Chemokine Receptor CCR2/CCR5 Polymorphism in Spanish Patients with Systemic Lupus Erythematosus

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ABSTRACT. **Objective.** To investigate the possible association of CCR2 and CCR5 chemokine receptor gene polymorphisms with the susceptibility, clinical features, and the outcome of systemic lupus erythematosus (SLE).

Methods. We studied 276 patients with SLE and 194 ethnically matched healthy controls. Patients were stratified according to their clinical features and outcome. Genotyping of 190 (A/G) CCR2 and Δ32CCR5 was performed using polymerase chain reaction techniques.

Results. No association between the polymorphisms studied and susceptibility to SLE was found. However, when patients were stratified according to their clinical features, an increase in the frequency of individuals bearing Δ32CCR5 among patients with anti-dsDNA antibodies was found [15.1% vs 6.4% in negative patients ($p = 0.03$, $p_{corr} > 0.05$, OR 2.61, 95% CI 1.04–7.40) and 8% in controls ($p = 0.04$, $p_{corr} > 0.05$, OR 1.97, 95% CI 0.97–4.11)]. Moreover, a significant increase in the frequency of Δ32CCR5 individuals was observed among patients with biopsy-proven nephritis [20.5% vs 8.5% in patients without nephritis ($p = 0.03$, $p_{corr} > 0.05$, OR 2.79, 95% CI 0.93–7.70) and 8% in controls ($p_{Fisher} = 0.04$, $p_{corr} > 0.05$, OR 2.87, 95% CI 0.97–7.82)]. Regarding the outcome, a higher median severity index was found among patients bearing Δ32CCR5 (33.5 ± 17.9 vs 26.6 ± 17.1 in CCR5/CCR5 individuals; $p = 0.04$).

Conclusion. Polymorphisms of CCR2 and CCR5 do not seem to be involved in susceptibility to SLE, although a slight contribution of the CCR5 polymorphism in the production of anti-dsDNA autoantibodies, in the development of lupus nephritis, and in the outcome of the disease could be postulated. (J Rheumatol 2003;30:1770–4)

Key Indexing Terms:
CCR2   CCR5   SYSTEMIC LUPUS ERYTHEMATOSUS   GENETIC POLYMORPHISM

Systemic lupus erythematosus (SLE) is a multiorgan disorder characterized by immune dysregulation that results in autoantibody production, immune complex (IC) generation, failure of clearance of IC, and consequent tissue deposition and activation. These stimuli cause an inflammatory response concluding in tissue damage[1], in which infiltrating mononuclear leukocytes may play a crucial role[2]. The migration of leukocytes through vessels and tissues is dependent in part on chemokines, and secretion of chemokines has been detected in a wide variety of diseases causing the accumulation and activation of leukocytes in tissues and acting as mediators in inflammation[3].

Chemokines are produced by a variety of cell types in response to different signals such as tumor necrosis factor-α, interleukin 1, interferon-γ, lipopolysaccharide, platelet derived growth factor, oxidized low density lipoproteins, and IC[3]. The receptors for chemokines are expressed in a cell-type fashion. Receptors CCR2 and CCR5 are expressed in basophils, monocytes, dendritic cells, natural killer cells, and activated T lymphocytes[3]. CCR2 binds to monocyte chemoattractant proteins (MCP) 1–4 in humans; MCP-1 concentrations were found to be increased in serum of patients with SLE[4]. CCR5 binds to RANTES, macrophage inflammatory protein 1α (MIP-1α), and MIP-1ß[3]; RANTES concentrations were found to be decreased in SLE patients, whereas no differences in the levels of MIP-1α and MIP-1ß were found in SLE sera compared with controls[4]. Both receptors play an important role in the recruitment of cells and are highly expressed in chronic inflammation[5]. Additionally, CCR5 and CCR2 act as receptor and coreceptor for human immunodeficiency virus (HIV), respectively[6,7].

