant and would be expected to affect leukocyte-endothelial and leukocyte-leukocyte interactions, but more studies are required to prove this hypothesis.

L-selectin mediates fast interactions that generally represent the initial event in adhesion cascades (Alon *et al.*, 1997). Recent studies have addressed the significant association between the F206L polymorphism and certain disorders, such as IgA nephropathy (Takei *et al.*, 2002) and insulin-dependent diabetes mellitus (IDDM) (Kretowski & Kinalska, 2002). On the other hand, the strength of the binding of L-selectin to its ligands on other cells, particularly endothelial cells, may be influenced by the 206Leu allele, as the EGF domain is important in maintaining the secondary structure of selectins and, accordingly, plays a significant role in cell-cell interactions (Revelle *et al.*, 1996). The mechanistic relationship of the association between L-selectin F206L polymorphism and brucellosis may relate to the recognition and activation roles of this molecule in cell-cell interactions. It would be interesting to know whether the L-selectin polymorphism affects these physiological functions differentially, and whether these functions have a role in the relationship between the L-selectin F206L polymorphism and brucellosis.

It might be assumed that an increased frequency of the 206Leu allele in patients with relapse results in inappropriate activation of *Brucella*-infected cells. It has been shown that L-selectin can act as a signalling molecule and induce tyrosine phosphorylation (Waddell *et al.*, 1995), as well as activating mitogen-activated protein kinases. In phagocytes, activation of L-selectin by antibody cross-linking induces a variety of responses, including calcium flux (Laudanna *et al.*, 1994), upregulation of surface-expressed Mac-1 (Crockett-Torabi *et al.*, 1995; Smolen *et al.*, 2000), and activation of the respiratory burst (Crockett-Torabi *et al.*, 1995). Therefore,

inability of infected phagocytes to eradicate *Brucella* in patients who carry the 206Leu allele might be explained by inappropriate signalling in the 206Leu variant. Another hypothesis could be that the abnormal binding function of the L-selectin 206Leu variant on lymphocytes could lead to impaired migration to sites of *Brucella* colonization. This hypothesis is supported by studies that show that memory T cells reexpress L-selectin after antigen encounter and home to sites of chronic inflammation (Ley & Kansas, 2004). Most of our patients who showed a relapse had at least a 206Leu dominant allele.

In summary, this is the first report of a direct association between L-selectin gene polymorphism and a complex age-dependent brucellosis, which suggests that this molecule plays different roles in the *Brucella* infection process. It is postulated that L-selectin might be involved in the severity of brucellosis and its complications. This concept is supported by the observation that the majority of our patients with relapse were homozygous or heterozygous for the Leu206 allele of L-selectin. Taking into account the polymorphism only, the 206Leu allele was associated with a 6.53-fold increased risk of relapse.

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