Corticotropin-Releasing Hormone Promoter Polymorphisms in Patients with Rheumatoid Arthritis from Northwest Spain

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ABSTRACT. ***Objective.*** To investigate the possible implications of polymorphism in the CRH promoter in rheumatoid arthritis (RA) susceptibility, we examined a series of patients with RA from a defined area of Northwest Spain.

Methods. A total of 177 patients with RA and 147 ethnically matched controls from the Lugo region of Northwest Spain were studied. Patients and controls were genotyped for CRH polymorphisms in the 5' regulatory region of the gene at position 1273 (alleles A1 and A2) and at position 225 (alleles B1 and B2) by PCR-restriction fragment length polymorphism. Patients were stratified for age at onset of disease and rheumatoid factor status.

Results. When the whole group of patients was examined, no significant differences in CRH allele or genotype frequency were found compared with controls. However, the CRH allele A2 was significantly increased in patients with late onset seronegative RA compared with the seronegative group with younger age of disease onset ($p = 0.03$). In addition, 4 (36.4%) of the 11 patients with late onset seronegative RA carried the CRH-A2 allele versus only 2 (6.6%) of 31 patients with seronegative RA beginning before age 61 (OR 8.3, 95% CI 1.4–47.0; $p = 0.015$).

Conclusion. In Northwest Spain, polymorphism in the CRH gene regulatory region may play a role as a disease susceptibility marker for late onset seronegative RA. (J Rheumatol 2003;30:913–7)

Key Indexing Terms:
RHEUMATOID ARTHRITIS AGE OF ONSET RHEUMATOID FACTOR
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Rheumatoid arthritis (RA) is a polygenic inflammatory disease characterized by both joint and systemic extraarticular manifestations. The major genetic contribution is provided by genes of the HLA region, particularly the MHC class II HLA-DRB1 alleles[1,2]. However, several studies have suggested that the contribution of genes within the major histocompatibility complex to RA only accounts for one-third to one-half the total genetic contribution[3]. Thus, other potential genes may also be implicated in the development of RA[4].

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RA is more common in women, especially before the menopause. Men may be protected by hormonal factors and require a stronger genetic component to develop disease. Reduced adrenal steroid concentrations have been observed in patients with RA. In these patients a subnormal secretion of cortisol in the setting of a sustained inflammatory disease has been observed[5].

Corticotropin-releasing hormone (CRH) regulates the immune response and helps maintain homeostasis during inflammatory stress. CRH is produced by the hypothalamus and this process is stimulated by the action of inflammatory cytokines such as interleukin 1 (IL-1), IL-6, or tumor necrosis factor-α. CRH is the best activator of the hypothalamic-pituitary-adrenal (HPA) axis through stimulation of ACTH release. The chromosomal locus of the human CRH gene has been assigned to chromosome 8q13 by somatic cell hybrids and *in situ* hybridization studies[6].

Susceptibility to streptococcal cell wall-induced arthritis in Lewis rats was observed to be due to defective inflammatory and stress mediator-induced activation of the HPA axis[7]. Lewis rats have markedly impaired plasma corticotropin and corticosterone responses to streptococcal cell wall, recombinant human IL-1α, and synthetic rat/human CRH. These rats have profoundly deficient paraventricular nucleus CRH mRNA levels and hypothalamic CRH content

in response to streptococcal cell wall. Thus, in these rats a hypothalamic defect in the synthesis and secretion of CRH yielded impaired CRH and corticosterone responses following inflammatory mediators and led to experimental arthritis. The primary defect was not found in the CRH gene, but was instead related to its inappropriate regulation[7].

CRH promoter region polymorphisms in the 5' regulatory region of the CRH gene have been reported in healthy controls and patients with RA[8]. Evidence for significant linkage and association with RA and the CRH genomic region was reported by Fife, *et al*[9]. However, the functional consequences of polymorphisms in CRH gene need further investigation.

Polymorphisms in the CRH promoter might contribute to variation in the functioning of the HPA axis in RA, and thus they might play a role in the pathogenesis of this condition. Indeed, Baerwald, *et al* suggested that CRH promoter polymorphism might constitute a new genetic marker for RA susceptibility[10].

To investigate the possible implications of CRH promoter polymorphisms in RA we examined a series of patients with RA from a defined population from Northwest Spain.

MATERIALS AND METHODS

Patients and controls. Patients (n = 177; 126 women, 51 men) fulfilling the 1987 American College of Rheumatology classification criteria for RA[11] were recruited from the Hospital Xeral-Calde, Lugo, in Galicia, Northwestern Spain. All cases and controls gave informed consent for the study. The cohort was constituted by a series of patients attending hospital outpatient clinics seen over about one year at the only rheumatology unit in the area.