CCR2 and CCR5 genes have been mapped very close to one another in human 3p21 chromosome[8]. This region shows weak but suggestive evidence of linkage to SLE in genome scan studies[9]. Genetic polymorphisms in both chemokine receptors have been described. The CCR2 190-

G/A transition leads to aVal64Ile amino acid change located at the first transmembrane domain of the protein[8]. For CCR5, a 32 bp deletion (Δ32) resulting in a nonfunctional receptor has been found[10]. Homozygosity for the deletion protects almost completely against HIV-1 infection and cells are nonresponsive to specific chemokines, while heterozygosity is associated with a reduced cell surface expression of CCR5 and has been correlated with a delayed progression to acquired immune deficiency syndrome[10].

We investigated the possible influence of both the 190 G/A transition of the CCR2 gene and the presence of the deletion of 32 bp of CCR5 in the susceptibility, clinical manifestations, and outcome of SLE.

MATERIALS AND METHODS

We studied 276 Caucasian Spanish patients (242 women and 34 men) with SLE according to the American College of Rheumatology (ACR) 1982 revised criteria for the classification of SLE[11] and 194 ethnically matched volunteer bone marrow donors as healthy controls (105 men, 89 women). Patient and control groups were formed by consecutive unrelated individuals. The median age of patients was 41.6 years (range 14–91), with a median disease duration of 15.3 years (range 4–42). The median age at diagnosis was 29.5 years (range 4–78). Patients were stratified according to clinical features defined by the ACR 1982 revised criteria[11] (Table 1). Nephritis was defined when one of the following criteria was observed: persistent proteinuria > 1 g/day; hematuria/leukocyturia > 10,000/min; creatinine increase over 50% of baseline in one year; or presence of a positive renal biopsy. Kidney biopsy was performed in 39 patients with lupus nephritis. WHO classification based on the nephritis pattern was applied[12]: WHO class I was present in 2 patients, class II in 6 patients, class III in 4 patients, class IV in 23 patients, and class V in 3 patients. Presence of anti-dsDNA antibodies in patient sera was investigated by indirect immunofluorescence (IIF) technique using *Crithidia lucilae* as substrate[13]. Antinuclear antibodies were studied by indirect immunodiffusion on the HEp-2 cell line.

Table 1. Clinical features of Spanish patients with SLE.

Clinical Manifestations	No. of Patients (%)
Musculoskeletal	
Polyarthritis	238 (86)
Cutaneous	
Malar rash	205 (74)
Discoid rash	81 (29)
Photosensitivity	129 (47)
Immunological	
Anti-DNA antibodies	166 (60)
Antinuclear antibodies, titer > 1/40	274 (99)
Hematologic	
Leukopenia, < 4000/mm^3	175 (63)
Lymphopenia, < 1500/mm^3	235 (85)
Thrombocytopenia, < 100,000 mm^3	100 (36)
Renal	
Nephritis	99 (36)
Serositis	
Pericarditis	55 (20)
Pleuritis	51 (18)
Neurological	
Seizures	21 (8)
Psychosis	18 (7)

Depending on the severity of the disease the treatment consisted of nonsteroidal antiinflammatory drugs (NSAID; 45 patients), corticosteroids only (76 patients), or corticosteroids and cytotoxic agents such as azathioprine, methotrexate or cyclophosphamide (155 patients). As well, to define whether the CCR2 and CCR5 genotypes were associated with more severe disease, we defined a severity index based on the 9 most affected organ systems. These 9 organ systems were grouped into 4 categories reflecting the importance of each one in the disease[14]. The following scores were assigned: 16 for central nervous system, vascular, or renal involvement; 8 for musculoskeletal and serosal damage; 4 for dermal, immunological, or constitutional alterations; and 1 for hematologic changes (maximum possible score was 77).

