# Immunogenetic Risks of Anti-Cyclical Citrullinated Peptide Antibodies in a North American Native Population with Rheumatoid Arthritis and Their First-degree Relatives

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***ABSTRACT.*** ***Objective.*** To determine the prevalence of anti-cyclic citrullinated peptide (anti-CCP) antibodies in unaffected relatives of North American Native probands with rheumatoid arthritis (RA); and the associations of the shared epitope (SE) and HLA-DRB1*0901 with RA and anti-CCP antibodies.

***Methods.*** The subjects were RA probands, affected relatives, unaffected first-degree (FDR) and more distant relatives, and unaffected controls from the same population. HLA-DRB1 typing was determined by DNA sequencing and anti-CCP antibodies were determined by ELISA.

***Results.*** DRB1*0901, SE, and SE/DRB1*0901 genotypes were all associated with RA. SE/DRB1*0901, but not other SE genotypes, was associated with disease onset at age < 16 years. The frequency of anti-CCP antibodies was 82% in RA probands, 17% in FDR, 11% in more distant relatives, and 3% in controls. Among unaffected relatives, a significant increased risk of anti-CCP was associated with SE/DRB1*0901 genotype, but not with SE.

***Conclusion.*** An independent association of the non-SE allele DRB1*0901 with RA was confirmed in this population, and this allele in combination with a SE allele was associated with younger age at disease onset. FDR of RA probands have a higher prevalence of anti-CCP antibodies than more distant relatives and unrelated controls, suggesting a gradient of risk for disease development. Immunogenetic risks may act early in disease pathogenesis at the level of initiation of RA autoantibody formation; however, it is not clear what additional genetic and environmental risks are involved in progression to clinical disease. (J Rheumatol First Release May 1 2009; doi:10.3899/jrheum.080855)

*Key Indexing Terms:*
IMMUNOGENETIC RISK ANTI-CYCLICAL CITRULLINATED PEPTIDE ANTIBODIES
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The Cree and Ojibway, a North American Native people in central Canada, have a prevalence of rheumatoid arthritis (RA) of 2%, twice that of the Caucasian population in this area[1]. At least 50% of Cree and Ojibway probands have affected relatives[2]. In previous studies we reported a frequency of the shared epitope (SE) of 59% in this population, 87% among patients with RA, and 93% among unaffected relatives[2,3]. Since HLA is the major contributor to disease risk in RA, the equally high frequency of the SE in unaffected relatives combined with the high familial rate of RA in this population suggests a high genetic risk of RA among relatives of probands. Identification of at-risk relatives may provide insights into the events surrounding the onset of RA. Further, since a severe RA phenotype has been reported for most North American Native patients, earliest possible diagnosis and treatment may help ameliorate disease severity[1,2,4-6].

In addition to disease susceptibility provided by the SE, DRB1*0901, a non-SE-bearing allele, has been associated with RA in selected populations, notably Koreans, Chileans, and Malaysian Chinese[7-9]. Further, a trend toward an association of DRB1*0901 with RA was reported in the Tlingit

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of Alaska, another North American Native group, and we previously found a possible association in North American Native patients with rheumatoid factor (RF)-positive polyarticular juvenile rheumatoid arthritis[3,10].

Anti-cyclical citrullinated peptide (CCP) antibodies have been shown to precede clinically diagnosed RA and may be responsible for the association of RA with the SE[11-15]. We postulated that anti-CCP antibodies may be found in unaffected first-degree relatives (FDR) of RA probands and that immunogenetic risks are associated with anti-CCP antibodies in both RA probands and their relatives. The objectives of our study were to confirm associations of DRB1*0901 with RA in a larger number of Cree and Ojibway subjects, to determine the prevalence of anti-CCP antibodies in relatives of RA probands, and to explore the association of both the SE and DRB1*0901 with anti-CCP antibodies in RA probands and their unaffected relatives.

