

Association Between HLA Class II Genes and Autoantibodies to Cyclic Citrullinated Peptides (CCPs) Influences the Severity of Rheumatoid Arthritis

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Objective. The functional role of HLA class II molecules in the pathogenesis of rheumatoid arthritis (RA) is unclear. HLA class II molecules are involved in the interaction between T and B lymphocytes required for long-lived B cell responses and generation of high-affinity IgG antibodies. We undertook this study to investigate the relationship between HLA class II gene polymorphisms and RA-specific IgG antibodies against cyclic citrullinated peptides (anti-CCP antibodies).

Methods. High-resolution HLA-DR and DQ typing and anti-CCP-2 antibody testing were performed on 268 RA patients from the Early Arthritis Clinic cohort at the Department of Rheumatology of the Leiden University Medical Center. The presence of anti-CCP antibodies was analyzed in carriers of the different DR and DQ alleles. Disease progression was measured over a period of 4 years by scoring radiographs of the hands and feet using the Sharp/van der Heijde method.

Results. Carriership of the individual alleles

HLA-DRB1*0401, DRB1*1001, DQB1*0302, and DQB1*0501 was associated with the presence of anti-CCP antibodies. Carriers of DQ-DR genotypes containing proposed RA susceptibility alleles were significantly more often anti-CCP antibody positive. Carriership of one or two HLA-DRB1 shared epitope (SE) alleles was significantly associated with production of anti-CCP antibodies (odds ratio [OR] 3.3, 95% confidence interval [95% CI] 1.8–6.0 and OR 13.3, 95% CI 4.6–40.4, respectively). An increased rate of joint destruction was observed in SE+, anti-CCP+ patients (mean Sharp score 7.6 points per year) compared with that in SE-, anti-CCP+ patients (2.4 points per year) ($P = 0.04$), SE+, anti-CCP- patients (1.6 points per year) ($P < 0.001$), and SE-, anti-CCP- patients (1.6 points per year) ($P < 0.001$).

Conclusion. HLA class II RA susceptibility alleles are associated with production of anti-CCP antibodies. Moreover, more severe disease progression is found in RA patients with both anti-CCP antibodies and SE alleles.

Rheumatoid arthritis (RA) is a chronic, inflammatory joint disease with autoimmune features. Previous studies have indicated that the risk of RA in siblings of affected individuals is 2–17 times higher than in siblings of unaffected individuals, pointing to a contribution of genetic factors in the pathogenesis of RA (1). An association between RA and the HLA complex, also known as the major histocompatibility complex (MHC), has long been observed in many different populations, and this is thought to account for approximately one-third of the genetic component of RA susceptibility (2).

Various paradigms have been put forward to

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explain the observed association between HLA and RA. It has been proposed that a conserved motif in the third hypervariable region (HVR3) of certain HLA-DR class II alleles plays a role in the presentation of "arthritogenic" antigens to T lymphocytes, the "shared epitope" (SE) hypothesis (3). Not only does carriage of SE alleles increase the risk of RA, but in RA patients, these alleles are also associated with a more severe disease type (4,5). However, since there are differences in the strength of association between SE alleles and RA, and since no convincing demonstration of the mechanisms underlying the associations is currently available, other paradigms that refine or complement the SE hypothesis have been put forward (6,7).

A prominent feature of RA is the presence of various types of disease-specific and non-disease-specific autoantibodies (8). Best known are the rheumatoid factors (RFs), which are antibodies to the Fc portion of IgG molecules. Although they are not disease specific, detection of IgM-RF is routinely used in the diagnosis of RA (sensitivity 60–70%, specificity 80–90%).

Recently developed assays detecting IgG antibodies against cyclic citrullinated peptides (anti-CCP antibodies) have a higher specificity of 98% at a similar sensitivity of 68–80% (9). Interestingly, using stored samples obtained from patients with RA, anti-CCP antibodies have been detected up to 9 years before the onset of arthritis (10). This suggests that production of these antibodies may be one of the earliest events in the disease. Indeed, we have recently shown that the presence of anti-CCP antibodies accurately predicts progression to RA in patients with undifferentiated arthritis (11).

Similar to IgM-RF, anti-CCP antibodies are a marker of severe disease. It has been shown that anti-CCP antibody-positive RA patients develop significantly more severe radiographic damage than do RA patients who are anti-CCP antibody negative (12).

