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FULL PAPER

Interleukin-1β polymorphisms in Colombian patients with autoimmune rheumatic diseases

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Interleukin-1 beta (IL-1β) exerts a range of inflammatory and immunomodulatory activities that are important in host defense and autoimmune response. The IL-1β gene, located on chromosome 2 (2q13), is polymorphic. The influence of its polymorphism on 355 patients with autoimmune rheumatic diseases was examined. To this effect, 172 patients with rheumatoid arthritis (RA), 114 with systemic lupus erythematosus (SLE), and 69 with primary Sjögren's syndrome (pSS) were studied. The control group consisted of 392 matched healthy individuals. Genotyping of IL-1β single-nucleotide polymorphisms (SNPs) at positions −511 (C/T) and +3953 (C/T) was performed by the polymerase chain reaction-restriction fragment length polymorphism technique. In addition, levels of IL-1β were measured by immunoassay in supernatants of lipopolysaccharide (LPS)-stimulated and nonstimulated peripheral blood monocytes (PBM) obtained from 19 homozygous individuals for the three most common IL-1β likely haplotypes, all belonging to the control group. Allele+3953T was protective for SLE (odds ratio (OR)=0.57, 95% confidence intervals (CI)=0.34–0.88, P=0.01) as was the haplotype −511C+3953T (OR=0.43, 95%CI=0.25–0.74, pc=0.006). The latter was associated with a lower LPS-stimulated-PBM IL-1β secretion. Results suggest that IL-1β polymorphism influences the susceptibility to acquire SLE in our population. The protective association might be explained by the observed inhibitory effect of IL-1β +3953T allele on the secretion of IL-1β under inflammatory circumstances.
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Introduction

Interleukin (IL)-1β, a proinflammatory pleiotropic cytokine, is a member of IL-1 family that possess the ability to stimulate the expression of genes associated with inflammation and immune response, including cyclooxygenase type 2, type 2 phospholipase A, and inducible nitric oxide synthase.[1] Additionally, another important proinflammatory property of IL-1β is its capacity to increase the expression on endothelial and other cell surfaces of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1).[1] IL-1α and IL-1β bind to the IL-1 receptor type I, eliciting signal transduction and the corresponding biological effects. Conversely, the IL-1 receptor antagonist (IL-1Ra) is a competitive inhibitor that does not initiate intracellular signaling when bound to the same receptor.[2]

The IL-1 gene cluster consists of 430-kb region with three genes encoding for IL-1α, IL-1β, and IL-1Ra; it is located on the long arm of human chromosome 2, at 2q13.[3] At least three single-nucleotide polymorphisms (SNPs) in the IL-1β gene have been reported, all representing a C/T base transition at positions −511[4] and −31[5] in the promoter, and at +3953 in the exon 5.[6]

IL-1β plays a key role in the pathogenesis of inflammatory and autoimmune diseases.[1,2,7] Rheumatoid arthritis (RA) is an autoimmune systemic disease characterized by persistent inflammation of the diartrodial joints with synovial hyperplasia and progressive joint destruction. IL-1β triggers the expression of metalloproteinase genes such as collagenases and elastase, adhesion molecules, and several proinflammatory mediators involved in joint destruction.[7] Injection of IL-1 into knee joints of rabbits induces leukocyte infiltration and produces cartilage degradation.[8] Antibodies against IL-1 ameliorate collagen-induced arthritis in another RA animal model.[9] IL-1β serum and synovial concentrations are higher in RA patients with active disease than in those in remission.[10] Furthermore, IL-1Ra has been successfully used in the treatment of RA patients.[11]

Primary Sjögren's syndrome (pSS), another autoimmune disease, mainly affects exocrine glands and is characterized by glandular lymphocytic infiltrate and the presence of anti-Ro/SSA and anti-La/SSB antibodies associated with glandular dysfunction. Several animal models suggest that elevated glandular levels of IL-1β may impair the lachrymal gland secretion.[12,13] Both infiltrating mononuclear cells and glandular epithelial cells from patients with pSS express high levels of

