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

Anikó Kapitány MD PhD[1,3,*], Zoltán Szabó MD PhD[2,*], Gabriella Lakos MD PhD[1], Magdolna Aleksza MD PhD[1], Anikó Végvári MD[2], Lilla Soós MD[2], Zsolt Karányi MD[4], Sándor Sipka MD PhD DSc[1], Gyula Szegedi MD PhD DSc[3] and Zoltán Szekanecz MD PhD DSc[2]

[1]Laboratory of Immunology and [2]Division of Rheumatology, Third Department of Medicine, University of Debrecen Medical Centre, Debrecen, Hungary

[3]Research Group of Autoimmune Diseases, Hungarian Academy of Sciences, Debrecen, Hungary

[4]First Department of Medicine, University of Debrecen Medical Centre, Debrecen, Hungary

**Key words:** rheumatoid arthritis, anti-CCP, rheumatoid factor, shared epitope, HLA-DR

## 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 = 5.828, $P < 0.01$). 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*01 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 is extremely useful in early RA when anti-CCP positivity may precede clinical symptoms by years [10,11]. 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

* A. Kapitány and Z. Szabó are co-primary authors who contributed equally to the study

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 Hybaid 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 MultiImage Light Cabinet (Alpha 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 ±

MHC = major histocompatibility complex
ELISA = enzyme-linked immunosorbent assay
HLA = human leukocyte antigen

PCR = polymerase chain reaction

**Table 1.** Associations between serum anti-CCP, CRP and RF concentrations and shared epitope alleles in RA patients

| | DRB1*01/04 (n=3) | DRB1*01/X (n=13) | DRB1*04/04 (n=3) | DRB1*04/X (n=11) | DRB1*X/X (n=23) | All DRB1*04 positive (n=17) | All DRB1*04 negative (n=36) | $P^1$* |
|---|---|---|---|---|---|---|---|---|
| Anti-CCP (U/ml) | 530.5 ± 174.2 | 12.0 ± 8.6 | 439.5 ± 182.8 | 591.0 ± 164.2 | 76.8 ± 56.2 | 530.0 ± 182.6 | 56.8 ± 27.4 | < 0.01 |
| CRP (mg/L) | 5.5 ± 1.2 | 9.5 ± 5.6 | 10.6 ± 2.8 | 6.9 ± 2.7 | 4.3 ± 3.1 | 7.1 ± 2.4 | 6.5 ± 2.2 | NS |
| RF (U/ml) | 16.5 ± 6.2 | 29.5 ± 9.8 | 85.5 ± 34.6 | 70.0 ± 46.2 | 71.0 ± 38.4 | 63.5 ± 28.4 | 57.5 ± 22.6 | NS |

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

174.2 U/ml; *04/*04: 439.5 ± 182.8 U/ml; *04/*X: 591.0 ± 164.2 U/ml; all DRB1*04 positive: 530.0 ± 182.6 U/ml) had significantly increased anti-CCP values compared to HLA-DR4-negative patients (DRB1*01/*X: 12.0 ± 8.6 U/ml; *X/*X: 76.8 ± 56.2 U/ml; all DRB1*04 negative: 56.8 ± 27.4 U/ml) (all DRB1*04 positive versus negative: $P < 0.01$) [Table 1, Figure 1]. There was no difference in serum anti-CCP antibody concentrations between patients carrying only one HLA-DRB1*01 allele but no HLA-DRB1*04 allele (DRB1*01/*X) (12.0 ± 8.6 U/ml) in comparison to SE-negative (DRB1*X/*X) patients (76.8 ± 56.2 U/ml) [Table 1]. Thus patients with HLA-DRB1*01/*04, DRB1*04/*04 or DRB1*04/*X genotypes exhibited an overall tenfold increase in serum anti-CCP antibody levels in comparison to HLA-DRB1*04-negative subjects [Table 1]. When comparing patients carrying one copy of HLA-DRB1*04 to those carrying two copies, we did not find any difference in anti-CCP antibody production (data not shown).