Anti-CCP antibodies are directed against antigens containing the nonstandard amino acid citrulline (13), for example, citrullinated fibrin (14), which is found in the rheumatoid joint (15,16). Although citrullination or deimination of arginine residues of proteins may result in the formation of epitopes that are targets of antibodies, help from T lymphocytes is likely required for long-term B cell responses and antibody isotype switching. Accordingly, in mice transgenic for the SE allele HLA-DRB1*0401, it was recently demonstrated that citrullination of peptides could lead to the activation of CD4+ T cells, most likely as a result of increased

binding of these citrullinated peptides to MHC class II molecules (17).

In RA patients, anticitrulline antibody production has been reported to be associated with the presence of certain HLA alleles (18,19). Using the antifilagrin antibody (AFA) test, which is less sensitive than current anti-citrullinated peptides antibody tests (18), Bas et al showed that RA patients with AFAs were more likely to be carriers of SE alleles DRB1*01 and *04 (19). That study, however, was restricted to the carrier status of these two SE alleles only.

Given these results and the notion that anti-CCP antibodies are highly specific for RA, a thorough analysis of HLA class II alleles involved in generating autoantibodies to citrullinated antigens is important to gain a better understanding of this particular immune response. In this study, we set out to determine whether the presence of certain HLA class II DR and DQ alleles in RA patients is associated with production of anti-CCP antibodies. Moreover, we analyzed the extent to which HLA genes and anti-CCP antibodies are associated with RA disease progression as measured by the extent of joint destruction.

PATIENTS AND METHODS

Patients. In 1993, after approval of the Institutional Review Board, a special Early Arthritis Clinic (EAC) was started at the Department of Rheumatology of Leiden University Medical Center, the primary referral center for patients with rheumatic disease in an area with ~300,000 inhabitants in the western part of The Netherlands. General practitioners were encouraged to refer patients directly when arthritis was suspected. Patients referred to the EAC could be seen within 2 weeks and were included in the program when the physician's examination of the patient revealed arthritis and the symptoms had lasted <2 years. Second opinions were excluded (20).

A standard diagnostic evaluation was performed at the first visit. This consisted of a patient history, physical and laboratory examinations, and radiographs of the hands and feet (20). Baseline laboratory examination included an enzyme-linked immunosorbent assay (ELISA) for IgM-RF as previously described (21) and an anti-CCP-2 antibody ELISA (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands) which was performed according to the manufacturer's instructions (sensitivity 74%, specificity 97–99%). Patients included in this study all had a definite diagnosis of RA according to the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria (22) 1 year after inclusion in the study.

Radiographic progression. Radiographs of the hands and feet at baseline, at 6 months, and at years 1, 2, 3, and 4 were available for the first 126 of the 268 RA patients included in this cohort (23,24). Radiographic damage was scored using the modified Sharp/van der Heijde method (25) in two sessions by one experienced rheumatologist. During the first session,

radiographs taken at baseline, 6 months, 1 year, and 2 years were randomly scored. The intraclass correlation coefficient for the assessor's scoring (in 52 patients) was 0.91. Radiographs taken at the third and fourth years were scored during the second session, with an intraclass correlation coefficient for the assessor's scoring (in 42 patients) of 0.98. The rheumatologist was blinded to the clinical data and unaware of the study questions. With 11% of radiographs not taken, not taken within an acceptable time frame, or not available for scoring, 5 radiographs on average (range 2–6) were scored per patient. Two patients (1.6%) had only 2 radiographs scored; both patients had a Sharp score of zero on both radiographs.

HLA typing. HLA-DQB1 and DRB1 typing and subtyping were performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides. DQA1 genotype was deduced from DRB1 typing based on the strong linkage disequilibrium of the genes in the Dutch population.

DRB1 alleles *0101, *0102, *0104, *0401, *0404, *0405, *0408, *0413, *0416, and *1001 are SE alleles (26). In the RA protection (RAP) hypothesis, DQB1*0301, *0302, *0303, *0401, or *0402 combined with DQA1*0301 or *0302 are defined as DQ3 molecules, and DQB1*0501 combined with DQA1*0101 or *0104 are defined as DQ5 molecules. DRB1 alleles bearing the DERAA motif in their HVR3 are *0103, *0402, *1102, *1103, *1301, and *1302 (27). DQ3/3 and DQ5/5 patients have two doses of DQ3 or DQ5, respectively, and DQ3/5 patients are DQ3/DQ5 heterozygous.

HLA typing performed on blood from 306 healthy, unrelated Dutch donors served as a control (28).

