New classification of the shared epitope in rheumatoid arthritis: impact on the production of various anti-citrullinated protein antibodies

Ágnes Gyetvai, Zoltán Szekanecz, Lilla Soós, Zoltán Szabó, Andrea Fekete, Anikó Kapitány, Marius Teodorescu, Sándor Sipka, Gyula Szegedi and Gabriella Lakos

Abstract

Objective. HLA-DR [shared epitope (SE)] alleles have recently been re-classified into S1, S2, S3P and S3D groups. S2 and S3P have been associated with increased risk for RA. We assessed the impact of S1, S2, S3P and S3D alleles on anti-citrullinated protein antibody (ACPA) production. Instead of comparing allele-carriers to non-carriers, we studied each allele group individually, using the X/X (non-SE) genotype as reference.

Methods. Serum and genomic DNA samples of 91 RA patients and 78 healthy controls were obtained. Various ACPAs and IgM RF were determined by ELISA. HLA-DRB1 genotyping and subtyping was performed by PCR. HLA-DRB1 alleles were re-classified as described above. Correlations between SE and ACPAs were determined.

Results. Not only S2 and S3P, but, to a lesser extent, S1 and S3D alleles also predisposed to anti-cyclic citrullinated peptide (CCP) production (P<0.0001, P=0.004, P=0.01 and P=0.027, respectively), with the following hierarchy of association: S2+S3P>S1+S3D>X/X. Similar associations were observed for anti-citrullinated vimentin. Anti-citrullinated fibrinogen (CF) exerted a different association pattern with the strongest correlation with S1 alleles [odds ratio (OR) 16.00; P=0.05]. In addition, HLA-DRB1*15 alleles may represent a special predisposing effect for anti-CF antibody production. Finally, in this study, RF production was associated only with the HLA-DRB1*0401 SE allele (P=0.04).

Conclusions. Our approach of comparing individual S allele carriers with X/X genotype patients allowed us to perform unequivocal analyses and demonstrate new associations. Thus, novel subgroups of RA could be identified with potential relevance for prognosis and therapy.

Key words: Rheumatoid arthritis, Shared epitope, Anti-citrullinated protein antibodies, Anti-cyclic citrullinated peptide, Anti-citrullinated vimentin, Anti-citrullinated fibrinogen, Rheumatoid factor.

Introduction

RA is an autoimmune inflammatory disease that may lead to progressive joint destruction and disability. There is an immense need to determine prognostic indicators, including genetic and autoimmune factors that may predict the long-term outcome of the disease. Genetic contribution accounts for ∼60% of the risk for developing RA [1], with the MHC being the most important factor [2]. The association between RA and the HLA-DRw4 antigen was first demonstrated three decades ago [3]. In 1987, Gregersen et al. [4] observed that HLA-DRB1 alleles reported to be associated with RA share the RAA (arginine, alanine and alanine) amino acid motif at positions 72–74 of their third hypervariable region, which they hypothesized to act as a functional unit and called shared epitope (SE). HLA-DRB1 SE alleles are associated with susceptibility to RA [4, 5] and also with structural...
severity of the disease [5, 6]. Recently, they were also linked to the production of anti-citrullinated protein antibodies (ACPAs) [7–9]. Although there is a general agreement about the predisposing effect of SE alleles, not every allele confers the same risk [5, 10]. Moreover, the SE hypothesis does not fully explain the influence of HLA-DRB1 alleles on disease susceptibility [11, 12]. The protective effect of specific DRB1 alleles [13, 14] and of the presence of isoleucine (I) at position 67 or aspartic acid (D) at position 70 has also been suggested [15, 16].

Aiming for reconsideration of the SE hypothesis, a new classification system has recently been proposed by du Montcel et al. [17]. Authors suggest that the risk represented by the RAA sequence is modulated by the amino acids at positions 70 and 71. At position 71, lysine (K) confers the highest risk, arginine (R) intermediate risk, whereas alanine (A) and glutamic acid (E) lower risk. At position 70, glutamine (Q) and R represent higher risk than D [17]. Based on the type of amino acids at positions 70 and 71, the new classification system divides SE alleles into S1, S2, S3P and S3D groups and denotes all non-RAA motifs as X. A positive association with RA susceptibility was found for S2 and S3P allele carriers, whereas S1, S3D and X are low-risk alleles pooled together as L alleles [17]. The new system has since successfully been validated on French [18] and also on a large international population including Caucasians, Asians, Amerindians and African-American subjects [19]. A recent evaluation study on a UK Caucasian cohort also demonstrated a highly significant association of S2 and S3P alleles with RA, however, the authors could not fully substantiate the new classification regarding the effect of DRB1*13 and *1001 [20].

