Associations between Serum Anti-CCP Antibody, Rheumatoid Factor Levels and HLA-DR4 Expression in Hungarian Patients with Rheumatoid Arthritis

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Abstract

Background: The presence of anti-cyclic citrullinated peptide autoantibody is highly specific for rheumatoid arthritis. Certain HLA-DR4 (HLA-DRB1*04) alleles, also known as the "shared epitope," are associated with increased susceptibility to RA. In addition, these alleles may also have relevance for disease outcome. Anti-CCP antibody positivity has been associated with the presence of HLA-DR4 alleles in patients with RA. However, there is little information regarding a relationship between quantitative anti-CCP production (serum anti-CCP concentrations) and the shared epitope.

Objectives: To determine the association between anti-CCP antibody production and various HLA-DRB1 alleles.

Methods: Serum anti-CCP, rheumatoid factor and C-reactive protein levels were assessed in 53 RA patients. All these patients underwent HLA-DRB1 genotyping.

Results: Of the 53 patients 33 (62%) were positive for anti-CCP antibody. We found significant correlations between anti-CCP and RF positivity (chi-square = 6.717, P < 0.01), as well as between anti-CCP and HLA-DRB1*04 positivity (chi-square = 5.628, P < 0.01). There was no correlation between RF positivity and serum levels, CRP serum levels and HLA-DRB1*04 positivity. When quantitatively comparing serum anti-CCP levels with shared epitope positivity, patients carrying one or two copies of HLA-DRB1*04 alleles had significantly higher anti-CCP concentrations (530.0 ± 182.6 U/ml) compared to DRB1*04-negative patients (56.8 ± 27.4 U/ml) (P < 0.01). There was no difference in serum anti-CCP antibody concentrations between patients carrying only one HLA-DRB1*04 allele but no HLA-DRB1*04 allele (12.0 ± 8.6 U/ml) compared to SE-negative patients (76.8 ± 56.2 U/ml). Regarding non-SE HLA-DRB1 genotypes, all 6 patients (100%) carrying DRB1*15 alleles and 6 of 7 (85%) patients carrying DRB1*13 were anti-CCP positive. In addition, patients with HLA-DRB1*13 (282.5 ± 23.8 U/ml) and DRB1*15 (398.7 ± 76.2 U/ml) produced significantly more anti-CCP than did any other non-SE HLA-DRB1 subtypes (P < 0.01).

Conclusions: There is significant association between anti-CCP and RF, as well as between anti-CCP and SE positivity in RA. In addition, the presence of one or two copies of HLA-DRB1*04 alleles has been associated with higher serum anti-CCP antibody levels. Thus, patients carrying HLA-DRB1*04 alleles exhibited an overall tenfold increase in serum anti-CCP antibody levels in comparison to HLA-DRB1*04-negative subjects. Increased anti-CCP production may also be associated with other non-SE HLA-DRB1 genotypes, such as DRB1*13 or DRB1*15. In reports by other investigators, both anti-CCP concentrations and SE positivity were related to more rapid disease progression and unfavorable outcome.

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Rheumatoid arthritis is a chronic autoimmune inflammatory disease that affects approximately 0.5–1% of the population [1]. Chronic synovitis in RA may eventually lead to joint destruction. The etiology of the disease is still unknown, but both genetic and environmental factors play an important role in the onset of RA [1-3].

The diagnosis of RA depends on clinical symptoms, laboratory investigations and imaging. Several autoantibodies have recently been associated with disease activity and/or prognosis of RA [4-6]. However, until recently, rheumatoid factor of the immunoglobulin M isotype has been the only laboratory marker routinely used in RA. The assessment of IgM RF has rather low specificity for RA as it can also be detected in sera of patients with other autoimmune diseases, infectious disorders, as well as in the healthy elderly population [7].

C-reactive protein is a convenient and sensitive marker of inflammatory activity in clinical practice. The control of inflammation by anti-rheumatic therapy usually leads to suppression of serum CRP levels [8].