Power calculation and statistical analysis. Approximately 60% of Dutch RA patients are carriers of one or two SE alleles (23). Anti-CCP antibodies are found in ~55–60% of patients with early RA (11,29). In an unmatched cohort, at a power of 80% and a confidence level of 95%, 222 RA patients (133 SE carriers and 89 non-SE carriers) are required to detect that SE carriership confers a risk for production of anti-CCP antibodies (odds ratio [OR] 2.25).

Study power, ORs with 95% confidence intervals (95% CIs), and *P* values obtained with the chi-square test were calculated using the Epi Info Statcalc computer program (Centers for Disease Control and Prevention, Atlanta, GA). When cell values were <5, results from the 2-tailed Fisher's exact test were used.

In the analysis of rate of joint damage, to correct for radiographs missing or taken at an irregular interval, the course of radiographic progression for each patient was fitted using simple linear regression. For all groups, the mean slope was calculated. Progression rates were calculated with the assumption that radiographic damage progresses at a constant rate (30). Differences between groups of interest were tested with a 2-tailed Mann-Whitney U test. For all tests, *P* values less than or equal to 0.05 were considered significant.

RESULTS

HLA-DRB1 and DQB1 carriership and anti-CCP antibodies. Of the RA patients included in this study, 53% (142 of 268) had anti-CCP antibodies at

Table 1. Baseline clinical features of the 268 patients with early rheumatoid arthritis in the study*

Age, mean (range) years	57 (17–93)
Female, %	66
Caucasian, %	100
IgM rheumatoid factor positive, %	54
Anti-CCP antibody positive, %	53
Erosions on radiographs of hands and feet, %	25
Duration of symptoms, median (range) weeks	11 (0–52)

* CCP = cyclic citrullinated peptide.

baseline (Table 1). Univariate analysis of the presence of anti-CCP antibodies in conjunction with HLA-DRB1 carriership was performed (Table 2). Not taking alleles with <10 carriers into account, carriership of two HLA-DRB1 alleles, DRB1*0401 and *1001, was associated with the presence of anti-CCP antibodies with the 95% CI for DRB1*0401 not including 1 (Table 2). Seven

Table 2. HLA-DRB1 allele carriership and anti-CCP antibody status in rheumatoid arthritis patients*

DRB1	No. of carriers	Anti-CCP+	Anti-CCP–	OR (95% CI)†
*0101	60	36	24	1.4 (0.8–2.5)
*0102	11	5	6	0.8 (0.2–2.9)
*0301	68	24	44	0.4 (0.3–0.8)
*0400	3	3	0	–
*0401	84	56	28	2.0 (1.2–3.4)
*0402	1	0	1	–
*0403	4	2	2	0.9 (0.1–8.9)
*0404	28	19	9	2.0 (0.8–4.8)
*0405	3	3	0	–
*0406	1	1	0	–
*0407	3	1	2	0.4 (0.0–6.3)
*0408	9	6	3	1.8 (0.4–9.2)
*0701	47	22	25	0.8 (0.4–1.5)
*0800	6	3	3	0.9 (0.1–5.6)
*0901	11	7	4	1.6 (0.4–6.5)
*1001	12	12	0	–
*1101	25	14	11	1.1 (0.5–2.8)
*1104	4	2	2	0.9 (0.1–8.9)
*1102	3	1	2	0.4 (0.0–6.3)
*1200	7	4	3	1.2 (0.2–6.8)
*1301	21	7	14	0.4 (0.2–1.2)
*1302	19	6	13	0.4 (0.1–1.1)
*1303	4	1	3	0.3 (0.0–3.2)
*1401	12	5	7	0.6 (0.2–2.3)
*1500	69	32	37	0.7 (0.4–1.3)
*1600	2	1	1	0.9 (0.0–32.9)
Total	517‡	273	244	

* CCP = cyclic citrullinated peptide; OR = odds ratio; 95% CI = 95% confidence interval.

† ORs were calculated comparing against all other groups.

‡ Of the 268 patients, 19 were homozygous for HLA-DRB1. HLA-DRB1*0400, *0800, *1200, *1500, and *1600 represent DR 4, 8, 12, 15, and 16, respectively. DRB1 shared epitope alleles *0104, *0409, *0410, *0412, *0413, *0416, *1402, and *1406, which are rare in the Dutch population, were not found in our cohort.