The new classification of HLA-DRB1 alleles has also been able to distinguish predisposing and protective alleles for RA-specific antibody production [21]. The presence of an S2 or S3P allele has been correlated with RF, anti-CCP and anti-citrullinated fibrinogen (CF) antibody production, whereas the presence of S3D and S1 alleles appeared to be protective [21]. These results, however, have not been supported by other papers. An earlier study [7] found significant association between anti-CF antibodies and DRB1*0404 alleles only, whereas in two cohorts of ACPA-positive patients with RA, SE alleles predisposed for the development of antibodies against citrullinated vimentin (CV), but not for the development of antibodies against CF [22]. A large study of North American RA patients reported that RF positivity was not significantly associated with the presence of SE alleles independent of anti-CCP antibodies [23], and these data were confirmed in a Dutch cohort [24].

In the present study, we wished to investigate the relevance of the du Montcel classification system in the production of RF and ACPAs including anti-CCP, anti-CF and anti-CV antibodies using a distinct approach. In previously published studies assessing the predisposing effect of DRB1 alleles, carriers have been compared with non-carriers. This type of analysis can be biased because of the adverse effect of other alleles. The du Montcel classification divides HLA-DRB1 alleles into five groups; consequently, a carrier of one particular HLA-DRB1 allele belonging to one of these groups can potentially have five different genotypes. For example, an S2 carrier’s genotype can be S2/S2, S2/S1, S2/S3P, S2/S3D and S2/X. The population that is used for comparison (non-carriers) can also carry four other types of allele combined into nine different genotypes.

In order to eliminate the modifying effect of other alleles, we examined the impact of S1, S2, S3P and S3D alleles on antibody production individually, always using the X/X genotype as reference. When analysing the effect of S1 alleles, for example, patients with S1/X and S1/S1 genotypes were compared with RA subjects with X/X genotype. This unique approach revealed that in contrast with previous findings, not only S2 and S3P, but also S1 and S3D alleles, predispose to the production of anti-CCP and also of anti-CV antibodies, with the following hierarchy regarding the strength of association: S2+S3P > S1+S3D > X/X. Anti-CF antibodies followed a different association pattern, and showed the strongest correlation with the S1 group of alleles. Finally, RF correlated with the presence of S2 alleles only, but probably not independently of ACPA production.

Patients and methods

Patients and controls

Ninety-one consecutive RA patients regularly seen by the Outpatient Clinic of the Department of Rheumatology, University of Debrecen, were involved in the study. All patients were Caucasians and met the ACR classification criteria for RA [25]. The clinical records of the patients were reviewed for classification. The group consisted of 76 females and 15 males. The mean (±s.d.) age of the patients was 53 (±11) years. For comparisons, we tested 78 age- and sex-matched healthy control subjects [67 females and 11 males; mean age 41 (±12) years]. Informed written consent was obtained from each participant. This study has been performed with the approval of the Institutional Review Board of the University of Debrecen Medical Center. This research has been carried out in compliance with the Declaration of Helsinki.

Autoantibodies

Serum samples were stored at −80°C until analysis. Anti-CCP and IgM RF were measured as described previously [26, 27] with QUANTA Lite™ CCP ELISA (Inova Diagnostics, San Diego, CA, USA) and with ImmuLisa™ RF IgM ELISA (Immco Diagnostics, Buffalo, NY, USA), respectively. Anti-CV antibodies were measured as anti-mutated CV (MCV) antibodies with Anti-MCV ELISA (Orgentec Diagnostika GmbH, Mainz, Germany). Anti-CF levels were assessed with in-house ELISA. In brief, plasminogen-depleted human fibrinogen was purchased from Calbiochem (EMD Chemicals, Gibbstown, NJ, USA) and IgG depleted on a protein G–sepharose column. Fibrinogen was then citrullinated.
using rabbit skeletal muscle peptidylarginine deiminase (Sigma-Aldrich, St Louis, MO, USA) at 3 U/mg fibrinogen ratio for 2 h at +37°C in 0.1 M Tris–HCl buffer (pH 7.4) with 10 mM CaCl₂ and 5 mM DTT. The reaction was terminated by adding 20 mM EDTA. CF (10 μg/ml) diluted in phosphate-buffered saline (PBS, pH 7.4) was coated on Nunc MaxiSorp microtitre plates overnight. Plates were subsequently blocked with StabiCoat (SurModics, Eden Prairie, MN, USA), and wells were incubated with sera diluted in the ratio 1:101 in PBS–Tween 20. Bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti-human IgG (Fcγ specific) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and TMB substrate solution. The cut-off was determined as the 99th percentile level of the results of 100 healthy blood bank donors. The analytical specificity of the system was verified by coating non-CF on ELISA plates. No binding of anti-CF-positive specimens occurred to non-CF under the same conditions. A positive specimen was assigned arbitrary units and was assayed as calibrator in each run together with positive and negative control specimens. Based on results obtained on 119 RA patients and 199 control subjects (including healthy controls and patients with other autoimmune diseases), the diagnostic sensitivity and specificity of the anti-CF ELISA were 54 and 99%, respectively. The sensitivity of the anti-CCP, anti-CV and RF assays were 75.8, 76.9 and 70.3%, respectively.