Patients with RA were considered seropositive if the rheumatoid factor (RF) (by nephelometry) was found to be positive in at least 2 determinations during the course of the disease; by this definition, 135 (76%) patients were positive for RF.

RA is classically considered to be a chronic disease, with onset in middle age. However, disease onset is variable. Patients with disease beginning above the age of 60 are defined as having a late or elderly onset[12]. Forty-three (24%) of the 177 RA patients were older than 60 years at disease onset. All were treated by the same group of rheumatologists (most by MAG-G and CG-P); the main clinical characteristics of these patients have been described[13].

Patients were from the area around the city of Lugo. Hospital Xeral-Calde is the single referral center for a mixed rural and urban caucasian population of almost a quarter of a million people[14]; ethnically matched controls were from the same area. This area has been geographically isolated from the rest of Galicia and the rest of Spain for many centuries. This population is relatively static, and no important migration has occurred in the area during the last decades. Due to this, special attention was paid to only select patients and controls whose families had lived in this area for several generations.

Patients who fulfilled the 1987 classification criteria for RA but who had symptoms of giant cell arteritis such as new onset headache, jaw claudication, scalp tenderness, abnormal temporal artery on physical examination, or visual manifestations were excluded. Patients who developed clinical manifestations of giant cell arteritis during the followup (at least 4 years) were also excluded.

Patients with RA were stratified according to age of onset (≤ 60 or > 60 years of age) and RF status. The choice of these subgroups was based on clinical differences and genetic associations previously observed in RA patients from Lugo. In Lugo, RA patients with disease onset under age 40 years were strongly associated with HLA-DRB1*0401 and *0404. In contrast, RA patients with disease onset above 60 years were not associated with HLA-DRB1*04, but they were associated with HLA-DRB1*01[15]. Moreover, the stratification of patients with late onset RA by RF status revealed further heterogeneity. Those late onset patients with positive RF showed an association with HLA-DRB1*01. In contrast, patients with late onset seronegative disease had increased frequency of HLA-DRB1*13/*14 alleles, which was also observed in cases with isolated polymyalgia rheumatica[15].

Molecular analysis of CRH promoter polymorphisms. We examined biallelic polymorphism in the 5' regulatory region of the CRH gene characterized as a T → C base substitution located at position 1273 (alleles A1 and A2). This substitution leads to the absence of the recognition site for *Afl*III (position 1273) restriction enzyme[8]. In addition, biallelic polymorphism in the 5' flanking region of the CRH gene characterized as a T → G base substitution located at position 225 (alleles B1 and B2) was also examined. This substitution leads to absence of the recognition site for *Xmn* I restriction enzyme[16]. Polymorphisms in CRH promoter region were examined using the following PCR primers: CRH-A: forward 5' GCT GTT CTT GTG ATA GTA AAT A 3', reverse 5' CCC CAG AGG AAG AGA AGC 3'; CRH-B: forward 5' TGA AGG TAC AAG GTG ATA CAA GTG ACA A 3', reverse 5' ACA CAA ACT GAG GTG AAA AGA TGA A 3'.

DNA was extracted from EDTA anticoagulated blood using a phenol-chloroform extraction procedure.

A total of 100 ng genomic DNA (5 µl) was amplified in a 25 µl PCR reaction containing 2.5 µl KCl buffer, 1.5 mM $MgCl_2$ for CRH-A, and 3.5 mM $MgCl_2$ for CRH-B, 2.5 µl dNTP (Bioline), 0.5 µl of each primer for CRH-A and 1.0 µl of each primer for CRH-B, 0.1 µl Taq polymerase (Bioline), Betaine (4 M) 6.0 µl for CRH-A, and distilled water (7.9 µl for CRH-A and 10.9 µl for CRH-B). Thermal cycling was performed using a Hybaid OmniGene PCR machine. Cycles consisted of 10 min denaturation at 95°C followed by 35 rounds of 95°C for 1 min each one, annealing at 52°C for 1 min for CRH-A and 66ºC for 1 min for CRH-B, 72°C for 1 min, and a final extension at 72°C for 10 min. The presence of product was verified on a 1% agarose gel stained with ethidium bromide. PCR products were digested with *Afl*III for CRH-A and *Xmn* I for CRH-B in a 10 µl final volume. PCR products were incubated overnight at 37°C and the products of the digest were then visualized on a 4% agarose gel stained with ethidium bromide.

Statistical analysis. Strength of association between RA and alleles or genotypes of CRH-A and CRH-B was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher's exact analysis. The same methods were used to examine the strength of associations between RA subgroups and CRH polymorphisms. Statistical significance was defined as $p \leq 0.05$. Except where noted, comparisons have not been corrected for multiple comparisons. Calculations were performed with the Stata v6 statistical package.