Anti-cyclic citrullinated peptide has been identified as a potential diagnostic and prognostic marker of RA. Citrulline is an unusual amino acid resulting from an enzymatically modified arginine residue present on certain human proteins. The presence of anti-CCP antibodies is highly specific and sensitive for RA [9]. Determination of anti-CCP helps to distinguish RA from other arthropathies; furthermore, as a prognostic marker it may predict persistent, erosive, more aggressive synovitis [10]. The assessment of anti-CCP antibody enzyme-linked immunosorbent assay testing has shown a specificity of 98% in sera from patients with established RA and 96% in sera from subjects with early RA [10]. The sensitivity

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RA = rheumatoid arthritis
CCP = cyclic citrullinated peptide
RF = rheumatoid factor
CRP = C-reactive protein
SE = shared epitope
IgM = immunoglobulin M
of this anti-CCP antibody ELISA was 68% in established RA and 48% in early RA [10]. Some investigators found a correlation between anti-CCP and RF positivity [12].

The major histocompatibility complex is encoded in human leukocyte antigen gene clusters on chromosome 6 [3]. Among MHC class II molecules, various HLA-DR alleles have been associated with susceptibility to RA in several racial groups [13-18]. In addition, some HLA-DRB1 alleles have also been related to the severity and outcome of RA [2,14]. These disease-associated HLA molecules share a common amino acid sequence in the third hypervariable region of the beta chain of the HLA-DR molecule (HLA-DRB1), hence the term “shared epitope” [3]. SE is a sequence of five amino acids. Among the SE variants, the QKRAA, QRRAA and RRRAA motifs have been described within various DRB1 variants [2,3].

There may be an association between SE positivity and the production of anti-CCP antibody. The presence of one or two shared epitope alleles has been associated with anti-CCP antibody positivity [19,20]. Moreover, unfavorable disease progression has been related to anti-CCP production and SE positivity [10,21,22]. HLA-DR3 has been related to anti-CCP-negative disease [20]. However, there is little information available regarding possible associations between serum anti-CCP antibody levels and HLA-DRB1 expression.

In the present study, we investigated associations of SE positivity with anti-CCP positivity and serum levels. This is the first report on the possible relationship between SE and anti-CCP in Hungarian RA patients.

**Patients and Methods**

**Patients**

Fifty-three RA patients (44 females and 9 males, all Caucasians) were included in this study. All patients fulfilled the 1987 revised classification criteria of the American College of Rheumatology [23]. The mean age of the patients was 50 ± 15 years (range 17–82 years). The mean disease duration at the time of the study was 6 ± 4 years (range 0.5–22). Informed consent was obtained from each RA patient. We also obtained local ethical committee approval at the University of Debrecen for this study.

**Polymerase chain reaction with sequence specific primers**

Peripheral blood was drawn from each patient for DNA isolation. Genomic DNA was isolated from buffy coats of EDTA-anticoagulated blood using QiAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Polymerase chain reaction-based HLA-DRB genotyping (DRB1*01-DRB1*16) was performed with the help of sequence specific primers (Olerup SSP, Genovision, Norway) [24]. HLA-DR1-DRB1*01 or HLA-DR4-DRB1*04-positive samples were further analyzed for DR1 and DR4 subtypes (Genovision). All samples were processed according to the manufacturer’s instructions using recombinant Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR amplification of DNA was performed using Hybrid PCR express thermal cycler (Basingstoke, UK). HLA genotypes were determined on the basis of the PCR product pattern obtained using 2% agarose gel electrophoresis. DNA bands were detected using Alpha Imager Multimage Light Cabinet (αlpha Innotech Corporation, San Leandro, CA, USA).

**Laboratory markers of RA**

Serum IgM RF and CRP were assessed by quantitative nephelometry (Cobas Mira Plus, Roche, Basel, Switzerland), using RF and CRP reagents, respectively (both Dialab, Budapest, Hungary). RF levels > 50 U/ml and CRP levels ≥ 5 mg/L were considered high.

Anti-CCP autoantibodies were detected in serum samples using Immunoscan-RA CCP2 ELISA test (Euro Diagnostica, Arnhem, The Netherlands). The assay was performed according to the manufacturer’s instructions. A concentration > 25 U/ml was considered positive.

**Statistical analysis**

Statistical analysis was performed using the SPSS 10.0 software. A chi-square test was used to detect differences between two different groups of patients. Median and 0.25/0.75 quartiles were used when appropriate. Clinical data were analysed using Kolmogorov-Smirnov two-sample test. P values < 0.05 were regarded as significant.

**Results**

Of the 53 serum samples 39 (73.6%) were RF positive and 43 (81.1%) were CRP positive. Anti-CCP antibody was present in 33 (62.2%) of the 53 samples. Seventeen of 53 patients (32.1%) carried one or two copies of the HLA-DRB1*04 allele (data not shown).