HLA-DRB1 genotyping

Genomic DNA was isolated from the peripheral blood of RA patients and controls using QIAamp Blood Mini Kit (Qiagen GmbH, Halden, Germany) according to the manufacturer’s instructions. HLA-DRB1 typing and subtyping was performed by PCR with sequence-specific primers (Olerup SSP; GenoVision, PA, USA and Dynal Biotech, Oslo, Norway), as described previously [28]. HLA-DRB1 alleles were grouped according to the new classification by du Montcel and colleagues [17]. The presence or absence of the RA-A2 amino acid sequence at positions 72–74 defines S and X alleles. S alleles can be subdivided into S1, S2 and S3 according to the amino acid at position 71 (S1: E-RAA or A-RAA; S2: K-RAA; and S3: R-RAA). Among S3 alleles, S3D and S3P alleles encode the D-R-RAA and the Q- or R-R-RAA sequence, respectively. S1 and S2 alleles had either Q or D at position 70.

Statistical analysis

In order to assess the risk for RA susceptibility, odds ratios (ORs) were calculated with 95% CIs for the presence of S1, S2, S3P, S3D and X alleles and the association was tested with Fisher’s exact test. Genotype risk analyses were conducted the same way. The correlations between HLA-DRB1 allele groups and RA-specific autoantibodies were first examined by comparing the distribution of positive and negative patients for the respective antibody among carriers and non-carriers with Fisher’s exact test. ORs (with 95% CI) were also calculated. Then the X/X genotype was selected as reference genotype and the risk for antibody development conferred by S1, S2, S3P and S3D alleles was calculated individually, comparing those carrying the respective allele only with the reference genotype. All P-values were two sided, and P < 0.05 was considered statistically significant. All statistical analyses were performed using the statistical package SPSS 11.0 (SPSS Institute, Chicago, IL, USA).

Results

HLA-DRB1 allele frequencies and the risk for RA

The frequencies of HLA-DRB1 alleles in RA patients and controls according to the du Montcel classification are shown in Table 1. The presence of S2 and S3P alleles was significantly associated with RA (P = 0.002 and 0.11, respectively), whereas the S1, S3D and X alleles were under-transmitted in RA subjects (P = 0.03 for S1; P = NS for S3D and X) (Table 1). As S1, S3D and X alleles represented similar low risk, they were pooled together as previously suggested [16, 17], and denoted with L. The corresponding genotype distribution analysis revealed that 59.3% of the RA patients carried one or two S2 and/or S3P alleles in comparison with 29.5% of controls (P < 0.0001). In addition, genotypes consisting of two predisposing alleles (S2/S3P, S3P/S3P or S2/S2) were found in RA patients only, but not in this cohort of controls (Table 2). S2/S3P, S3P/S3P and S2/S2 genotypes were associated with the greatest risk for RA (P < 0.0001), followed by the S2/L and S3P/L genotypes (P = 0.03 and 0.02, respectively), with the reference genotype being L/L (Table 2).

HLA-DRB1 allele carrier status and risk for RA according to ACPA status

Anti-CCP, anti-CV and anti-CF antibodies were present in the sera of 75.8, 76.9 and 51.6% of the RA patients, respectively. Six patients with anti-CCP antibodies were negative for anti-CV, and seven patients with anti-CF antibodies were negative for anti-CCP, whereas all anti-CF-positive cases carried either anti-CCP or anti-CV antibodies among carriers and non-carriers with Fisher’s exact test. All bold values show significant differences or correlations.

<table>
<thead>
<tr>
<th>Allele</th>
<th>RA patients, n = 91 (%)</th>
<th>Controls, n = 78 (%)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>21 (23.1)</td>
<td>31 (39.7)</td>
<td>0.45 (0.23, 0.88)</td>
<td>0.03</td>
</tr>
<tr>
<td>S2</td>
<td>26 (28.6)</td>
<td>7 (9.0)</td>
<td>4.06 (1.65, 9.98)</td>
<td>0.002</td>
</tr>
<tr>
<td>S3D</td>
<td>33 (36.3)</td>
<td>36 (46.2)</td>
<td>0.66 (0.36, 1.23)</td>
<td>0.45</td>
</tr>
<tr>
<td>S3P</td>
<td>35 (38.5)</td>
<td>16 (20.5)</td>
<td>2.42 (1.21, 4.84)</td>
<td>0.01</td>
</tr>
<tr>
<td>X</td>
<td>48 (52.7)</td>
<td>48 (61.5)</td>
<td>0.69 (0.38, 1.29)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