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mRNA for IL-1β.[14] Likewise, IL-1β mRNA has been detected in significant concentrations in lung lesions and peripheral blood mononuclear cells (PBMC) from these patients.[15] In addition, the number of IL-1β-secreting PBMC is significantly higher in pSS patients (mainly in those with recurrent parotid swelling) than in healthy controls.[16]

Systemic lupus erythematosus (SLE) is characterized by the production of pathogenic autoantibodies, B-cell hyperactivation, and defective clearance of immune complexes, affecting several organs. Although the role of IL-1β in SLE has not been fully examined, there is evidence suggesting that IL-1 is crucial for the synthesis of IgG autoantibodies in these patients.[17] IL-1β secretion by PBMC is higher in patients with SLE than in healthy controls.[18] Lower levels of IL-1Ra have also been detected in lupus nephritis thus adding further evidence of the IL-1β cascade involvement in the pathogenesis of SLE.[19]

Given that IL-1β plays an important role in autoimmune rheumatic diseases as summarized, and that previous studies have evaluated a susceptibility role for IL-1 locus in autoimmune rheumatic diseases,[20–31] the present study aimed at evaluating simultaneously IL-1β −511, and +3953 SNPs in SLE, RA, and pSS patients, all belonging to the same ethnic group, primarily of Spanish ancestry with no significant admixture with Amerindian or Black populations.[32] In addition, functional analysis were performed in healthy homozygous subjects in order to evaluate the influence of IL-1β haplotypes on the *in vitro* protein secretion.

Results

Allelic and genotype frequencies corresponding to *IL-1β* −511 and +3953 SNPs are shown in Table 1. Allele +3953T was protective for SLE (Table 1). There were no significant differences concerning genotype frequencies between patients and controls. There was linkage disequilibrium (LD) between *IL-1β* −511 and +3953 SNPs in both the control group and RA patients. Hardy–Weinberg equilibrium was observed for −511 SNP in all groups. For +3953, we observed an excess of homozygous in both the control and pSS groups. The −511T + 3953C was the commonest haplotype in the control group (42%), followed by −511C + 3953C (37%), −511C + 3953T (15%), and −511T + 3953T (5%). Haplotype −511C + 3953T was protective for SLE (7 *vs* 15%, odds ratio (OR) = 0.43, 95% confidence intervals (CI) = 0.25–0.74, $P = 0.0016$, pc = 0.006).

Kinetics of the *in vitro* IL-1β secretion

Under steady conditions (without inflammatory stimulus), two peaks of IL-1β secretion, at 12 and 48 h were observed, whereas under lipopolysaccharide (LPS) stimulus a single peak of IL-1β was present after 24 h (Figure 1).

The levels of IL-1β during the 96 h culture (area under the curve) in function of the most common haplotypes are shown in Table 2. Haplotype TC was found to be associated with a mild response ($\Delta = 31 \pm 43\%$), whereas CC was associated with a significant response to LPS ($\Delta = 19 \pm 35\%$). The carriers of CT haplotype were considered as nonresponders because the induced IL-1β secretion rate was even lower to that present at basal conditions ($\Delta = -23 \pm 26\%$) (Figure 1 and Table 2).

Influence of *IL-1β* polymorphism on clinical variables

There were no significant differences on genotypes or alleles in function of sex. Influence of *IL-1β* polymorphisms on clinical and immunologic characteristics of patients was not found (data not shown).

Table 1 *IL-1β* −511 +3953 SNP allele and genotype frequencies

Allele	*RA* $n = 344$ (%)	*SLE* $n = 228$ (%)	*PSS* $n = 134$ (%)	*Healthy controls* $n = 768$ (%)
−511				
C	165 (48)	114 (50)	74 (55)	404 (53)
T	179 (52)	114 (50)	60 (45)	364 (47)
+3953				
C	289 (84)	201 (88)*	109 (81)	622 (81)
T	55 (16)	27 (12)**	25 (19)	146 (19)
Genotype	$n = 172$ (%)	$n = 114$ (%)	$n = 69$ (%)	$n = 392$ (%)
−511				
CC	36 (21)	29 (25)	17 (25)	110 (28)
CT	95 (55)	59 (52)	43 (62)	192 (49)
TT	41 (24)	26 (23)	9 (13)	90 (23)
+3953				
CC	122 (71)	89 (78)***	49 (71)	262 (67)
CT	44 (26)	23 (20)	13 (19)	106 (27)
TT	6 (3)	2 (2)	7 (10)	24 (6)