Genomic DNA from patients and controls was extracted from peripheral blood as described[15]. Genotyping of position 190 of the CCR2 gene was performed using a polymerase chain reaction (PCR) amplification refractory mutation system (ARMS). Primers were designed according to the published sequence for CCR2 human gene obtained from GeneBank (accession number X_M 002924). CCR2-F1 5'TTGTGGGCAACAT-GCTGGTCA3' and CCR2-F2 5'TTGTGGGCAACATGCTGGTCG3' were used as forward primers and CCR2-R 5'ACTGTGAATAATTTTG-CACATTGC3' as reverse. For each sample, 2 separate PCR were carried out, one with the primers CCR2-F1 and CCR2-R and the other with CCR2-F2 and CCR2-R. PCR were performed in a Perkin Elmer 9600 thermal cycler with mixes consisting of 1 µl genomic DNA, 10× buffer (20 mM Tris-HCl, pH 8.3, 3.0 mM MgCl$_2$, 100 mM KCl, 0.005% gelatine), 400 µM of each dNTP, 20 ng cresol red, 10% glycerol, 2 pmol of each primer (Perkin Elmer, Foster City, CA, USA), 0.4 U of Taq polymerase (Pharmacia, Uppsala, Sweden) and dd H$_2$O to a final volume of 10 µl. The following thermal profile was run: initial denaturation at 95ºC for 2 min, 10 cycles consisting of 94ºC for 10 s and 67ºC for 1 min, and finally 20 cycles consisting of 94ºC for 10 s, 62ºC for 50 s, and 72ºC for 30 s. PCR products were resolved in 2% agarose gels stained with ethidium bromide. After resolving, samples showing the expected size fragment in only one tube were genotyped as homozygous, whereas samples showing amplification in both tubes were genotyped as heterozygous.

The presence of Δ32CCR5 was tested by PCR using primers previously described spanning the region of the Δ32 deletion[16] with mixes consisting of 1 µl genomic DNA, 10× buffer (10 mM Tris-HCl, pH 9.0, 2.0 mM MgCl$_2$, 50 mM KCl; Pharmacia), 250 µM of each dNTP (Pharmacia), 1.0 mM MgCl$_2$ 2 pmol of each primer (Perkin Elmer), 0.4 U of Taq polymerase (Pharmacia), and dd H$_2$O to a final volume of 10 µl. PCR consisting of 30 cycles at 94ºC for 1 min, 58ºC for 1 min, and 72ºC for 1 min was carried out. After a final extension at 72ºC for 10 min, PCR products were run in 2% agarose gel. The presence of the 32 bp deletion (Δ32CCR5) generates a product of 212 bp, whereas the wild-type allele (CCR5) produces a fragment of 244 bp.

Allelic and genotypic frequencies were determined by direct counting. Statistical analysis to compare genotypic distributions was by chi-square test calculated on 2×3 or 2×2 contigency tables using the Statcalc program (Epi-Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). Fisher's exact test was used when expected cell value was less than 5. The p values were corrected (p$_{corr}$) by multiplying by the number of comparisons. Odds ratio (OR) with 95% confidence intervals (95% CI) was calculated using the same software. Differences in age at diagnosis and severity index among groups were tested by ANOVA (Epi-Info 2002).

RESULTS

The frequencies of all genotypes were found to be not significantly different from those predicted from Hardy-Weinberg equilibrium in both SLE patients and controls. No significant differences in the genotypic distributions of the dimorphism Val64Ile in CCR2 gene and the deletion of 32 bp in CCR5 gene were found when patients and controls

were compared (Table 2). No significant difference attributable to sex was observed in the distribution of these polymorphisms in either patient or control groups (data not shown).