The n (%) values are for RA cases and controls. OR (95% CI) and P-value for Fisher’s exact test. All bold values show significant differences or correlations.
anti-CCV antibodies. Altogether, the frequency of ACPAs was 83.5%. It has previously been suggested that SE alleles show association only with anti-CCP-positive RA [6]. To test this hypothesis, patients were divided according to their antibody status, and the association between DRB1 alleles and RA was examined separately in the antibody-positive and -negative groups. The presence of S2 and/or S3P alleles associated strongly with anti-CCP- and anti-CCV-positive RA \( (P < 0.0001 \text{ for both}) \) but not with anti-CCP- or anti-CCV-negative disease (Table 3). Anti-CCF-positive and -negative RA, however, showed the same degree of positive association with S2 and S3P alleles \( (P = 0.001 \text{ and 0.004, respectively}) \).

**Relationship between HLA-DRB1 alleles and RA-specific antibodies**

When various HLA-DRB1 allele carriers in the RA group were compared with non-carriers (an approach previously used by other studies), a trend was found towards association between S2 and S3P alleles and anti-CCP and anti-CCV antibodies \( (OR 3.17 \text{ and 1.93 for anti-CCP, and OR 2.94 and 1.77 for anti-CCV antibodies}) \). Anti-CCF antibodies and RF followed a distinct trend: anti-CCF associated only with S3P alleles \( (OR 2.10) \), but not with S2 alleles, whereas RF correlated with S2 alleles \( (OR 2.15) \) (Table 4). S1 and S3D alleles have been previously designated as protective ones regarding antibody production. Our results did not fully reflect this protective effect, except for the negative association between RF and S3D alleles and between anti-CCF and S1 alleles (Table 4). Moreover, we found discrepancy between S1 and S3D alleles when compared with each other and also with X alleles regarding the tendencies they represented for antibody production, suggesting that pooling them together might mask some individual associations. Indeed, when X/X and L/L genotypes were compared with the rest of the patients, the protective effect of the X/X genotype proved to be stronger than that of the L/L genotype for each APCA (Table 4). No difference was seen, though, regarding IgM RF, suggesting that X1, S3D and X alleles may represent similar risk for the production of this antibody.

In order to eliminate the modifying effect of other DRB1 alleles occurring when carriers are compared with non-carriers, we next examined the impact of S1, S2, S3P and S3D alleles on antibody production individually, using the X/X genotype as reference. This analysis demonstrated a very strong predisposing effect of S2 and S3P alleles for the production of both anti-CCP \( (P < 0.0001 \text{ and 0.004}) \) and anti-CCV antibodies \( (P = 0.002 \text{ and 0.007}) \) (Table 5). Moreover, in contrast with earlier findings, S1 and S3D alleles were also found to predispose to the development of anti-CCP antibodies \( (P = 0.01 \text{ and 0.027}) \) and to a lesser extent to anti-CCV antibodies (Table 5). Because of their similar impact, S2 and S3P alleles, as well as S1 and S3D alleles were pooled together, and the resulting two groups were examined regarding their predisposing effects. The analysis revealed the following hierarchy: the S2+S3P group of alleles represented the highest degree of risk for

### Table 2 The effect of HLA-DRB1 genotype on RA susceptibility

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RA patients, ( n = 91 ) (%)</th>
<th>Controls, ( n = 78 ) (%)</th>
<th>OR (95% CI)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3P/S3P, S2/S3P and S2/S2</td>
<td>13 (14.3) 0 (0) ( ^* )</td>
<td>( &lt;0.0001 )</td>
<td>3.19 (1.18, 8.57) 0.03</td>
<td>2.42 (1.14, 5.11) 0.02</td>
</tr>
<tr>
<td>S2/L</td>
<td>26 (28.6) 16 (20.5)</td>
<td>( 2.42 (1.14, 5.11) 0.02 )</td>
<td>( 2.42 (1.14, 5.11) 0.02 )</td>
<td>( 0.31 (0.12, 0.79) 0.01 )</td>
</tr>
<tr>
<td>S3P/L</td>
<td>27 (30.0) 17 (21.8)</td>
<td>( 2.42 (1.14, 5.11) 0.02 )</td>
<td>( 2.42 (1.14, 5.11) 0.02 )</td>
<td>( 0.31 (0.12, 0.79) 0.01 )</td>
</tr>
<tr>
<td>L/L</td>
<td>37 (40.7) 55 (70.5) 1</td>
<td>( 2.42 (1.14, 5.11) 0.02 )</td>
<td>( 2.42 (1.14, 5.11) 0.02 )</td>
<td>( 0.31 (0.12, 0.79) 0.01 )</td>
</tr>
</tbody>
</table>

The L alleles are the low-risk alleles S1, S3D and X. S2/S3P, S3P/S3P and S2/S2 genotypes were pooled together because of low frequency. The reference genotype is L/L. \( P \)-values were calculated with Fisher’s exact test. All bold values show significant differences or correlations. \( ^* \) OR cannot be calculated because of lack of corresponding genotypes in controls.