*OR = 1.7, 95% CI = 1.13–2.7, $P = 0.01$.
**OR = 0.57, 95% CI = 0.34–0.88, $P = 0.01$.
***OR = 1.8, 95% CI = 1.1–2.9, $P = 0.03$, pc = 0.06.

Discussion

In the present study, we examined simultaneously IL-1β polymorphisms in Colombian patients with autoimmune rheumatic diseases, as well as in clinically healthy individuals. In our study, we noted that *IL-1β* polymorphism influences the susceptibility to acquire SLE.

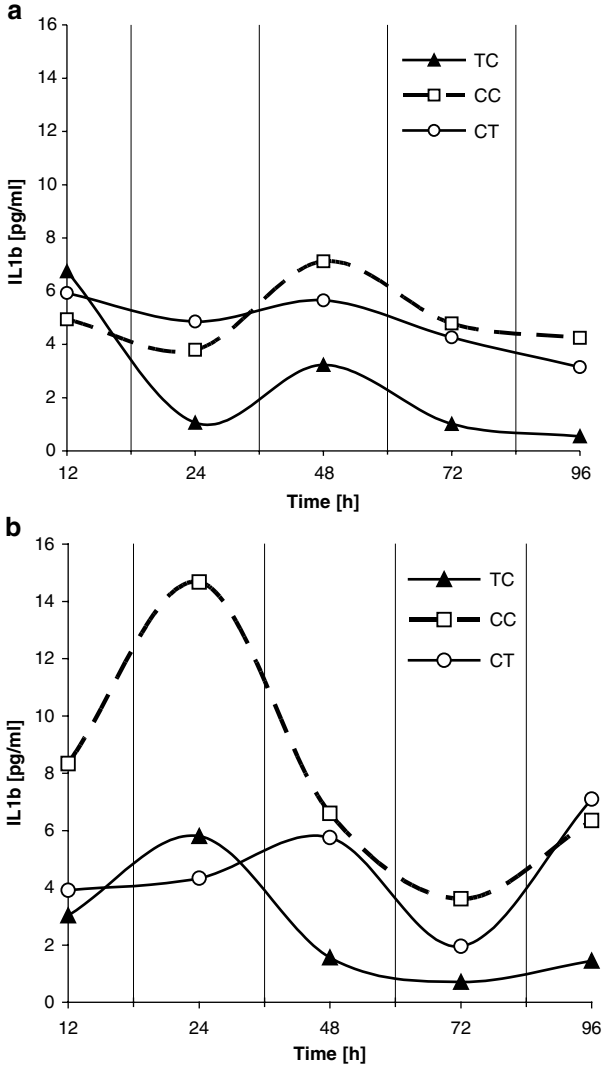

Figure 1 Levels of IL-1β secreted by peripheral blood monocytes in homozygous healthy individuals for −511 (C/T) and +3953 (C/T) haplotypes. (**a**) IL-1β levels under steady conditions time plot. (**b**) IL-1β levels under LPS stimulus time plot.

The protective role of +3953T allele and −511C+3953T haplotype on SLE susceptibility might be explained by an inhibitory *in vitro* effect of IL-1β +3953T allele on the secretion of IL-1β under inflammatory circumstances.

Evidence indicating a linkage of the IL-1 cluster genes to RA and a possible role for this region in erosive disease was reported by Cox *et al*,[20] who examined 195 nuclear families with RA. However, association studies of IL-1β polymorphism in RA are controversial. While some studies have found that +3953T SNP is associated with severe RA in French, Chinese, and Sweden patients,[21–24] others have failed to confirm this association in RA patients from Netherlands[25] and also from China.[26] Genevay *et al*[27] observed that IL-1β −511T was associated with a milder erosive disease in Swiss patients. Our study fails to observe any influence of IL-1β −511 and +3953 SNPs on RA susceptibility.