## MATERIALS AND METHODS

*Patients, relatives, and controls.* Patients were selected if they met the following criteria: a diagnosis of RA based on fulfillment of American College of Rheumatology criteria[16] or the polyarthritis rheumatoid (RF)-positive type of juvenile idiopathic arthritis by International League of Associations for Rheumatology criteria[17], and self-identified North American Indian descent, regardless of quantum. Controls were randomly collected healthy adults with no history of RA and with self-identified North American Indian descent from the same communities. Patients, relatives, and controls were recruited from the University of Manitoba Arthritis Centre Clinics and during field trips to community health clinics on reserves in Manitoba and Saskatchewan. Relatives of RA probands were recruited on the basis of availability and willingness to participate in the study. Relatives were classified as being clinically unaffected by RA after a rigorous exclusion process that included the following elements: (1) no history of RA as documented by a physician; (2) no subjective history of joint swelling, particularly in the hands; (3) no history of morning stiffness > 30 minutes; and (4) no evidence of synovitis on clinical examination by a rheumatologist (HEG, JM). Relatives with well characterized rheumatic diseases such as systemic lupus erythematosus were excluded from the study.

North American Native tribes in Manitoba and Saskatchewan belong mainly to Cree and Ojibway linguistic groups, with fewer from the Na-Dene group. HLA typing of controls and patients from previously published studies were incorporated into the present analyses provided the subjects met our inclusion criteria[2,3,18].

The study was approved by research ethics boards at the universities of Manitoba and Saskatchewan. Approval was also obtained from the Assembly of Manitoba Chiefs. All subjects gave informed consent before participation.

*Assays.* HLA-DRB1 typing was performed by polymerase chain reaction using sequence-specific oligonucleotide primers, and sequence-based typing. Anti-CCP antibodies were assayed by ELISA using a second-generation anti-CCP kit (CCP2; Inova Diagnostics, San Diego, CA, USA). Positive tests were defined as those with values ≥ 20 units and strongly positive were ≥ 60 units. Results of RF tests were obtained from patients' medical records. RF values ≥ 20 international units (IU) were considered positive.

The following DRB1 alleles were included as SE-bearing alleles: DRB1*0101, 0102, 0401, 0404, 0405, 0408, 0410, 1001, and 1402[19]. Other SE-bearing alleles were not found in this population[18].

*Statistical analyses.* Analyses were performed by SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Two-sided chi-square or Fisher's exact tests were used to compare frequencies, 2-tailed t tests to compare means, and Mann-Whitney U tests to compare median values. Odds ratios (OR) with 95% confidence limits (95% CI) were estimated. A p value of 0.05 was accepted as significant for confirmation of previously reported associations of the SE with RA or anti-CCP antibody and of DRB1*0901 with RA[7-9,15]. An association of DRB1*0901 with anti-CCP antibody has not previously been reported. Therefore in univariate analyses, significance was set at $p < 0.01$ to adjust for 2 comparisons, DRB1*0901 and SE/DRB1*0901 genotype. Binary logistics regression analyses were performed entering variables by a backward conditional method, and significance was set at a p value of 0.05.

## RESULTS

*SE and DRB1*0901 in RA.* Two hundred sixty-six probands with RA, 165 unaffected FDR, and 42 unaffected second-degree or more distant relatives were studied. Thirty-seven probands and 2 affected relatives were < 16 years of age at disease onset. Patient characteristics are shown in Table 1. Probands were significantly older than unaffected relatives. Most probands had prolonged disease duration. HLA typing was available for 250 probands, all relatives, and 176 controls. Among these, HLA typing results for 101 probands and 105 controls were available from previous studies[2,3,18]. There was a nearly 2-fold higher risk of RA for SE/*0901 and a slightly higher risk for SE/SE genotypes than for genotypes with a single SE (SE/X) (Table 2).