### Table 3 Relationship between HLA-DRB1 allele carrier status and ACPA-positive and -negative RA

<table>
<thead>
<tr>
<th>S2 and/or S3P carrier</th>
<th>Yes, ( n ) (%)</th>
<th>No, ( n ) (%)</th>
<th>OR (95% CI)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CCP-positive RA, ( n = 69 )</td>
<td>46 (50.5) 23 (25.3)</td>
<td>( 4.78 (2.38, 9.61) )</td>
<td>( &lt;0.0001 )</td>
<td></td>
</tr>
<tr>
<td>Anti-CCP-negative RA, ( n = 22 )</td>
<td>8 (8.8) 14 (15.4)</td>
<td>( 1.37 (0.51, 3.70) )</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Anti-CV-positive RA, ( n = 70 )</td>
<td>45 (49.5) 25 (27.5)</td>
<td>( 4.30 (2.16, 8.58) )</td>
<td>( &lt;0.0001 )</td>
<td></td>
</tr>
<tr>
<td>Anti-CV-negative RA, ( n = 21 )</td>
<td>9 (9.9) 12 (13.2)</td>
<td>( 1.79 (0.67, 4.84) )</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Anti-CF-positive RA, ( n = 47 )</td>
<td>29 (31.9) 18 (19.8)</td>
<td>( 3.85 (1.80, 8.27) )</td>
<td>( &lt;0.0001 )</td>
<td></td>
</tr>
<tr>
<td>Anti-CF-negative RA, ( n = 44 )</td>
<td>25 (27.5) 19 (20.9)</td>
<td>( 3.15 (1.46, 6.80) )</td>
<td>( 0.004 )</td>
<td></td>
</tr>
</tbody>
</table>

The \( n \) (\%) values of RA cases and controls in each category. OR (95% CI) and \( P \)-value for Fisher’s exact test. All bold values show significant differences or correlations.
### Table 4: Association between RA-specific antibodies and HLA-DRB1 allele carrier status according to the du Montcel classification in RA patients

<table>
<thead>
<tr>
<th>S1 allele, OR (95% CI)</th>
<th>S2 allele, OR (95% CI)</th>
<th>S3P allele, OR (95% CI)</th>
<th>S3D allele, OR (95% CI)</th>
<th>X allele, OR (95% CI)</th>
<th>X/X genotype, OR (95% CI)</th>
<th>L/L genotype, OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CCP 0.74 (0.25, 2.22)</td>
<td>3.17 (0.85, 11.81)</td>
<td>1.93 (0.68, 5.54)</td>
<td>1.30 (0.47, 3.59)</td>
<td>0.43 (0.16, 1.18)</td>
<td>0.04** (0.004, 0.35)</td>
<td>0.029* (0.011, 0.78)</td>
</tr>
<tr>
<td>Anti-CV 0.37 (0.13, 1.08)</td>
<td>2.94 (0.78, 10.99)</td>
<td>1.77 (0.61, 5.10)</td>
<td>1.18 (0.42, 3.31)</td>
<td>0.79 (0.42, 3.31)</td>
<td>0.09*** (0.02, 0.53)</td>
<td>0.42 (0.15, 1.13)</td>
</tr>
<tr>
<td>Anti-CF 1.04 (0.39, 2.76)</td>
<td>0.74 (0.29, 1.83)</td>
<td>2.10 (0.88, 4.98)</td>
<td>0.99 (0.42, 2.33)</td>
<td>0.73 (0.30, 2.12)</td>
<td>0.14 (0.02, 1.19)</td>
<td>0.82 (0.35, 1.19)</td>
</tr>
<tr>
<td>RF 0.80 (0.28, 2.28)</td>
<td>2.15 (0.71, 6.47)</td>
<td>1.09 (0.43, 2.76)</td>
<td>*<em>0.39</em> (0.16, 0.99)</td>
<td>1.05 (0.43, 2.59)</td>
<td>0.53 (0.11, 2.56)</td>
<td>0.65 (0.26, 1.60)</td>
</tr>
</tbody>
</table>

Allele carriers were compared with non-carriers. *P < 0.05; **P = 0.001; ***P = 0.007. All bold values show significant differences or correlations.