Till date, a few studies of IL-1β polymorphism in patients with SLE and pSS are available. Huang *et al*[28] observed no association between IL-1β polymorphisms and SLE in Chinese patients. On the contrary, the IL-1β −511 CT genotype and −511T allele were shown to be associated with SLE in African Americans but not in Whites from the Southeastern US.[29] IL-1β +3953 polymorphism was not associated with pSS in Finnish patients.[30] In Japanese, genotypes −511 CC and −31 TT were significantly less frequent in pSS patients than in healthy controls and SLE patients.[31] In our population, we observed a protective role of −511C+3953T haplotype on the susceptibility to SLE.

The diverse results summarized above may be due to differences in the origin of the studied populations, LD with other IL-1 cluster genes, or insufficient sample size. On this regard, our results are twice pertinent. Firstly, this is the only report of IL-1β polymorphism in a large Colombian population. Secondly, although the association between *IL-1β* polymorphism and autoimmune diseases has been previously examined, our results show for the first time the influence of *IL-1β* on SLE.

The protective role of +3953T allele on SLE could be explained to some extent by the influence of this allele on IL-1β secretion. We explored the effect of the most common haplotypes on the *in vitro* cytokine secretion. Our results are somehow different from those previously reported (Table 3).[6,16,21,33–37] As stated by Hall *et al*[37] differences in assay methods, population origin and their health status, reagents, sample size, and monocyte numbers might all contribute to the differences observed among studies (Table 3). Our functional study was carried out in healthy homozygous individuals, who had not suffered from infection disease and were free of medication.

Table 2 Area under the curve for IL-1β peripheral blood monocytes secretion in function of the most common haplotypes found in homozygous healthy individuals

Haplotype	*Steady state (pg/ml/96 h)*	*LPS stimulation (pg/ml/96 h)*	*Δ %*[a]	*p1*[b]	*p2*
−511T+3953C ($n=7$)	149.2±224.5	168.2±171.7	31±43	0.03	0.6
−511C+3953C ($n=9$)	326.1±192.8	516.1±386.2	19±35	0.01	0.16
−511C+3953T ($n=3$)	309.6±122.4	265.4±136.5	−23±26	0.17	0.25

[a]The ratio between nonstimulated and stimulated PBM IL-1β secretion was defined as delta (Δ) and given in percentages.
[b]By Wilcoxon signed-ranks test. p1 = one-tailed, p2 = two-tailed.

Table 3 Influence of *IL-1β* polymorphisms on IL-1β levels

Author, year (ref.)	*SNP or haplotype*	*Effect on IL-1β levels*	*Methods*	*Observations*
Pociot *et al*, 1992[6]	+3953 TT > TC > CC	Enhances production	Monocyte culture	IDDM patients. LPS-induced protein secretion
Santtila *et al*, 1998[33]	−511T+3953C	Slightly higher	Serum levels	Finnish population
Dorffel *et al*, 1999[34]	None[a]	Significantly increased	Monocyte culture	Hypertensive patients *vs* healthy individuals
Hefler *et al*, 2001[35]	+3953C/T	None	Serum levels	Idiopathic recurrent miscarriage. Case–control study
Buchs *et al*, 2001[21]	−511+3953	None	Serum levels	French patients with RA
Dominici *et al*, 2002[36]	+3953C/T	None	Monocyte culture	Healthy control samples. LPS-induced protein secretion
Willeke *et al*, 2003[16]	None[a]	Systemic increased	ELISPOT	pSS patients
Hall *et al*, 2004[37]	−511T−31C	2–3-fold increased	Whole-blood *ex vivo* assay	LPS/ATP-induced protein secretion. No-effect of +3953 SNP. RA patients and drug trial volunteers
Present study, 2004	−511C+3953T	Decreased levels	Monocyte culture	Colombian homozygous healthy individuals

IDDM: insulin-dependent diabetes mellitus, LPS: lipopolysaccharide, RA: rheumatoid arthritis, pSS: primary Sjögren's syndrome.
[a]Although these studies did not investigate for *IL-1β* polymorphism, they are cited because the levels of cytokine were analyzed in pathology.