We investigated possible associations of serum RF, CRP and anti-CCP antibody concentrations with other HLA-DRB1 genotypes (HLA-DRB1*03, *07, *08, *11, *13, *14, *15 and *16). The number of patients in each group was rather low for statistical analysis. We did not find any notable associations between serum RF or CRP levels and any HLA-DR subtype. Interestingly, all six patients (100%) carrying DRB1*15 alleles and 6 of 7 (85%) carrying DRB1*13 were anti-CCP positive. In addition, apart from HLA-DRB1*01 and *04 described above, patients with HLA-DRB1*13 (282.5 ± 23.8 U/ml) and DRB1*15 (398.7 ± 76.2 U/ml) had significantly higher serum anti-CCP concentrations than any other HLA-DR subtypes ($P < 0.01$). In contrast, only 45–55% of patients carrying DRB1*03, *07, *08, *11, *14 or *16 had elevated serum anti-CCP antibody levels [Figure 2].

## 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

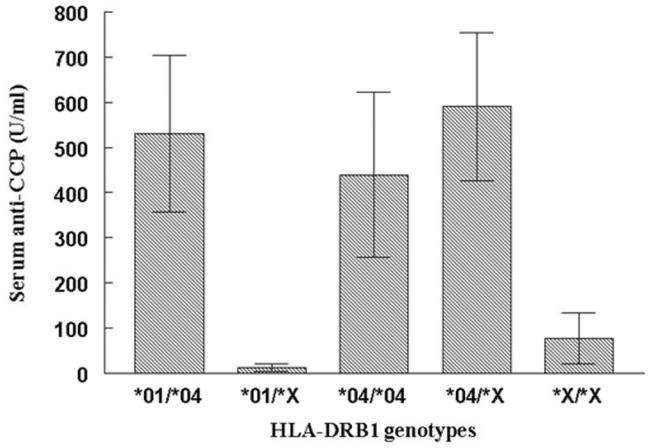


**Figure 1.** Serum anti-CCP antibody concentrations associated with various HLA-DR1 and HLA-DR4 genotypes

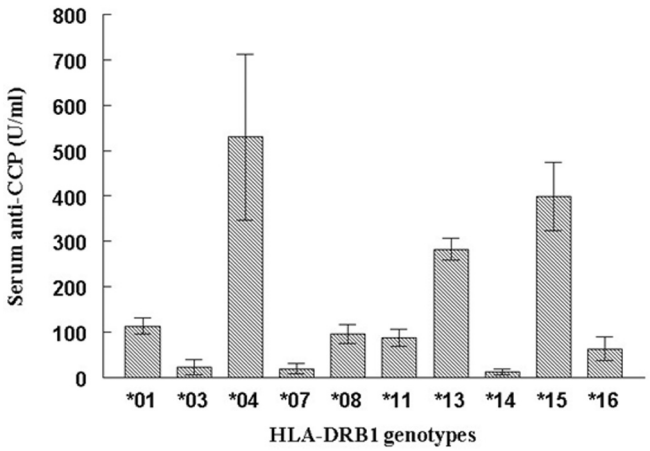


**Figure 2.** Serum anti-CCP antibody levels associated with various HLA-DRB1 genotypes

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 [25]. 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