When patients were stratified according to the clinical manifestations listed in Table 1, the following results were found. The median age at diagnosis was lower in 2 groups: patients with anti-dsDNA antibodies and patients with nephritis compared with the rest (26.9 ± 13.0, $p = 0.0001$, and 26.8 ± 14.1, $p = 0.01$, respectively). Other differences between clinical and demographic features were not observed. No significant differences between the CCR2 and CCR5 genotypes and the median age at diagnosis were observed. When the distribution of CCR2 and CCR5 genotypes among patients with different clinical features was analyzed, a significant deviation was found in the distribution of CCR5 genotypes among SLE patients showing anti-dsDNA antibodies compared with anti-dsDNA negative patients and controls. This different distribution corresponded to a significant increase in the frequency of individuals bearing $\Delta32CCR5$ among patients showing anti-dsDNA antibodies [15.1% vs 6.4% in negative patients ($p = 0.03$, $p_{corr} > 0.05$, OR 2.61, 95% CI 1.04–7.40) and 8% in controls ($p = 0.04$, $p_{corr} > 0.05$, OR 1.97, 95% CI 0.97–4.11)] (Table 3a). Significant deviations in the distribution of CCR5 genotypes were also found in the subgroup of 39 patients with biopsy-proven nephritis compared with both non-nephritis patients and control individuals. This different distribution corresponded to a significant increase of the frequency of individuals bearing $\Delta32CCR5$ among the biopsy-proven nephritis patient group [20.5% vs 8.5% in patients without nephritis ($p = 0.03$, $p_{corr} > 0.05$, OR 2.79, 95% CI 0.93–7.70) and 8.0% in controls ($p_{Fisher} = 0.04$, $p_{corr} > 0.05$, OR 2.87, 95% CI 0.97–7.82)] (Table 3b). For CCR2, no significant differences in the genotypic distributions and clinical features were observed (data not shown). As for severity according to the different CCR2 and CCR5 genotypes, a higher median severity index was found among patients bearing $\Delta32CCR5$ (33.5 ± 17.9 vs 26.6 ± 17.1 in CCR5/CCR5 individuals; $p = 0.04$). When patients were stratified according to treatment, no significant differences among the different CCR2 and CCR5 genotypes were

Table 2. Genotypic distribution of the 190 (G/A) CCR2 and $\Delta32CCR5$ polymorphisms in Spanish patients with SLE and healthy controls.

	SLE Patients, n = 276 (%)	Controls, n = 194 (%)
+190 CCR2 genotypes		
GG	226 (82)	157 (81)
AG	47 (17)	35 (18)
AA	3 (1)	2 (1)
$\Delta32CCR5$ genotypes		
CCR5/CCR5	244 (88)	178 (92)
CCR5/$\Delta32CCR5$	31 (11)	16 (8)
$\Delta32CCR5/\Delta32CCR5$	1 (0.4)	0 (0)

Table 3a. Frequency of CCR5 genotypes in Spanish patients with SLE releasing and not releasing anti-dsDNA antibodies. $\Delta32CCR5/CCR5 + \Delta32CCR5/\Delta32CCR5$ versus CCR5/CCR5, $p = 0.03$, OR 2.61, 95% CI 1.04–7.40.

CCR5	Anti-dsDNA, n = 166 (%)	No Anti-dsDNA, n = 110 (%)
CCR5/CCR5	141 (84.9)	103 (93.6)
$\Delta32CCR5/CCR5 + \Delta32CCR5/\Delta CCR5$	25 (15.1)	7 (6.4)

Table 3b. Frequency of the CCR5 genotypes in Spanish patients with SLE with biopsy-proven nephritis and without nephritis. $\Delta32CCR5/CCR5 + \Delta32CCR5/\Delta32CCR5$ versus CCR5/CCR5, $p = 0.03$, OR 2.79, 95% CI 0.93–7.70.

CCR5	Byopsy-Proven Nephritis, n = 39 (%)	No Nephritis, n = 177 (%)
CCR5/CCR5	31 (79.5)	162 (91.5)
$\Delta32CCR5/CCR5 + \Delta32CCR5/\Delta CCR5$	8 (20.5)	15 (8.5)

found. Nevertheless, a certain tendency in the distribution of the CCR5 genotypes was observed ($p = 0.1$), since individuals bearing $\Delta32CCR5$ required cytotoxic drugs more frequently (65% vs 55% of the CCR5/CCR5) and responded less frequently to NSAID (6% vs 18% of the CCR5/CCR5).