Table 3. HLA-DQB1 allele carriership and anti-CCP antibody status in rheumatoid arthritis patients*

DQB1	No. of carriers	Anti-CCP+	Anti-CCP-	OR (95% CI)†
*0201	60	23	37	0.5 (0.3–0.9)
*0202	27	12	15	0.7 (0.3–1.5)
*0301	75	41	34	1.0 (0.6–1.7)
*0302	79	57	22	2.5 (1.4–4.4)
*0303	20	12	8	1.3 (0.5–3.5)
*0304	1	1	0	–
*0402	5	3	2	1.3 (0.2–10.0)
*0501	76	49	27	1.6 (1.0–2.8)
*0502	3	2	1	1.7 (0.1–46.8)
*0503	12	9	3	2.6 (0.6–12.1)
*0601	0	0	0	–
*0602	64	30	34	0.7 (0.4–1.2)
*0603	18	3	15	0.2 (0.0–0.6)
*0604	17	8	9	0.7 (0.3–2.1)
*0609	3	1	2	0.8 (0.0–5.9)
Total	460‡	251	209	

* See Table 2 for definitions.

† ORs were calculated comparing against all other groups.

‡ Data are based on results from 237 patients. Of these 237 patients, 14 were homozygous for HLA-DQB1.

DRB1*0401 carriers were homozygotes, with 6 of them having anti-CCP antibodies (not shown). HLA-DRB1*1001 homozygotes were not found in our cohort (not shown). Carriership of HLA-DRB1*0301 was inversely correlated with production of anti-CCP antibodies with the 95% CI not including 1. Anti-CCP antibodies

were not detected in the 5 HLA-DRB1*0301 homozygotes.

Carriership of HLA-DQB1*0302 and DQB1*0501 was associated with the presence of anti-CCP antibodies (Table 3). All 5 HLA-DQB1*0302 homozygotes and all 6 DQB1*0501 homozygotes had anti-CCP antibodies (not shown). HLA-DQB1 alleles for which carriership was inversely correlated with anti-CCP antibodies (with 95% CIs not including 1) were DQB1*0201 and *0603, and no anti-CCP antibodies were detected in the 3 HLA-DQB1*0201 homozygotes (not shown).

Anti-CCP antibodies and DQ-DR genotypes.

HLA-DR and HLA-DQ alleles are in strong linkage disequilibrium. Therefore, to gain a detailed knowledge of the presence of anti-CCP antibodies in combination with HLA-DRB1 and HLA-DQB1 gene variants, we combined the data from the two loci into HLA DQ-DR genotypes. Due to the large number of possible combinations for these two genes, we focused on specific HLA-DRB1 and DQB1 alleles.

HLA-DRB1 SE alleles and specific HLA-DQB1 molecules called DQ3 and DQ5 have been proposed to confer susceptibility to RA (7,26), whereas HLA-DRB1 alleles encoding the motif DERAA, when present in combination with DQ3 or DQ5, have been proposed to provide protection against severe RA (31). Therefore, to reduce the complexity of combinations of HLA-DRB1 and HLA-DQB1, patients were grouped based on the

Table 4. HLA-DQ and HLA-DR rheumatoid arthritis susceptibility genotypes and association with the presence of anti-CCP autoantibodies*

DQ-DR genotype†	No. of carriers	Anti-CCP+	Anti-CCP-	OR (95% CI)‡	P
3/3-SE/SE	19	17	2	23.4 (4.4–165.8)	<0.001
3/3-SE/x	6	4	2	5.5 (0.8–48.7)	0.06
3/3-x/x	1	1	0	–	
3/5-SE/SE	13	9	4	6.2 (1.5–28.3)	0.007
3/5-SE/x	1	1	0	–	
5/5-SE/SE	6	6	0	–	
3/x-SE/x	59	36	23	4.3 (1.9–10.1)	<0.001
3/x-x/x	6	3	3	2.8 (0.4–19.7)	0.3
5/x-SE/x	43	27	16	4.6 (1.9–11.8)	<0.001
3/x-SE/DERAA	8	3	5	1.7 (0.3–9.4)	0.7
3/x-x/DERAA	3	1	2	1.4 (0.0–21.6)	1.0
5/x-x/DERAA	12	6	6	2.8 (0.7–11.6)	0.2
x/x-x/x	60	16	44		
Total	237	130	107		

* SE = shared epitope; DERAA = motif carried by DRB1 alleles in their third hypervariable region (see Table 2 for other definitions).