1. Harris ED Jr. Rheumatoid arthritis: pathophysiology and implications for therapy. N Engl J Med 1990;332:1277–87.
2. Weyand CM, Hicok KC, Conn DL, Goronzy JJ. The influence of HLA-DRB1 genes on disease severity in rheumatoid arthritis. Ann Intern Med 1992;117:801–6.
3. Gregersen PK, Silver J, Winchester RF. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987;30:1205–13.
4. Hassfeld W, Steiner G, Graninger W, Witzmann G, Schweitzer H, Smolen JS. Autoantibody to the nuclear antigen RA33; a marker for early rheumatoid arthritis. Br J Rheumatol 1993;32:199–203.
5. Deprés N, Boire G, Lopez-Longo FJ, Ménard HA. The SA system: a novel antigen-antibody system specific for rheumatoid arthritis. J Rheumatol 1994;21:1027–33.
6. Nienhuis RLF, Mandema EA. A new serum factor in patients with rheumatoid arthritis: the antiperinuclear factor. Ann Rheum Dis 1964;23:302–5.
7. Visser H, Gelinck LB, Kampfraath AH, Beedveld FC, Hazes JM. Diagnostic and prognostic characteristics of the enzyme linked immunosorbent rheumatoid factor assay in rheumatoid arthritis. Ann Rheum Dis 1996;55:157–61.
8. Pepys MB. C-reactive protein: the role of an ancient protein in modern rheumatology. Clin Exp Rheumatol 1983;1:3–7.
9. Schellenkens GA, de Jong BAW, van den Hoogen FHJ., van de Putte LBA, van Verooij WJ. Citrulline is an essential constituent of antigenic determinants recognised by rheumatoid arthritis-specific autoantibodies. J Clin Invest 1998;101:273–81.
10. Schellekens GA, Visser H, de Jong BA, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum 2000;43:155–63.
11. Nielen MM, Van Schaardenburg D, Reesink HW, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum 2004;50:380–6.
12. Forslind K., Ahlmén M., Eberhardt K., Hafström I., Svensson B; for the BARFOT Study Group. Prediction of radiological outcome in early rheumatoid arthritis in a clinical practice: role of antibodies to citrullinated peptides (anti-CCP). Ann Rheum Dis 2004;63:1090–5.
13. Gorman JD, David-Vaudey E, Pai M, Lum RF, Criswell LA. Particular HLA-DRB1 shared epitope genotypes are strongly associated with rheumatoid arthritis. Arthritis Rheum 2004;50:3476–84.
14. Seidl C, Koch U, Buhleier T, et al. HLA-DRB1*04 subtypes are associated with increased inflammatory activity in early rheumatoid arthritis. Br J Rheumatol 1997;36:941–4.
15. Varga É, Palkonyai É, Temesvári P, Tóth F, Petri IB. The role of HLA-DRB1*04 alleles and their association with HLA-DQB genes in genetic susceptibility to rheumatoid arthritis in Hungarian patients. Acta Microbiol Immunol Hung 2003;50:33–41.
16. Stastny P. Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. N Engl J Med 1978;298:869–71.
17. Watanabe Y, Tokunaga K, Matsuki K, et al. Putative amino acid sequence of HLA-DRB chain contributing to rheumatoid arthritis susceptibility. J Exp Med 1989;169:2263–8.
18. Kapitany A, Zilahi E, Szanto S, et al. Association of rheumatoid arthritis with HLA-DR1 and HLA-DR4 in Hungary. Ann NY Acad Sci 2005;1051:263–70.
19. van Gaalen FA, van Aken J, Huizinga TW, et al. Association between HLA class II genes and autoantibodies to cyclic citrullinated peptides (CCPs) influences the severity of rheumatoid arthritis. Arthritis Rheum 2004;50:2113–21.
20. Irigoyen P, Lee AT, Wener MH, et al. Regulation of anti-cyclic citrullinated peptide antibodies in rheumatoid arthritis: contrasting effects of HLA-DR3 and the shared epitope alleles. Arthritis Rheum 2005;52:3813–18.

21. van Zeben D, Hazes JM, Zwinderman AH, et al. Association of HLA-DR4 with a more progressive disease course in patients with rheumatoid arthritis. *Arthritis Rheum* 1991;34:822–30.
22. Visser H, le Cessie S, Vos K, Breedveld FC, Hazes JMW. How to diagnose rheumatoid arthritis early: a prediction model for persistent (erosive) arthritis. *Arthritis Rheum* 2002;46:357–65.
23. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315–24.
24. Ollerup O, Zetterquist H. HLA typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992;39:225–35.
25. Fries JF, Wolfe F, Apple R, et al. HLA-DRB1 genotype associations in 793 white patients from a rheumatoid arthritis inception cohort: frequency, severity, and treatment bias. *Arthritis Rheum* 2002;46:2320–9.

**Correspondence:** Dr. Z. Szekanecz, Third Department of Medicine, Rheumatology Division, University of Debrecen Medical Centre, 22 Móricz Street, Debrecen, H-4004, Hungary.
Phone: (36-52) 314-091; Fax: (36-52) 414-489
email: szekanecz@iiibel.dote.hu