DISCUSSION

Our results revealed no association between the CCR2 190-G/A (Val64Ile) and $\Delta32CCR5$ polymorphisms and susceptibility to SLE in our population. However, a slight increase in individuals bearing $\Delta32CCR5$ among patients with anti-dsDNA antibodies and having biopsy-proven nephritis was found. These results would require confirmation because p values became nonsignificant after correction. The CCR2 190 G/A (Val64Ile) polymorphism has been investigated in several pathologies. In some, such as Crohn's disease and myocardial infarction, no association was found[17,18], whereas an association was found in insulin dependent diabetes mellitus (IDDM)[19] and pulmonary sarcoidosis[20,21], but with a different allele.

Increased concentrations of MCP-1 have been found in sera from patients with SLE[4], suggesting a possible role of ligand/receptor pair in the inflammatory response. However, in a previous investigation with the same SLE patient group, we found an association between the MCP-1 promoter polymorphims and SLE only in patients with cutaneous vasculitis[22]. Thus, our studies support that the role played by the polymorphisms in both ligand and receptor are unimportant in susceptibility to SLE, as well as in the clinical manifestations and outcome.

For CCR5, our study reveals a lack of association with SLE susceptibility. Similar results were obtained by Gomez-

Reino, *et al*[16]. The Δ32CCR5 polymorphism has been studied in several pathologies with different results: positive association with pulmonary sarcoidosis[21] and myocardial infarction[18], and no association with Crohn's disease[17], IDDM[19], inflammatory bowel disease[23], and polymyalgia rheumatica[24]. Finally, some conflicting results regarding rheumatoid arthritis[16,25] and asthma[26-28] have been reported.

Our results suggest the existence of a weak association between the allele Δ32CCR5 and anti-dsDNA production, nephritis development, and more severe outcome among patients with SLE. Although this difference may be due to a type I error, alternatively it may reflect true association between these clinical features and outcome. The hallmark of the immunologic abnormality in SLE is hyperactivity of B cells, leading to excessive autoantibody production. Among these autoantibodies, IgG anti-dsDNA is thought to play an important role in lupus nephritis. It is difficult to speculate about the exact basis of the contribution of the presence of Δ32CCR5 and the anti-dsDNA production. Patients releasing these autoantibodies could represent a more homogeneous group in this multifaceted disease. Recently, it has been reported that renal tissue infiltrates from patients with lupus nephritis expressed CCR4 but not CCR5[29]. Thus, it is possible that cells both from Δ32CCR5/Δ32CCR5 individuals who do not express functional CCR5 and from CCR5/Δ32CCR5 individuals with a reduced cell surface expression have increased expression of other chemokine receptors, migrating mainly to kidney. This could be the basis of our finding.

Patients releasing anti-dsDNA antibodies and lupus nephritis had a lower median age at diagnosis than the others. Nevertheless, we observed no influence of any of the studied polymorphisms on patient's age at diagnosis. Thus, Δ32CCR5 could influence clinical features at the age at diagnosis in an independent way. Regarding outcome, the worst evolution of patients bearing Δ32CCR5 could be caused by the score attributed to renal involvement or to a greater number of important organ systems affected. We cannot clarify this point because only 23 patients presented the most important organ systems affected, although 22% of them bore Δ32CCR5 versus 10% of the rest.

Although the contribution of CCR5 polymorphism to SLE seems to be weak, genomic screening in human SLE clearly indicates that multiple genes contribute to the disease, as in other autoimmune diseases. Thus, there would be many susceptibility genes with extremely variable effects acting in SLE at different levels. Although weak, the contribution of each individual gene may be important in susceptibility to the disease as well as in its clinical course.

Our findings suggest that CCR2 and CCR5 polymorphisms are not involved in the susceptibility to SLE, although an influence of CCR5 polymorphism on some clinical features cannot be excluded.

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