### Table 5: Predisposing effect of HLA-DRB1 alleles to RA-specific antibody production

<table>
<thead>
<tr>
<th></th>
<th>Anti-CCP</th>
<th></th>
<th>Anti-CV</th>
<th></th>
<th>Anti-CF</th>
<th></th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P-values</td>
<td>OR (95% CI)</td>
<td>P-value</td>
<td>OR (95% CI)</td>
<td>P-value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>S2, n = 11</td>
<td>33.00 (2.46, 443.60)</td>
<td>&lt;0.0001</td>
<td>30.00 (2.19, 410.99)</td>
<td>0.007</td>
<td>13.50 (1.20, 152.41)</td>
<td>NS</td>
<td>-a</td>
</tr>
<tr>
<td>S3P, n = 13</td>
<td>58.00 (5.12, 657.43)</td>
<td>&lt;0.0001</td>
<td>37.50 (4.25, 330.63)</td>
<td>0.001</td>
<td>8.77 (0.94, 81.67)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S2/S3P, n = 32</td>
<td>27.00 (1.98, 368.38)</td>
<td>0.01</td>
<td>11.25 (1.19, 106.12)</td>
<td>0.049</td>
<td>16.00 (1.32, 194.62)</td>
<td>0.05</td>
<td>2.00 (0.27, 14.78)</td>
</tr>
<tr>
<td>S1, n = 11</td>
<td>13.20 (1.24, 140.68)</td>
<td>0.027</td>
<td>7.50 (1.02, 54.99)</td>
<td>0.06</td>
<td>6.00 (0.58, 61.84)</td>
<td>NS</td>
<td>1.25 (0.21, 7.62)</td>
</tr>
<tr>
<td>S3D, n = 16</td>
<td>16.50 (1.71, 159.13)</td>
<td>0.007</td>
<td>8.21 (1.30, 51.99)</td>
<td>0.025</td>
<td>7.85 (0.84, 73.46)</td>
<td>NS</td>
<td>1.50 (0.28, 8.04)</td>
</tr>
</tbody>
</table>

ORs, 95% CI and P-value are for Fisher’s exact test. Reference genotype: X/X. All bold values show significant differences or correlations. NA: not applicable; S2: S2/S2 and S2/X genotypes; S3P: S3P/S3P and S3P/X genotypes; S2/S3P: S2/S2, S2/X, S3P/S3P, S3P/X and S2/S3P genotypes; S1: S1/S1 and S1/X genotypes; S3D: S3D/S3D and S3D/X genotypes; S1/S3D: S1/S1, S1/X, S3D/S3D, S3D/X and S1/S3D genotypes. aOR cannot be calculated; all subjects in the group are positive for the respective antibody.
producing anti-CCP (OR 58.00; \( P < 0.0001 \)) and anti-CV antibodies (OR 37.50; \( P = 0.001 \)), followed by the S7+S3D group of alleles (OR 16.50; \( P = 0.007 \) and OR 8.21; \( P = 0.025 \)), being the X/X group as reference genotype (OR 1.0). The presence of anti-CF antibodies correlated with each SE allele group, but the pattern was different from what was seen for anti-CCP and anti-CV antibodies. The strongest association was observed with S7 alleles. It is noteworthy that in the S7 group (nine S1/X and two S1/S7 genotypes), eight RA patients carried one or two copies of the HLA-DRB1*15 allele, and seven of them were anti-CF antibody positive. Overall, the S2+S3D group of alleles represented a similar risk for the development of anti-CF antibodies (OR 8.77; \( P = 0.04 \)) to the S1+S3D group (OR 7.85; \( P = 0.09 \)). IgM RF showed significant association with S2 alleles only (\( P = 0.04 \)). This association, however, may not be independent of the presence of ACPAs, as all RA patients in the group had not only RF, but also anti-CCP antibodies. In this case, because of the striking difference in predisposing effects between S2 and S3P alleles, they were not pooled together.

Regarding anti-CCP isotypes other than IgG, we also calculated the correlations with IgM anti-CCP according to the new SE classification, but only demonstrate a non-significant trend for association between IgM anti-CCP antibodies and S2, S3P, S1 and S3D alleles (data not shown).

Classifying the RA patients according to their genotype (S2/L, S3P/L, L/L, S2/S2, S3P/S3P and S2/S3P) revealed that a double dose of S2 and/or S3P allele did not significantly strengthen the risk for anti-CCP or anti-CV production, as the majority of the patients was positive even in the presence of a single dose of S2 and/or S3P. In the case of anti-CF and RF, some additive effect was observed in those with a double dose of S2 and/or S3P allele; however, the association did not reach statistical significance when compared with those with a single dose or no SE allele at all (data not shown).