When stratified by age at disease onset, the odds of disease were significantly increased by DRB1*0901 for those with onset at age < 16 years, and the OR for SE/DRB1*0901 genotypes was greater than the sum of the individual OR for each [SE: 29/37 (78%), OR 1.9, 95% CI 0.8, 4.4, not significant (NS); DRB1*0901: 8/37 (22%), OR 3.8, 95% CI 1.4, 10.0, p = 0.005; SE/*0901: 6/37 (16%), OR 11.2, 95% CI 2.7, 47.0, p < 0.0001, compared with controls, respectively]. Further, in stepwise logistic regression, only SE/*0901 remained significant, while *0901 and SE were both eliminated, suggesting that both SE and DRB1*0901 together increase the risk of RA in this age group (Table 3).

The same effect was not apparent among those with onset at age ≥ 16 years [SE: 135/155 (87%), OR 3.5, 95% CI 2.0, 6.1, p < 0.0001; DRB1*0901: 16/155 (10%), OR 1.6, 95% CI 0.7, 3.4, NS; SE/0901: 11/155 (7%), OR 4.4, 95% CI 1.2, 16.1, p = 0.015, compared with controls, respectively]. In regression analysis the SE and DRB1*0901 each remained significant and no interaction between the 2 was evident (Table 3).

*Anti-CCP antibodies.* Anti-CCP antibody tests were available for 125 probands, 6 affected relatives, 134 unaffected FDR, 34 distant relatives, and 91 controls. The frequency of anti-CCP antibodies was 81.7% in probands and affected relatives, 17.2% in unaffected FDR, and 11.8% in unaffected distant relatives. Only 5 controls had positive values (5.4%). There was a higher frequency of a positive anti-CCP test among unaffected FDR (p = 0.009), but not in more distant relatives (p = 0.227), compared with controls. The median for positive anti-CCP values was higher in RA (150

*Table 1.* North American Native population with RA, relatives, and controls.

| | RA Probands | Affected Relatives | Unaffected FDR | Unaffected Distant Relatives | Unrelated Controls |
|---|---|---|---|---|---|
| N | 266 | 6 | 165 | 42 | 176 |
| Female, % | 80 | 67 | 75 | 67 | 64 |
| Age at study, mean ± SD, yrs | 44.3 ± 13.8 | 30.5 ± 9.6 | 37.6 ± 12.2* | 26.8 ± 9.1* | 31.9 ± 12.4* |
| Age at RA onset, mean ± SD, yrs | 32.7 ± 13.8 | 15.0 + 4.5 | | | |
| Age at RA diagnosis, yrs | 36.3 ± 14.3 | 21.2 ± 8.3 | | | |
| RA disease duration, mean ± SD, yrs | 11.7 ± 9.3 | 11.5 ± 9.0 | | | |
| RF-positive (%) | 223/255 (88) | 5/6 (83.3) | | | |
| ANA-positive (%) | 154/201 (77) | 2/6 (33.3) | | | |
| Receiving DMARD (%) | 196/210 (93) | 4/4 (100) | | | |

ANA: antinuclear antibody; DMARD: disease modifying antirheumatic drug; FDR: first-degree relatives; RF: rheumatoid factor. * $p < 0.0001$ compared with RA probands.

*Table 2.* Shared epitope and DRB1*0901 in RA probands and controls.