† DQ3 = DQB1*0301, *0302, *0303, *0401, or *0402 combined with DQA1*0301 or *0302; DQ5 = DQB1*0501 combined with DQA1*0101 or *0104; SE = DRB1*0101, *0102, *0401, *0404, *0405, *0408, and 1001; DERAA = DRB1*0103, *0402, *1102, *1103, *1301, and *1302.

‡ ORs were calculated comparing against the no-DQ and no-DR (x/x-x/x) group.

Table 5. Association of HLA-DRB1 shared epitope (SE) alleles and anti-CCP autoantibodies in rheumatoid arthritis*

SE status†	No. of carriers	Anti-CCP+	Anti-CCP–	OR (95% CI)‡	P
SE/SE	40	34	6	13.3 (4.6–40.4)	<0.001
SE/x	141	82	59	3.3 (1.8–6.0)	<0.001
x/x	87	26	61		
All	268	142	126		

* See Table 2 for other definitions.

† SE alleles include DRB1*0101, *0102, *0401, *0404, *0405, *0408, and *1001.

‡ ORs were calculated comparing double-dose SE carriers (SE/SE) and single-dose SE carriers (SE/x) against carriers of no SE alleles (x/x).

presence of proposed RA-predisposing SE, DQ3, and DQ5 alleles and on the presence of DERAA-encoding alleles proposed to protect against RA (Table 4).

Anti-CCP antibodies were found in 84% of patients (32 of 38) with two susceptibility alleles at both DQB1 and DRB1 (combinations 3/3–SE/SE, 3/5–SE/SE, and 5/5–SE/SE). Compared against individuals having no susceptibility alleles, carriership of genotypes 3/3–SE/SE and 3/5–SE/SE was significantly associated with the presence of anti-CCP antibodies (OR 23.4 [95% CI 4.4–165.8], $P < 0.001$ and OR 6.2 [95% CI 1.5–28.3], $P = 0.007$, respectively). Of the few patients having genotypes with two predisposing DQ alleles and one SE allele (3/3–SE/x and 3/5–SE/x), 71% (5 of 7) had anti-CCP antibodies, and these genotypes combined were significantly associated with the presence of anti-CCP antibodies (OR 6.7 [95% CI 1.0–57.8], $P = 0.02$).

Anti-CCP antibodies were detected in 62% of patients (63 of 102) with genotypes bearing one DQ and one SE allele (3/x–SE/x and 5/x–SE/x). Both genotypes were associated with anti-CCP antibodies (OR 4.3 [95% CI 1.9–10.1], $P < 0.001$ and OR 4.6 [95% CI 1.9–11.8], $P < 0.001$, respectively).

Proposed protective DRB1 alleles encoding DERAA were infrequent. DERAA allele carriership was not inversely correlated with anti-CCP antibodies when compared with individuals with no susceptibility alleles (not shown).

However, anti-CCP antibodies were found in 43% of patients (10 of 23) with one DQ3 or DQ5 allele and one DERAA allele (3/x–x/DERAA, 3/x–SE/DERAA, and 5/x–x/DERAA) compared with 61% of patients (66 of 108) with one DQ3 or DQ5 allele but no DERAA allele (3/x–SE/x, 3/x–x/x, and 5/x–SE/x). Carriership of DERAA in combination with one DQ3 or DQ5 allele appeared to be inversely correlated with the presence of anti-CCP antibodies (OR 0.5, 95% CI 0.2–1.3), but this was not significant ($P = 0.1$).

Subsequently, we analyzed which of the two models (RAP or SE hypothesis) described the best association with the presence of anti-CCP antibodies. To do this, the two models were simplified.

Both models essentially distinguish 3 categories: two susceptibility factors (two SE alleles or two times DQ3 or DQ5), one susceptibility factor (one SE allele or one dose of DQ3 or DQ5), or no susceptibility factor/dominant protective factors (no SE or DERAA/DQ5, DERAA/DQ3, and no DQ [DQ x/x]). Using these categories, a 2×3 table was constructed for both models. The results of the chi-square tests for both models were similar, although the chi-square value for the RAP model was slightly higher than that for the SE model ($\chi^2 = 39.6$, 2 degrees of freedom [2df], $P < 0.001$ and $\chi^2 = 32.5$, 2df, $P < 0.001$, respectively). This was no great surprise, given that we found no protective effect of the DERAA motif and given that the proposed susceptibility alleles in both models are highly linked (Table 4).