RESULTS

Allele and genotype frequencies of CRH-A and CRH-B polymorphisms in RA patients. In controls no evidence of departure from Hardy-Weinberg equilibrium was observed (p = 0.98). When the whole group of RA patients was examined for allele frequencies and genotype distribution for each CRH polymorphism, no significant differences were found compared with ethnically matched controls (Table 1).

Allele and genotype frequencies of CRH polymorphisms in RA patients according to age at disease onset and RF status. Among the patients with disease onset before age 61 years

Table 1. CRH-A and CRH-B marker allele and genotype frequencies in RA patients and controls. No statistically significant differences among the different groups were found. Values are percentages.

CRH	Controls	RA
Allele	2 N = 294	2 N = 354
A1	90.8	90.1
A2	9.2	9.9
Genotype	N = 147	N = 177
A1/A1	82.3	81.9
A1/A2	16.3	16.9
A2/A2	1.4	1.1
Allele	2 N = 284	2 N = 352
B1	94.7	94.6
B2	5.3	5.4
Genotype	N = 142	N = 176
B1/B1	89.4	89.8
B1/B2	10.6	9.7
B2/B2	0	0.6

an increased frequency of the A2 allele and the CRH A1/A2 and CRH A2/A2 genotypes was observed in patients who were RF positive compared with those RF negative (Table 2). However, the increase of allele A2 in seropositive RA patients with disease onset before the age of 61 years needs to be interpreted in the context of the frequency of the A allele in the control population (9.2%), which was similar to that in this group with earlier onset (≤ 60 years) seropositive RA. Further, the increase in the frequency of CRH A1/A2 and CRH A2/A2 genotypes compared with CRH A1/A1 genotype in seropositive patients was not statistically significant when a Yates correction was applied (p = 0.08). In contrast, among the RA patients with late onset disease the carriage of allele A2 appeared to be marginally increased in those who were seronegative (p = 0.067, OR 4.5, 95% CI 0.9–22.8).

When patients with late onset seronegative RA were compared with seronegative patients with disease onset before age 60, some differences were found — allele A2 was significantly increased in the group of late onset seronegative patients compared with the younger disease onset seronegative group (p = 0.03) (Table 2). In addition, 4 (36.4%) of the 11 late onset seronegative patients carried the CRH-A2 allele versus only 2 (6.5%) of the 31 patients with early onset seronegative RA [OR 8.3, 95% CI 1.4–47.0, p = 0.015, corrected p (Bonferroni) = 0.030] (Table 2). This genotype difference was also statistically significant when a Yates correction was applied. These observations, although based on a small group of patients, suggest a possible role of CRH-A2 allele as a marker for late onset seronegative RA. However, no CRH genotype or allele differences were observed between men compared to women. Finally, the haplotype distribution exhibited differences between late onset and early onset seronegative RA (p = 0.05). In addition, 4 of the 11 late onset seronegative patients carried A1 A2/B1 B2 or A1 A2/B1 B2 haplotypes versus only 2 of the 31 early onset seronegative patients (p = 0.015, OR 8.3, 95% CI 1.4–47.0) (Table 3).

DISCUSSION

CRH has been implicated in the regulation of endocrine and autoimmune processes. This peptide promotes the release of ACTH, which stimulates the adrenal cortex to produce cortisol. Thus, CRH is in fact the regulator of antiinflammatory glucocorticoid release from the adrenal gland[17]. In patients with RA the HPA axis activity is not different from

Table 2. CRH-A and CRH-B marker allele and genotype frequencies in RA patients stratified by age at the onset of disease and rheumatoid factor (RF) status. Values are percentages.

CRH	Age of Onset ≤ 60 yrs		Age of Onset > 60 yrs	
	RF +	RF –	RF +	RF –
Allele	2 N = 206	2 N = 62	2 N = 64	2 N = 22
A1	87.4	96.8	95.3	81.8
A2	12.6	3.2*	4.7	18.2*
Genotype	N = 103	N = 31	N = 32	N = 11
A1/A1	77.7	93.5	90.6	63.6
A1/A2	20.4	6.5**	9.4	36.4**
A2/A2	1.9	0	0	0
Allele	2 N = 206	2 N = 62	2 N = 62	2 N = 22
B1	92.7	98.4	98.4	90.9
B2	7.3	1.6	1.6	9.1
Genotype	N = 103	N = 31	N = 31	N = 11
B1/B1	86.4	96.8	96.8	81.8
B1/B2	12.6	3.2	3.2	18.2
B2/B2	1	0	0	0

* Allele A2 in patients with seronegative late onset RA versus those with early onset disease: p = 0.03, OR 6.7, 95% CI 1.1–39.4. ** A1/A2 genotype in seronegative late onset RA compared to early onset RA: p = 0.015, corrected p (Bonferroni) = 0.030, OR 8.3, 95% CI 1.4–47.0.