**Discussion**

In spite of accumulating evidence on the significance of SE alleles in RA, results are controversial. The SE hypothesis does not fully explain the influence of MHC alleles on disease susceptibility [11, 12], and different SE alleles have been associated with variable effect on risk for RA [5, 10], disease severity [5, 6, 29] or ACPA production [7–9, 21].

A new classification of SE alleles by du Montcel et al. [17] attempted to stratify these alleles according to the risk they confer to the development of RA. Based on amino acid sequence at positions 70 and 71, the new classification system divided SE alleles into S1, S2, S3P and S3D groups, and demonstrated association with RA susceptibility for S2 and S3P allele carriers [17]. Subsequent validation studies also supported the risk conferred by S2 and S3P alleles for RA [18, 19]. In our present study, we were able to confirm the predisposing effect of S2 and S3P alleles for RA, and in agreement with previous results, we demonstrated similar low risk associated with the presence of S1, S3D and X alleles. Although we could not examine separately the effect of S2/S3P, S3P/S3P and S2/S2 genotypes because of the low number of subjects, we demonstrated the same risk hierarchy as earlier studies: S2/S3P, S3P/S3P and S2/S2 genotypes represented the greatest risk for RA, followed by the S2/L and S3P/L genotypes.

Earlier data suggest that the presence of HLA-DRB1 SE alleles is associated only with a particular phenotype of RA, namely, anti-CCP antibody-positive disease [7]. However, RA patients’ sera may contain antibodies against several citrullinated epitopes present on different antigenic targets. Besides anti-CCP, anti-CV and anti-CF have been associated not only with the presence of RA, but also with more severe disease [30–32]. Nonetheless, the genetic background of anti-CV- and anti-CF-positive and -negative RA has not been investigated so far. Our results revealed that the S2 and/or S3P carrier status is strongly associated with not only anti-CCP-positive RA, but also with anti-CV positive RA, whereas no correlation was found with anti-CCP- or anti-CV-negative disease. On the contrary, the ratio of S2+S3 carriers and non-carriers was similar in anti-CF-positive and -negative RA, but different from what was found in the controls. These data suggest that S2+S3 carriers have the same risk for developing anti-CF antibody-positive RA as non-carriers. Although the high level of agreement between various ACPAs is well documented, it is also known that disagreement between various ACPA tests does occur [27, 31, 33]. In our cohort, the agreement rate between anti-CCP and anti-CV antibodies was >90%, and all anti-CF-positive cases carried either anti-CCP or anti-CV antibodies. Nonetheless, we demonstrated important differences regarding the association of various ACPAs with S2 and S3P alleles, suggesting that different ACPAs may associate with different subtypes of RA and may carry different clinical/prognostic information.

Recent publications based on the du Montcel classification system has differentiated between predisposing and protective alleles regarding structural severity [29], and also regarding RF, anti-CCP and anti-CF antibody production [21]. In these studies, allele carriers were compared with non-carriers. When utilizing the same approach, our results supported the findings of Gourrad et al. [21] regarding anti-CCP, but not regarding anti-CF and RF. Available data on genetic susceptibility for the development of anti-CF antibodies are controversial. Separate studies demonstrated association with S2 and S3P alleles [21], with DRB1*0404 allele (part of the S3P group) only [8], or lack of association with SE [22]. RF production has also been linked to S2 and S3P alleles previously [21], whereas other studies did not find association between the presence of SE alleles and RF [23, 24]. These inconsistent data—together with our findings pointing towards differences between S1, S3D and X alleles regarding their effect on antibody production—suggest that the adverse effect of other alleles may introduce a potential bias in analyses comparing carriers...
with non-carriers, and may lead to controversial results. To eliminate the modifying effect of other DRB1 alleles, we examined the impact of S1, S2, S3P and S3D allele groups on antibody production individually, using the X/X genotype as reference. This approach confirmed the previously described predisposing effect of S2 and S3P alleles for the production of anti-CCP antibodies, but in contrast with earlier findings, revealed significant association with S1 and S3D alleles, too. Correlation with anti-CV antibodies followed the same pattern. Overall, the S2+S3P group of alleles represented the highest degree of risk for producing anti-CCP and anti-CV antibodies, followed by the S1+S3D group of alleles, with the X/X group as reference genotype. The Gourraud study [21] demonstrated significant negative association between anti-CCP and S3D alleles even after controlling for the adverse effect of S2 and S3P in the analysis of S3D; however, as the S2+S3P group confers higher risk than the S1+S3D group, comparing them with each other can lead to the false impression that S1 or S3D alleles are protective compared with S2 or S3P alleles.