We found a close association between anti-CCP and RF positivity. Thirty of the 53 patients (56.6%) were both RF and anti-CCP positive, while 9 (17%) were double negative (chi-square = 6.717, P < 0.01). In addition, there was a significant association between anti-CCP positivity and the presence of HLA-DRB1*04 alleles (chi-square = 5.829, P < 0.01). In contrast, we could not find any correlation between CRP or RF positivity and the presence of HLA-DRB1*04 alleles (data not shown).

When further analyzing these laboratory markers, patients were divided into two groups: those who carry one or two copies of the shared epitope alleles (SE-positive patients) and those who do not (SE-negative patients). Altogether, 16 patients were HLA-DRB1*01 positive, 17 patients were DRB1*04 positive, and 23 patients did not carry any of these alleles (Table 1). Patients carrying neither of these alleles were assigned the ‘X,X’ genotype. We did not find any differences in serum RF or CRP levels between SE-positive and negative patients (Table 1). In contrast, regarding anti-CCP antibody production, patients carrying one or two copies of HLA-DRB1*04 alleles (DRB1*01/*04: 530.5 ± 48% in early RA [10]. Some investigators found a correlation between anti-CCP and RF positivity [12].
Table 1. Associations between serum anti-CCP, CRP and RF concentrations and shared epitope alleles in RA patients

<table>
<thead>
<tr>
<th>DRB1*</th>
<th>DRB1*04 (n=3)</th>
<th>DRB1*01/X (n=13)</th>
<th>DRB1*04/04 (n=11)</th>
<th>DRB1*04/04 (n=23)</th>
<th>All DRB1*04 positive (n=17)</th>
<th>All DRB1*04 negative (n=36)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CCP (U/ml)</td>
<td>930.5 ± 174.2</td>
<td>120 ± 8.6</td>
<td>439.5 ± 182.8</td>
<td>591.0 ± 164.2</td>
<td>76.8 ± 56.2</td>
<td>530.0 ± 182.6</td>
<td>56.8 ± 27.4</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>5.5 ± 1.2</td>
<td>9.5 ± 5.6</td>
<td>10.6 ± 2.8</td>
<td>6.9 ± 2.7</td>
<td>4.3 ± 3.1</td>
<td>7.1 ± 2.4</td>
<td>6.5 ± 2.2</td>
</tr>
<tr>
<td>RF (U/ml)</td>
<td>16.5 ± 6.2</td>
<td>29.5 ± 9.8</td>
<td>85.5 ± 34.6</td>
<td>70.0 ± 46.2</td>
<td>71.0 ± 38.4</td>
<td>63.5 ± 28.4</td>
<td>57.5 ± 22.6</td>
</tr>
</tbody>
</table>

*P values indicate differences between all DRB1*04-positive versus negative patients.
NS = not significant

Discussion

We analyzed possible associations of both the positivity and serum concentrations of RF, CRP, anti-CCP antibody and the expression of shared epitope in patients with RA. In a previous study we showed that the frequency of HLA-DRB1*04 (HLA-DR4) alleles was significantly increased in Hungarian RA patients compared to healthy subjects (31.3% vs. 10.9%, P < 0.05). In addition, HLA-DRB1*01 (HLA-DR1) exhibited a tendency to be more frequent in RA patients than in controls (32.5% vs. 18%) [18].

Our results showing an association between anti-CCP and RF positivity reflect findings published by other researchers [12]. In the present study 56% of the patients were both RF and anti-CCP positive, while neither RF nor anti-CCP could be detected in 17% of the patients.

Previously, Seidl et al. [14] reported correlations between HLA-DRB1*01 or HLA-DRB1*04 genotypes and inflammatory activity indicated by serum CRP in German patients. We could not confirm these findings in Hungarian patients as we did not find any correlation between CRP positivity and the presence of HLA-DRB1*01 or DRB1*04 alleles. Although serum CRP concentrations were somewhat increased in RA patients carrying the SE, the differences were not significant. There was also no correlation between RF production and SE positivity. We must
bear in mind that serum CRP and RF levels may change during the treatment, so the association between increased CRP or RF production and HLA-DRB1 genotypes may be influenced by anti-rheumatic therapy.