Anti-CCP antibodies and SE alleles. Since both HLA-DRB1 alleles for which carriership was associated with anti-CCP positivity (HLA-DRB1*0401 and *1001) were SE alleles, we separately tested the association of SE carriership with the presence of anti-CCP antibodies. In our cohort, 15% of patients (40 of 268) had two SE alleles (SE/SE), 53% (141 of 268) had one SE allele (SE/x), and 32% (87 of 268) had no SE alleles (x/x) (Table 5). Anti-CCP antibodies were found in 85% of SE/SE patients (34 of 40), 58% of SE/x patients (82 of 141), and 30% of x/x patients (26 of 87). Compared with carriership in x/x patients, SE/SE and SE/x carriership was significantly associated with production of anti-CCP antibodies (OR 13.3 [95% CI 4.6–40.4], $P < 0.001$ and OR 3.3 [95% CI 1.8–6.0], $P < 0.001$, respectively).

To determine whether the association between SE and anti-CCP antibodies was entirely due to the DRB1*0401 and DRB1*1001 alleles, we retested the

association after excluding these two alleles. SE carrier-ship, however, was still associated with the presence of anti-CCP antibodies (OR 2.9 [95% CI 1.6–5.6], $P < 0.001$).

Given the strong association between SE alleles and anti-CCP antibodies in patients with RA, we compared the distribution of SE alleles in anti-CCP+ and anti-CCP– RA patients with the distribution of SE alleles in controls. SE alleles were more common in anti-CCP+ RA patients than in controls. SE/SE carrier-ship was found in 24% of anti-CCP+ RA patients and in 6% of controls ($P < 0.001$). SE/x carrier-ship was found in 58% of anti-CCP+ RA patients and in 36% of controls ($P < 0.001$). In contrast, SE carrier-ship did not differ between controls and anti-CCP antibody–negative RA patients. SE/SE carrier-ship was found in 5% of anti-CCP– RA patients and in 6% of controls ($P = 0.9$). SE/x carrier-ship was found in 46% of anti-CCP– RA patients and in 36% of controls ($P = 0.07$). These data suggest that the association between the SE and RA is mainly due to the underlying association between the SE and the production of anti-CCP antibodies found in RA patients.

Investigators in several studies have reported on SE carrier-ship and IgM-RF in RA (5,32). In our cohort, in line with previous reports, carrier-ship of SE alleles was associated with the presence of IgM-RF (OR 1.7 [95% CI 1.0–2.3], $P = 0.04$). However, this association was due to the association of SE/SE carrier-ship with IgM-RF (OR 2.8 [95% CI 1.2–6.4], $P < 0.001$), but not to the association of SE/x with IgM-RF (OR 1.5 [95% CI 0.9–2.7], $P = 0.1$).

Anti-CCP antibodies and HLA class II genes as disease progression markers. HLA class II SE alleles and production of anti-CCP antibodies are both markers of the presence of joint erosions (33). We analyzed the interaction between the two factors in predicting the extent of joint damage progression. Figure 1 shows the rate of joint damage per year as measured on radiographs of the hands and feet using the modified Sharp/van der Heijde method.

The highest rate of joint damage over 4 years in early RA was found in patients with both anti-CCP antibodies and SE alleles. The mean \pm SD rate in this group was 7.6 ± 10.4 Sharp points per year (Figure 1).

The mean \pm SD rate of joint damage in patients with early RA without anti-CCP antibodies and without SE alleles was 1.6 ± 3.9 Sharp points per year, which was significantly lower ($P < 0.001$) than that in anti-CCP+, SE+ RA patients. Patients with anti-CCP antibodies but without SE alleles had a rate of 2.4 ± 2.8 Sharp points