Table 3. Haplotype distribution in patients with seronegative RA according to age of disease onset. The haplotype distribution exhibited differences between late onset and "classic" onset seronegative RA (p = 0.05). Four of the 11 patients with late onset seronegative RA carried A1 A2/B1 B2 or A1 A2/B1 B2 haplotypes versus only 2 of the 31 seronegative patients with disease onset before age 60 years (p = 0.015, OR 8.3, 95% CI 1.4–47.0).

CRH	Age of Onset ≤ 60 yrs, N (%)	Age of Onset > 60 yrs, N (%)
A1 A1/B1 B1	29 (93.5)	7 (63.6)
A1 A2/B1 B1	1 (3.2)	2 (18.2)
A1 A2/B1 B2	1 (3.2)	2 (18.2)

that observed in healthy people. However, in patients with RA there is an inability of the HPA axis to undertake an appropriate cortisol response to the inflammatory challenge[18,19].

Sanger, *et al* performed direct sequencing of the 5' regulatory region of the CRH gene spanning 3625 nucleotides[20]. In 1996, Baerwald, *et al* described the first nucleotide polymorphism in the 5' flanking region of the human CRH gene[16]. More recently, Baerwald, *et al* sequenced 5 patients with RA and 2 controls and found additional polymorphisms in the 5' regulatory region leading to a T → C substitution at positions 1273 and 2942 and a C → G substitution at position 95[8]. Further studies revealed a complete co-segregation of the 3 polymorphisms as haplotypes. The structural organization of the 5' promoter region of the CRH gene has several binding sites for different transcription factors. Thus, it is possible that the CRH promoter polymorphism of the 1273 T → C SNP might affect RA susceptibility, possibly through a defective hypothalamic response to inflammatory signals in patients with RA.

Information about the role of CRH genetic polymorphisms in RA is limited. Baerwald, *et al* reported the distribution of CRH alleles in UK caucasians and black South African patients with RA[10]. They observed that the CRH-A2B1 haplotype was protective against development of RA in the UK[10]. Also, this haplotype correlated with a later onset of disease among these patients[10]. In contrast, in the South African black population the CRH-A1B1 haplotype was associated with RA[10].

In the Lugo region the susceptibility to RA was associated with different HLA-DRB1 alleles compared to other areas of Spain. Just as in the UK population, RA was associated with shared epitope (SE) alleles, primarily with HLA-DRB1*0401[21]. However, despite strong HLA-DRB1 similarities with RA patients from the UK, our results do not confirm an association of CRH alleles with unselected RA patients in Lugo. In contrast, we found that in elderly patients with seronegative RA a possible association may exist between CRH-A2 allele, unlike seronegative RA patients with early onset disease. In Lugo an association with the CRH allele A2 also appeared to exist in those elderly patients with biopsy proven giant cell arteritis who developed ischemic visual complications[22].

Healey has proposed late onset seronegative RA as being a discrete subset condition within RA identified in the elderly that is seronegative, frequently has a polymyalgia-like onset, is largely non-erosive and responsive to low dose steroids, and has a good prognosis[12,23]. In this regard, we recently described different HLA-DRB1 associations in RA patients according to age at disease onset and a further clear differentiation between RF positive and negative patients with late disease onset. Unlike patients with early onset or seropositive late onset RA, no association with the RA SE was observed in patients in Lugo with seronegative late onset RA[15]. This negative association with HLA-DRB1 alleles carrying the SE and the increased frequency of CRH A2 allele may suggest that in patients with late onset seronegative RA the CRH polymorphism may play an important role in disease susceptibility. Thus, CRH polymorphism may have its biggest effect in older patients, where HLA-DRB1*04 or HLA-DRB1*01 alleles, and the associated RF, are not a risk factor for the disease. This might also explain the good response to low dose prednisone reported by Healey in many patients in this category[12,23]. Thus, the allelic variations within the CRH may modulate proinflammatory effects in elderly people regardless of the presence of other traditional genetic risk factors for disease susceptibility and severity[2]. Thus, it is possible that the allelic variations within the CRH promoter may count as independent risk factors for disease, regardless of HLA-DRB1 status, in patients with autoimmune diseases. Knowledge of possible implications of the CRH gene polymorphisms may elucidate the pathogenesis of RA. Due to the small size of the sample assessed in this study, additional studies with a larger number of cases are needed to confirm the relationship between CRH polymorphism and age of disease onset.

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