| HLA-DRB1 Genotype | Controls (n = 176) N | All RA (n = 250) N | OR (95% CI) Controls vs RA | CCP+RA (n = 101) N | OR (95% CI) Controls vs CCP+RA | CCP–RA (n = 22) N | OR (95% CI) Controls vs CCP–RA |
|---|---|---|---|---|---|---|---|
| Any SE | 116 | 210 | 2.72 (1.72, 4.30) $p < 0.0001$ | 84 | 2.56 (1.39, 4.69) $p = 0.002$ | 17 | 1.76 (0.62, 5.00) p = NS |
| SE/X | 71 | 124 | 2.62 (1.60, 4.30) $p < 0.0001$ | 45 | 2.24 (1.16, 4.31) $p = 0.015$ | 13 | 2.20 (0.74, 6.52) p = NS |
| SE/SE | 45 | 86 | 2.87 (1.67, 4.91) $p < 0.0001$ | 39 | 3.06 (1.54, 6.09) $p = 0.001$ | 4 | 1.07 (0.27, 4.20) p = NS |
| Any *0901 | 12 | 33 | 2.08 (1.04, 4.15) $p = 0.035$ | 15 | 2.38 (1.07, 5.32) $p = 0.030$ | 2 | 1.37 (0.29, 6.55) p = NS |
| SE/*0901 | 3 | 18 | 4.47 (1.30, 15.43) $p = 0.010$ | 7 | 4.29 (1.09, 17.0) $p = 0.025$ | 0 | 0.98 (0.96, 1.0) p = NS |

CCP: cyclic citrullinated peptide; N = number with genotype; SE: shared epitope. Any SE: genotypes with SE in combination with any DRB1 allele including SE. Any *0901: genotypes with *0901 in combination with any DRB1 allele. SE/X: genotypes with SE in combination with non-SE DRB1 allele. SE/*0901: genotypes with SE in combination with *0901. Odds ratios for RA were calculated for any SE, SE/X, and SE/SE genotypes versus genotypes without SE, or any *0901 genotypes versus genotypes without *0901, and for SE/*0901 genotypes versus any other genotypes.

units, range 21–530) than in FDR (27 units, range 20–284) ($p < 0.0001$), unaffected distant relatives (24 units, range 22–43) ($p = 0.002$), and controls (47 units, range 23–142) ($p = 0.008$; Figure 1). It should be noted that of the 5 positive controls, 2 had strongly positive titers (> 60 units) and the remaining 3 had weak to moderate positive values, while in the FDR, 8/23 had strongly positive titers.

*SE and DRB1*0901 in anti-CCP-positive RA.* Similar correlations with the SE were found for anti-CCP-positive RA as for the unselected cohort of RA patients (Table 2). In addition, there were trends for associations with HLA-DRB1*0901 and SE/DRB1*0901 genotypes. There were 22 patients with anti-CCP-negative RA and no significant correlations were detected for this group (Table 2).

*Immunogenetic risk for anti-CCP antibodies.* To assess the effect of the SE and DRB1*0901 on the risk of having a positive anti-CCP test, comparisons were made among unaffected relatives. The question was whether these alleles were risk factors for the development of anti-CCP antibodies within families. As shown in Table 4, in univariate analyses DRB1*0901 alone and DRB1*0901 in combination with a SE allele showed trends to significant associations (OR 3.86, $p = 0.059$, and OR 4.66, $p = 0.04$, respectively). However, in a stepwise conditional regression analysis, only SE/DRB1*0901 remained significant, suggesting that the combination of a SE allele and DRB1*0901 increases the risk of anti-CCP antibody in this population, although the 95% confidence intervals were wide (Table 5).

## DISCUSSION

North American Native people can be separated according to ancestral migrations across the Bering Strait[20]. Ancestors of present-day Amerindian tribes are believed to have belonged to the most ancient migration, whereas the Na-Dene and Eskimo-Aleut people descend from later migrations[20]. Interestingly, prevalent rheumatic diseases among Native people also distribute along these lines. For example, high prevalence and incidence rates of RA are found prima-