We did not observe a significant influence of *IL-1β* polymorphism on the immune response or on the course of the diseases studied, although there was an inherent loss of statistical power in these subgroup analyses. Autoimmune diseases are observed in genetically susceptible individuals in whom clinical expression is modified by permissive and protective environments occurring over time. From a genetic point of view, these are complex diseases, meaning that their inheritance does not follow a single-gene dominant or single-gene recessive Mendelian law, and thus they are polygenic. Autoimmune phenotype varies among populations. Since the effects of mutations (genotype) on phenotype in a particular population may vary depending upon environment and the length of its exposure, there is a need to explore genetic associations in diverse populations.

In conclusion, polymorphism and function/tissue expression make *IL-1β* gene a candidate that confers protection to SLE in our population.

Patients and methods

Patients and controls

In this study, we analyzed 355 consecutive patients with autoimmune rheumatic diseases. Patients were seen by the same team in the Rheumatology Unit at the Clínica Universitaria Bolivariana, in Medellin, Colombia, and their clinical and immunological characteristics were systematically recorded using standard protocols as previously reported.[38–40] There were 153 women and 19 men with RA,[41] their mean age ± standard deviation was 48.5 ± 14.2 years, the mean duration of disease was 11 ± 8.1 years, and 80% tested positive for rheumatoid factor. Extra-articular manifestations were registered in 35% of cases, and comorbidity in 70%. There were 110 women and four men with SLE,[42] their mean age was 34 ± 11.9 years, the mean duration of disease was 15.5 ± 13 years, and antinuclear antibodies and anti-DNA antibodies were positive in 98 and 74%, respectively. Musculoskeletal manifestations were recorded in 94% of cases, mucocutaneous involvement in 88%, renal involvement in 40%, neurological involvement in 42%, cardiopulmonary involvement in 30%, and hematological involvement in 74%. A total of 69 women patients with primary SS were examined,[43] their mean age was 52.5 ± 13.5 years, and the mean duration of pSS was 6.4 ± 5.2 years. During the course of the disease, all patients presented with exocrine involvement, 63% had vascular-inflammatory involvement, and 16% immune-mediated manifestations. Anti-Ro and anti-La antibodies were detected in 74 and 32% of pSS patients, respectively.

The control group was composed of 392 healthy individuals without history of chronic inflammatory, autoimmune or infectious disease; they were matched to patients' group by gender, ethnicity, and socioeconomic status, and were unrelated to patients. Their mean age was 47 ± 9 years, and 90% were female. This research was conducted in compliance to Resolution No 008430 of 1993 from the Ministry of Health of the Republic of Colombia, and was classified as research with minimal risk. The local Ethics Committee approved the present study.

Genotyping for IL-1β polymorphisms

Genomic DNA was extracted from a 10 ml EDTA-anticoagulated blood sample using the standard salting-out technique. Genotyping of −511 SNP was performed by the polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) technique as described by Buchs *et al*.[21] A 304 bp fragment was amplified using a forward primer 5′-TGG CAT TGA TCT GGT TCA TC-3′ and a reverse primer 5′-GTT TAG GAA TCT TCC CAC TT-3′ (University of Texas Health Science Center—UTHSC—core facility, San Antonio, TX, USA). PCR conditions were as follows: (95°C, 1 min) × 1; (95°C, 1 min; 50°C, 1 min; 72°C, 1 min) × 35 cycles; (72°C, 5 min) × 1. PCR products were digested by incubation with *Ava*I and *Bsu*361 (Promega, Madison, WI, USA) at 37°C for 4 h. The restriction fragments were analyzed on a 2% agarose gel and stained with ethidium bromide. *Ava*I products yielded 190 and 114 bp fragments when allele 1 (C) was present, whereas for allele 2 (T) a single

304 bp product was observed. Conversely, *Bsu*361 digestion produced 190 and 114 bp bands for allele 2 and a 304 bp band (uncut product) for allele 1.