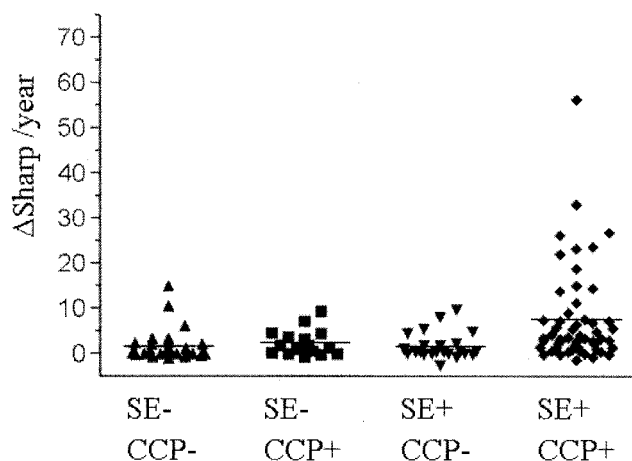


Figure 1. Rate of joint damage per year in patients with recent-onset rheumatoid arthritis over a period of 4 years. Patients were grouped by shared epitope (SE) status and anti-cyclic citrullinated peptide (anti-CCP) antibody positivity. At baseline, no differences were seen in joint damage between groups. SE+, anti-CCP+ patients ($n = 54$) had a significantly higher rate of joint damage than did SE–, anti-CCP– patients ($n = 33$) ($P < 0.001$), SE–, anti-CCP+ patients ($n = 16$) ($P = 0.04$), and SE+, anti-CCP– patients ($n = 23$) ($P < 0.001$). Horizontal bars indicate the mean change in Sharp score per year for each group. The SE alleles are DRB1*0101, *0102, *0401, *0404, *0405, *0408, and *1001. Results could not be explained by differences in treatment or differences in the presence of IgM rheumatoid factor (see Results for details).

per year, and patients without anti-CCP antibodies but with SE alleles had a rate of 1.6 ± 2.8 Sharp points per year ($P = 0.04$ and $P < 0.001$, respectively, versus anti-CCP+, SE+ patients).

Treatment strategy (23), mean age, and sex were approximately equally distributed among the 4 groups (data not shown). Each year an average of 75% of patients were treated with disease-modifying antirheumatic drugs (DMARDs). In the anti-CCP+, SE+ group, this value was 81%, which was not significantly different from the percentages in the other groups (data not shown). At the end of the study, the cumulative number of DMARDs used was higher in the anti-CCP+, SE+ group (mean 2.2, range 0–5) than in the anti-CCP–, SE– group (mean 1.5, range 0–5) ($P = 0.01$) or in the anti-CCP–, SE+ group (mean 1.3, range 0–4) ($P = 0.005$), but did not differ from the number used in the anti-CCP+, SE– group (mean 2.2, range 0–4) ($P = 0.9$).

However, IgM-RF was more common in groups with anti-CCP antibodies (86%) than in groups without anti-CCP antibodies (25%). Nonetheless, in a regression analysis model with joint damage progression as the dependent variable and IgM-RF and anti-CCP antibod-

ies as predictors, there was a significant relationship between anti-CCP antibodies and joint damage progression (β 0.32, standard error of the regression coefficient 0.13, $P = 0.02$), but not between IgM-RF and joint damage progression (β 0.17, standard error of the regression coefficient 0.14, $P = 0.21$). Taken together, these data indicate that the increased rate of joint destruction observed in patients with anti-CCP antibodies and SE alleles cannot be explained by differences in treatment or differences in the presence of IgM-RF.

In contrast to what was initially assumed, the rate of joint destruction did not progress in a linear manner over the 4-year period of the study. A significantly higher mean \pm SD rate of joint damage was observed in the first year than in years 2–4 (7.5 ± 12.6 Sharp points per year versus 4.3 ± 8.3 Sharp points per year; $P = 0.003$). In both time periods, however, the anti-CCP+, SE+ group had a significantly higher rate of joint damage compared with all the other groups (data not shown).

DISCUSSION

To investigate the immunogenetic background of the antibody response against citrullinated antigens, high-resolution DQB1 and DRB1 typing and anti-CCP antibody testing were performed on 268 Caucasian patients with early RA. Carriership analysis revealed that HLA-DRB1*0401, DRB1*1001, DQB1*0302, and DQB1*0501 were associated with the presence of anti-CCP antibodies. Moreover, associations were found between DQ-DR genotypes bearing proposed RA susceptibility alleles and the production of anti-CCP antibodies.

Carriership analysis such as that shown in Tables 2 and 3 appears to be fairly straightforward but has several disadvantages. Given the polymorphic nature of HLA genes, large numbers of patients are needed in order to assess the association for each allele. In this study, this is reflected in the wide CIs for the less frequent alleles. In addition, carriership analysis does not take into account the allele on the other chromosome. For highly polymorphic genes, this cannot be circumvented by homozygote analysis, since these are generally rare. Both issues can be illustrated by our findings for the DRB1*0901 allele. This allele, which is thought to be a neutral allele in RA susceptibility (34), seemed to be associated with anti-CCP antibodies. Eleven carriers of the allele were found in the cohort, and 7 had anti-CCP antibodies, giving an OR of almost 2; however, the CI included 1. More importantly, the possible association of this allele with anti-CCP antibod-

ies is likely due to the fact that 5 of 10 anti-CCP antibody-positive DRB1*0901 carriers had an SE allele on the other chromosome, and these are significantly associated with anti-CCP antibodies.