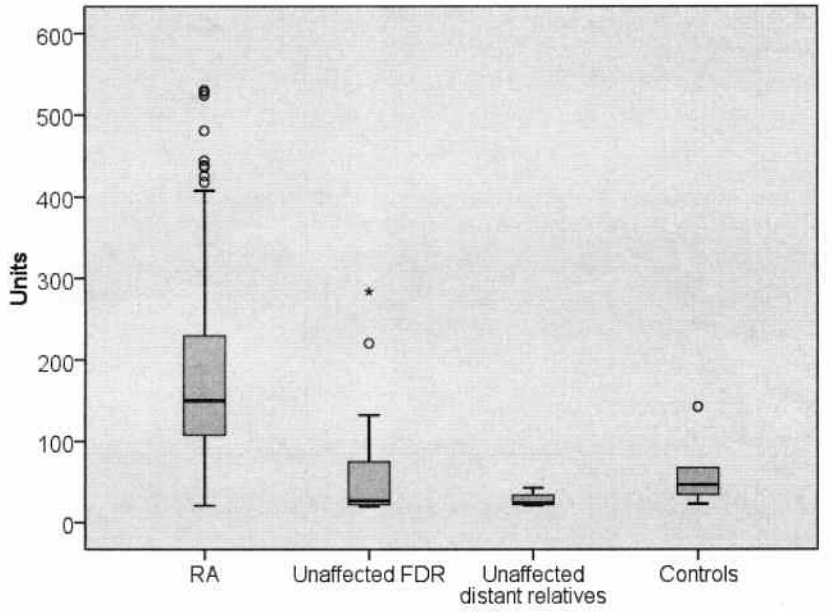


*Figure 1.* Positive anti-CCP antibody levels. FDR: first-degree relative.

*Table 3.* Regression analyses for risk of RA due to shared epitope and DRB1*0901 according to age at onset.

| Population Tested | Explanatory Variables | OR (95% CI) | p |
|---|---|---|---|
| Probands with onset at < 16 yrs (n = 37) | *0901 | Eliminated | |
| vs controls (n = 176) | Any SE | Eliminated | |
| | SE/0901 | 11.16 (2.65, 47.00) | 0.001 |
| | Constant | 0.179 | < 0.0001 |
| Probands with onset at ≥ 16 yrs (n = 160) | *0901 | 2.50 (1.06, 5.89) | 0.037 |
| vs controls (n = 176) | Any SE | 4.02 (2.22, 7.27) | < 0.0001 |
| | SE/*0901 | Eliminated | |
| | Constant | 0.276 | < 0.0001 |

The Cox and Snell R square values for the regressions were 0.052 for onset age < 16 years, and 0.074 for onset ≥ 16 years, respectively. RA probands and controls were entered in the regression.

rily among Amerind tribes, while seronegative spondyloarthropathies including undifferentiated types or reactive arthritis predominate in Na-Dene and Eskimo-Aleut[1]. The exception is the Tlingit, a Na-Dene people with a high prevalence of RA[4,21]. In these relatively genetically isolated people, high population frequencies of the SE among certain Amerind correlate with high rates of RA, whereas the frequencies of HLA-B27 are highest in Na-Dene and Eskimo-Aleut groups[1,22-25]. In North American Native populations studied to date, RA occurs at a young age, carries a severe phenotype, and has a high familial occurrence, all factors suggesting a large genetic contribution[1,4-6]. While the SE contributes to disease risk for these populations as a whole, additional genetic risks likely promote the development of RA within these populations, and more specifically within families with one or more affected members. Hence, these families provide an opportunity to search for non-SE and non-HLA genetic risks. Moreover, as the risk of disease is high among members of these families, a significant proportion is likely to be in a predisease state in which RA autoantibodies have recently been shown to occur[11-13].

In our studies of the Cree and Ojibway Native American population, SE was shown to be a risk factor for anti-CCP antibody-positive RA, as has been reported in other populations[15]. However, the odds ratios of 2.56 and 3.06 for SE and SE/SE, respectively, that were observed in our study were modest compared with results published in a Dutch early RA cohort and in the North American RA Consortium (NARAC) cohort of sibling pairs with established RA, for whom the odds ratios were 4.37 and 3.5 for SE, and 11.79 and 7.7 for SE/SE, respectively[15]. This likely relates to the high background prevalence of SE alleles in this population, which in the control group was 66%, a frequency substantially higher than in most other populations. We have previ-