For IL-1β +3953, a 250 bp fragment was amplified using a forward primer 5′-GTT GTC ATC AGA CTT TGA CC-3′ and a reverse primer 5′-TTC AGT TCA TAT GGA CCA GA-3′ (UTHSC core facility, San Antonio, TX, USA). PCR conditions were as follows: (95°C, 2 min) × 1; (95°C, 1 min; 60°C, 1 min; 72°C, 1 min) × 35 cycles; (72°C, 5 min) × 1. PCR products were digested by incubation with *Taq*I (Promega, Madison, WI, USA) at 65°C for 4 h. The restriction fragments were analyzed on a 3% agarose gel and stained with ethidium bromide. *Taq*I products yielded 39, 97, and 114 bp fragments when allele 1 (C) was present, whereas for allele 2 (T) 39 and 211 bp products were observed.

Human PBM isolation and stimulation

From the three most common IL-1β haplotypes, 19 homozygous individuals were selected, as follows, IL-1β −511T + 3953C ($n = 7$), IL-1β −511C + 3953C ($n = 9$), and IL-1β −511C + 3953T ($n = 3$), in order to study the influence of IL-1β polymorphism on the protein secretion using an *in vitro* model consisting in PBM challenged with LPS.

Peripheral blood was layered over Ficoll–Hystopaque (Pharmacia AB, specific gravity: 1.077) and centrifuged for 30 min at 3000 rpm. Cells harvested from the interphase were resuspended in RPMI 1640 (Sigma, St Louis, MO, USA) with 10% fetal bovine serum (FBS), layered on 75 cm^3 culture flasks and incubated for 1 h (37°C). Nonadherent cells were discarded from culture flasks by rising throughly with prewarmed (37°C) RPMI 1640. Most of the adherent cells (monocytes) were collected by rinsing with cold (4°C) RPMI 1640 and by gentle mechanical action; afterwards, these were resuspended in RPMI 1640 supplemented medium (10% FBS, Penicillin 100 U/ml, Streptomicin 100 mg/ml, 20 mM Hepes and Tylosin 8 μg/ml) for cell culture. Cell count and viability was determined by Trypan blue stain.

PBM were cultured and stimulated in 24-well plates (Nunc, Roslike, Denmark) (1 ml/well) at a concentration of 10^5 cells/ml with 10 ng/ml LPS from *Escherichia coli* 055.B5 (Sigma, St Louis, MO, USA) in RPMI 1640 supplemented medium. Stimulated and nonstimulated supernatants were collected at 12, 24, 48, 72, and 96 h after stimulation and stored at −20°C until cytokine assay.

IL-1β cytokine assay

Levels of IL-1β were measured by enzyme-linked immunosorbent assay using the optEIA Human IL-1β Kit (Pharmigen, San Diego, CA, USA). Cytokine assay was performed in supernatants from LPS-stimulated and nonstimulated PBM.

Statistical analysis

Data were managed and stored using the SPSS program (V9.05. for Windows, Chicago, IL, USA). Differences between allele and genotype frequencies were determined using χ^2 and Fisher exact test as appropriate. Bonferroni correction was applied as appropriate. Linkage disequilibrium testing and likely haplotype frequencies were calculated using Arlequin® software.[44] Since the gametic phase was unknown, designed haplotypes in this study are based on maximum likelihood. Area under the curve was calculated for IL-1β secretion. The ratio between nonstimulated and stimulated PBM IL-1β secretion was defined as delta (Δ) and given in percentages, using the following formula: 1−(nonstimu-nonstimulated/stimulated). The difference between IL-1β secretion before and after LPS stimulus was calculated by Wilcoxon signed-ranks test. The influence of *IL-1β* SNP on the clinical and immunological characteristics of patients was analyzed in 2×2 and 2×3 contingency tables. Crude OR were calculated with 95% confidence intervals (CI). A P-value < 0.05 was considered as statistically significant.

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