We examined, for the first time to our knowledge, the genetic background of anti-CV antibody production and revealed that anti-CF antibodies tended to associate with each SE allele group, especially with S1 alleles. This association pattern was clearly different from the pattern followed by anti-CCP and anti-CV antibodies, and resulted in similar overall impact of S2+S3P and S1+S3D alleles on anti-CF antibody development. This finding explains the comparable distribution of S2+S3P carriers and non-carriers in our CF-positive and -negative RA cohort, and also the lack of association between anti-CF antibodies and SE alleles (categorized later as S2 and S3P) in a previously published analysis [22]. It is of particular interest that 8 out of 11 patients in the S1 group carried one or two copies of the DRB1*15 allele and 7 of them developed anti-CF antibodies. Scarce reports [34–36] have previously suggested an association between the DRB1*15 allele and RA and also with the production of anti-CCP antibodies [37]. As all anti-CF-positive patients in our study population were either anti-CCP or anti-CV antibody positive, the association between the DRB1*15 allele and anti-CF antibodies could not be evaluated independently from other ACPAs. However, our results point to the special predisposing effect of DRB1*15 alleles for anti-CF production.

Another surprising result was the association of RF with S2 alleles and the lack of association with other allele groups. As all but one patient in the S2 group carried one or two copies of the *0401 allele, this practically means association with this particular allele. The RF-positive and anti-CCP-positive populations are known to largely overlap in RA. We could not examine the susceptibility for RF production independently from ACPAs, as all patients in the S2 group produced not only RF, but also anti-CCP antibodies. However, no other group of alleles correlated with RF production in spite of their strong association with anti-CCP antibodies.

This result is in agreement with our earlier statement demonstrating a similar effect of X/X and L/L (S1+S3D+X) genotypes on RF production.

Why could particular alleles be associated with particular citrullinated protein antibodies? SE forms one of the peptide-anchoring pockets of MHC class II molecules (known as P4). The interaction of antigenic peptides with this MHC anchoring pocket may be critical in initiating the autoimmune processes. Hill et al. [38] demonstrated that the conversion of arginine to citrulline at the peptide side chain position interacting with the SE significantly increases peptide–MHC affinity and leads to the activation of CD4+ T cells in DR4-IE transgenic mice. Only MHC class II molecules with the SE had an increased affinity for the citrulline-containing peptide. The authors suggested that this phenomenon is caused by the different charge interactions made between the P4 pocket and citrullinated antigens. However, Rosloniec et al. [39] showed that there are amino acid sequences with the same charge that have different binding affinities. Therefore, physico-chemical properties other than the electric charge of the peptide-binding pocket are probably involved in the peptide-binding affinity of the HLA-DR heterodimer.

**Conclusions**

In summary, our approach of comparing individual S allele carriers with X/X genotype patients allowed us to perform unequivocal analyses and demonstrate new associations. We show that the S2+S3P carrier status is strongly associated with anti-CCP- and also with anti-CV-positive RA, but not with anti-CCP- or anti-CV-negative disease. On the contrary, no difference was seen in the number of S2+S3D carriers between anti-CF-positive and -negative RA. We revealed that the presence not only of S2 and S3P, but also of S1 and S3D alleles predisposes for the production of anti-CCP antibodies, and established the following hierarchy regarding the strength of association: S2+S3P >S1+S3D > X/X. We also demonstrated the same association pattern for anti-CV antibodies. Anti-CF antibodies, however, displayed a different association pattern characterized by the strongest correlation with S1 alleles, resulting in a similar degree of correlation of S2+S3P and S1+S3D alleles with anti-CF antibody development. Based on our results, we propose that the presence of DRB1*15 alleles represents a special predisposing effect for anti-CF antibody production. Our data also suggest that the genetic susceptibility for developing RF segregates from the susceptibility for ACPAs as RF associates with only one of the SE alleles, specifically the DRB1*0401 allele. These results may facilitate the identification of risk patterns and subclassification of RA.

---

**Rheumatology key messages**

- According to the new SE classification, S2 and S3P carry increased risk for RA.
- S2, S3P and, to a lesser degree, S1 and S3D may predispose to ACPA production.

---

31
Acknowledgements

The authors are grateful to the RA patients and control subjects for their participation, as well as Ms Krisztina Biróne Barna, Ms Andrea Nagy and Ms Györgyne Deák for the essential technical help in the genotyping. Authors also thank Ms Katalin Hodosi for data analysis and technical assistance.

Funding: This work was supported by OTKA research grant T 048541 (Z.S.), as well as by the Hungarian Academy of Sciences Autoimmune Diseases Research Group (G.S.).

Disclosure statement: The authors have declared no conflicts of interest.

References


7 van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Huizinga TW, Toes RE, de Vries RR. The HLA-DRB1 shared epitope alleles are primarily a risk factor for anti-cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. Arthritis Rheum 2006;54:1117–21.