*Table 4.* Shared epitope and DRB1*0901 in anti-CCP-positive and anti-CCP-negative unaffected family members. Unaffected family members included are first-degree relatives (n = 132) or more distant relative (n = 34) who had HLA typing and anti-CCP antibody assay results. Odds ratios for risk of positive anti-CCP antibodies due to SE and DRB1*0901 were calculated by comparison of anti-CCP antibody-negative and anti-CCP-positive unaffected relatives.

| HLA-DRB1 Genotype | Anti-CCP-negative (n = 139) N | ANTI-CCP-positive (n = 27) N | OR (95% CI) | p |
|---|---|---|---|---|
| Any SE | 106 | 22 | 1.37 (0.48, 3.90) | 0.554 |
| SE/X | 72 | 15 | 1.38 (0.46, 4.10) | 0.567 |
| SE/SE | 34 | 7 | 1.36 (0.39, 4.71) | 0.628 |
| *0901 | 6 | 4 | 3.86 (1.01, 14.73) | 0.059 |
| SE/*0901 | 5 | 4 | 4.66 (1.16, 18.66) | 0.04 |

*Table 5.* Regression analysis for risk of anti-CCP antibody due to shared epitope and DRB1*0901 in unaffected family members. One hundred thirty-nine unaffected relatives with negative and 27 with positive anti-CCP antibody tests were entered into the regression.

| Population Tested | Explanatory Variables | OR (95% CI) | p |
|---|---|---|---|
| Unaffected family members | *0901 | Eliminated | |
| | Any SE | Eliminated | |
| | SE/*0901 | 4.66 (1.16, 18.66) | 0.030 |
| | Constant | 0.17 | < 0.0001 |

The Cox and Snell R-square value for the regression was 0.025.

ously shown that HLA-DRB1*0404 and *1402 are the most prevalent SE alleles in this population[2,3]. In the current study, we also confirmed an association of a non-SE allele, DRB1*0901, with RA in this population. There appeared to be an age effect for SE and DRB1*0901 associations, as the 2 together increased the risk of RA when onset was at age < 16 years. In contrast, for those with onset at age ≥ 16 years, the effects of these alleles were independent of each other. These results support the view that younger age at onset of disease is associated with a greater genetic component.

The substantially higher frequency of anti-CCP antibodies detected among unaffected relatives compared with controls suggests that these results are not due to a background population prevalence of these antibodies, but may truly represent a higher risk for anti-CCP formation within affected families. In other studies we have demonstrated limited isotype usage and different fine specificities of anti-citrulline antibodies in unaffected relatives compared to RA patients in this population[26]. Since SE alleles are highly prevalent in the population and confer a modest risk for the development of anti-CCP-positive RA, these findings suggest that other genetic influences may modify antibody characteristics and disease susceptibility. Our studies have previously suggested that polymorphisms of the IL-10 gene play a role in protection from developing RA in the face of increased susceptibility imparted by the SE alleles[2].

Studies of predisease sera from patients with RA have indicated that anti-CCP antibodies are detectable in some cases years prior to the onset of clinically detectable disease[11-13]. These studies have also suggested that the presence of anti-CCP and SE together is much more predictive of future RA development that either variable alone. Further, smoking has been shown to be an environmental factor that is clearly linked to the development of anti-CCP-positive RA, but only in the context of SE-positive individuals[27,28]. Thus, a compelling hypothesis has been proposed linking smoking and SE to the development of anti-CCP antibodies, and ultimately anti-CCP-positive RA[28]. It will be of considerable interest to follow anti-CCP and SE-positive unaffected relatives for the development of clinically detectable synovitis, potentially representing the onset of RA, particularly in relation to their smoking exposure. One illustrative case from this population has recently been published[29].

In summary, our study confirms an association of DRB1*0901 with RA in this population, demonstrates a higher frequency of anti-CCP antibodies in unaffected relatives of RA probands compared to controls from the same population, and suggests the possibility that DRB1*0901 in association with the SE may increase the risk of anti-CCP antibody in the absence of clinical RA.

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