Furthermore, when there is linkage disequilibrium between alleles of different loci, such as for HLA-DRB1 and DQB1, the contribution of individual alleles can be difficult to establish. For instance, HLA-DRB1*0401 and HLA-DQB1*0302, which were both associated with anti-CCP antibodies, are a part of the extended DQB1*0301;DQA1*0300;DRB1*0401 (DQ3-SE) haplotype. To provide insight into the relationship between HLA-DRB1 and DQB1 alleles, DQ-DR genotypes were analyzed. The analysis showed that anti-CCP antibodies were commonly found in patients with genotypes containing proposed susceptibility alleles from the RAP model (DQ3 and DQ5) and, consistent with previous reports (18,19), from the SE model. Patients with the protective DERA motif (according to the RAP hypothesis) were infrequent, as expected, and when this motif was present, it was not significantly inversely correlated with anti-CCP antibody production even after we attempted to correct for the presence of susceptibility alleles.

The presence of HLA susceptibility alleles and anti-CCP antibodies heralded a more severe disease type. Anti-CCP+, SE+ patients at baseline had a significantly higher rate of joint destruction than did all other patients. Although an increased rate of joint destruction was not found in every anti-CCP+, SE+ patient in our cohort, patients in this category constituted 92% (12 of 13) of those with joint damage progression above the 90th percentile (11.2 Sharp points per year). Surprisingly, the rate of joint destruction did not differ between anti-CCP+, SE- patients and anti-CCP-, SE+ patients ($P = 0.01$) or between anti-CCP-, SE+ patients and anti-CCP-, SE- patients ($P = 0.09$). This means that a higher rate of joint damage was only found when both anti-CCP antibodies and SE alleles were present.

A possible explanation might be the higher mean \pm SD levels of anti-CCP antibodies in anti-CCP+, SE+ (SE/SE and SE/x) patients than in anti-CCP+ and SE- (x/x) patients ($1,033 \pm 1,028$ arbitrary units [AU] versus 666 ± 594 AU). However, although this difference was significant ($P = 0.03$), it is unclear what it means, given that both mean values are well above the cutoff level of anti-CCP antibody positivity (36 times and 22 times the cutoff level, respectively). Currently, we are investigating whether these differences in the rate of joint destruction are due to a different composi-

tion of IgG subclasses, since these subclasses have different properties, such as the ability to activate complement (35).

It has been shown in mouse models of arthritis that citrullination occurs in the synovium. In spite of citrullination of several proteins under inflammatory conditions, these mice do not develop antibodies to citrullinated proteins (16). Moreover, in RA patients both with and without anticitrulline antibodies, citrullinated proteins have been detected in the synovium (36). Although it is conceivable that only citrullination of specific sequences of proteins leads to an immune response, these data suggest that the presence of citrullinated proteins in synovium by itself is not sufficient for persistent production of anticitrulline antibodies, but that additional factors are required.

The peptidylarginine deiminases type 4 (PADI4) gene locus on chromosome 1, which encodes an enzyme capable of citrullinating proteins, has been reported to be a susceptibility locus for RA. A haplotype of the PADI4 locus associated with increased stability of PADI4 transcripts was found more often in RA patients, and this haplotype was associated with the presence of antibodies to citrullinated proteins. Based on these findings, the authors speculated that a genetically determined, increased ability to citrullinate proteins is a risk factor for RA (37).

Moreover, given that during the conversion of arginine to citrulline the charged imino side-chain group is changed into an uncharged carbonyl group, citrullination is likely to affect the ability of peptides to bind to HLA molecules. In DRB1*0401 transgenic mice, citrullination of peptides not only increased peptide-MHC affinity, but also led to activation of CD4⁺ T cells (17). Since activation of T cells is important in B cell responses, we propose that the observed association between HLA class II genes and anti-CCP antibodies is based on an increased ability of citrulline-containing peptides to bind to certain HLA class II molecules, and that this increased binding ability promotes an antibody response against citrulline-containing antigens (17).

In summary, anti-CCP antibodies are associated with HLA class II RA susceptibility alleles, and the presence of these two factors is indicative of a severe disease course.

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