In contrast to CRP and RF, we did find associations between the anti-CCP positivity and expression of the HLA-DRB1*04 gene complex in our RA patients. In addition to these correlations also published previously by other groups [19,20], in the present study not only the presence of anti-CCP antibodies but also the serum levels of this antibody could be significantly associated with the expression of one or two copies of HLA-DRB1*04 alleles in our RA patients. RF still remains an important marker in RA diagnostics. However, the association of highly specific anti-CCP autoantibody levels and HLA DRB1*04 alleles could provide a valuable “disease marker” combination in evaluating future disease progression since no correlation was found between serum RF levels and the SE.

Earlier studies suggested that DRB1*0401 acts as a dominant gene in terms of disease susceptibility, while others, such as HLA-DRB1*0404 or DRB1*10, act as recessive genes and require two copies of the SE to increase susceptibility [29]. Here we could not find significant differences between anti-CCP levels comparing groups with one or two copies of the HLA-DRB1*04 allele.

We also studied possible associations of serum RF, CRP and anti-CCP antibody levels with HLA-DRB1 genotypes other than the SE. Although the number of patients in the studied groups was rather low for statistical analysis, we could not find any relationship between serum RF or CRP levels and the specific HLA-DRB1 subtypes. In contrast, most patients carrying either DRB1*13 or DRB1*15 were anti-CCP positive. In addition, patients expressing HLA-DRB1*13 or DRB1*15 produced significantly more anti-CCP than did patients with HLA-DRB1*03, *07, *08, *11, *14 or *16. These results seem to be rather interesting, as the HLA-DRB1*13 and DRB1*15 alleles have not been previously associated with RA.

In conclusion, our results support the findings reported by other investigators that anti-CCP antibodies and HLA-DRB1*04 genes may be present simultaneously in RA patients. Previous reports suggested an association between anti-CCP positivity and SE positivity in RA. Here we confirmed that not only anti-CCP positivity but, as a novel finding, also serum anti-CCP concentrations are associated with the SE in RA. The strong association between HLA-DRB1 genes and autoantibody production may influence future disease development and outcome. Anti-CCP antibody production may be associated with certain other HLA-DRB1 genotypes, such as HLA-DRB1*13 and DRB1*15, but this needs further confirmation in larger patient cohorts.

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References


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**Capsule**

**Prion diseases and strains**

Mammalian prion diseases are infectious lethal neurodegenerative disorders. The pathogenic agent of prion diseases is thought to lack nucleic acids, but instead relies on the propagation of a pathological conformation of the prion protein, an endogenous glycoprotein. Collinge and Clarke reviewed several aspects of prion diseases, including how prion strains can be generated and maintained and suggest a model for how prion propagation involves the generation of toxic intermediates.

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**Capsule**

**Deep-brain stimulation and decisions**

Deep brain stimulation of the subthalamic nucleus and dopaminergic medication are successful therapies for Parkinson’s disease. However, both interfere with normal cognitive strategies, such as decision-making in conflict situations. Frank and collaborators provide evidence for two distinct computational roles of these treatments in decision-making. Dopaminergic medication altered patients’ relative tendency to learn from positive versus negative outcomes without impacting conflict-induced slowing. In contrast, deep-brain stimulation sped up high conflict choices without affecting learning biases. Both of these findings were predicted in a computational model of the basal ganglia in learning and decision-making.

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**Capsule**

**Regulatory T cells**

What overarching factors regulate the regulatory T (Treg) cells that guide our immune systems to fight infectious diseases while leaving ourselves unharmed? By scrutinizing molecular events inside Treg cells, Tao and collaborators implicate the activity of chromatin remodeling proteins. Initial evidence emerged from the observation that a histone deacetylase (HDAC) inhibitor increased the level of the chief transcription factor in Treg cells, Foxp3, as well as the number and activity of Treg cells in mice. Furthermore, in culture-stimulated Treg cells, HDAC9 was the most prominently expressed member of the deacetylase family, and Treg cell function was enhanced in its absence.

Acetylation of the Foxp3 protein itself was also observed and corresponded with an increase in Foxp3 binding to its target genes. Finally, HDAC inhibition helped curtail inflammatory T cell responses, both to transplanted grafts and in a model of inflammatory bowel disease. The finding that regulating acetylation/deacetylation has such a measurable influence on Treg function and that it operates at the level of both the Foxp3 locus and the transcription factor suggests that different avenues might be open for testing HDAC inhibition as a form of immunotherapy.

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