Prevalence of Anti-Peptidylarginine Deiminase Type 4 Antibodies in Rheumatoid Arthritis and Unaffected First-degree Relatives in Indigenous North American Populations

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ABSTRACT. ***Objective.*** To determine whether anti-peptidylarginine deiminase type 4 (PAD4) antibodies were present in first-degree relatives (FDR) of patients with rheumatoid arthritis (RA) in 2 indigenous North American populations with high prevalence of RA.

Methods. Participants were recruited from 2 indigenous populations in Canada and the United States, including patients with RA (probands), their unaffected FDR, and healthy unrelated controls. Sera were tested for the presence of anti-PAD4 antibodies, anticyclic citrullinated peptide (anti-CCP) antibodies, and rheumatoid factor (RF). HLA-DRB1 subtyping was performed and participants were classified according to number of shared-epitope alleles present.

Results. Antibodies to PAD4 were detected in 24 of 82 (29.3%) probands; 2 of 147 (1.4%) relatives; and no controls ($p < 0.0001$). Anti-CCP was present in 39/144 (27.1%) of the relatives, and there was no overlap between positivity for anti-CCP and PAD4 in the relatives. In RA patients, anti-PAD4 antibodies were associated with disease duration ($p = 0.0082$) and anti-CCP antibodies ($p = 0.008$), but not smoking or shared-epitope alleles.

Conclusion. Despite a significant prevalence of anti-CCP in FDR, anti-PAD4 antibodies were almost exclusively found in established RA. The prevalence of anti-PAD4 antibodies in RA is similar to the prevalence described in other populations and these autoantibodies are associated with disease duration and anti-CCP in RA. (First Release Aug 1 2013; J Rheumatol 2013;40:1523–8; doi:10.3899/jrheum.130293)

Key Indexing Terms:
ARTHRITIS RHEUMATOID AUTOANTIBODIES PEPTIDYLARGININE DEIMINASE

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Autoantibodies directed against citrullinated proteins are highly specific for rheumatoid arthritis (RA)[1]. Anti-citrullinated protein antibodies have been shown in multiple studies to be present prior to the onset of RA, with broadening of the autoantibody response as disease onset approaches[2,3,4,5,6,7]. The family of enzymes known as peptidyl arginine deiminases (PAD) catalyze the citrullination of proteins, and polymorphisms in the gene encoding PAD4 have been associated with RA in some populations[8,9,10]. Studies have described the presence of autoantibodies directed against peptidyl arginine deiminase type 4 (PAD4) in a subset of patients with RA[11,12,13,14]. In established RA, anti-PAD4 antibodies have been associated with anticyclic citrullinated peptide (anti-CCP) antibodies and more severe disease[11,12,13].

First-degree relatives (FDR) of people with RA have a 3-fold or higher risk of developing RA compared to individuals without a family history, and this risk is further increased in multiplex families[15]. Indigenous North American populations have high rates of RA, with evidence of familial clustering of disease[16,17,18,19]. Because multiple

studies have documented the presence of preclinical RA-related autoantibodies years prior to the onset of RA and high rates of RA in indigenous North American populations, we enrolled unaffected FDR from these populations in a study focused on early identification of RA. In previous studies, we have shown a high prevalence of anti-CCP and/or rheumatoid factor (RF) in FDR without clinical RA in the Cree/Ojibway population of Central Canada[20].

Anti-PAD4 antibodies have also been detected prior to the clinical diagnosis of RA in a small subset of patients. In a study by Kolfenbach, *et al*[21], 18% of people who developed RA had PAD4 antibodies present in preclinical serum, with a mean duration of positivity prior to diagnosis of 4.7 years. In the majority of patients, anti-PAD4 antibodies were detected after the development of anti-CCP antibodies. We hypothesized that anti-PAD4 antibodies might be present in a subset of FDR, especially those with anti-CCP antibodies. In this study we tested for anti-PAD4 antibodies in indigenous North American people with RA, their FDR without RA, and in healthy controls and found that despite the high prevalence of anti-CCP in the FDR, anti-PAD4 antibodies were almost exclusively found in people with established RA.

MATERIALS AND METHODS

Recruitment of participants. Participants were recruited from 2 indigenous North American populations in Canada and the United States. This included both urban and rural locations in Manitoba and Alaska. The study population in Manitoba has been described[22,23], and in Alaska, participants were recruited in the largest city (Anchorage), as well as in 2 communities in Southeast Alaska. We invited the following 3 groups to participate: (1) patients with RA (probands); (2) their unaffected FDR; and (3) healthy controls without a personal or family history of RA or other autoimmune disease. All probands met the 1987 American College of Rheumatology classification criteria for RA[24]. The probands and FDR were all First Nations or Alaska Native people, while we included both indigenous and white controls. All study participants provided written informed consent to participate in the study. The study was approved by the Research Ethics Board of the University of Manitoba, the Alaska Area Institutional Review Board, the Band Councils of the individual study communities in Manitoba, and the tribal health organizations of the study communities in Alaska.

Study visit and clinical data. Participants completed a detailed questionnaire including demographics, environmental exposures, joint symptoms, and family history of autoimmune diseases. After completing the questionnaire, all participants underwent a joint examination by a rheumatologist to determine whether inflammatory arthritis was present, including a 66/68 swollen/tender joint count. At the study visit, whole blood was drawn and serum was obtained by centrifugation, following standard operating procedures. Sera and whole blood were stored at –80°C until testing.

Cyclic citrullinated peptide (CCP) antibody testing. Sera were tested for the presence of anti-CCP antibodies using a commercial anti-CCP2 enzyme-linked immunosorbent assay (ELISA; Inova Diagnostics). As described[22], a cutoff of ≥ 40 units was used to maximize specificity. In addition, all FDR sera with positive CCP antibodies were retested for confirmation of positivity.

Rheumatoid factor (RF) testing. IgM RF was tested by an ELISA calibrated with a standard of known IU measured by nephelometry. Because cutoff levels have not been established in this population and the smoking prevalence is high, the cutoff for a positive RF was set at ≥ 50 IU, based on a level where 95% of white controls were seronegative, as described[22].

Anti-PAD4 antibody testing. Anti-PAD4 antibodies were detected in serum by immunoprecipitation, as described[11,21]. Antibody testing was done at the Johns Hopkins University Rheumatic Disease Research Core Center (RDRCC). Briefly, ^{35}S-methionine (Perkin Elmer) labeled PAD4 was generated by *in vitro* transcription and translation (IVTT) of the full-length human cDNA cloned from HL-60 cells (NCBI accession number NP 036519.1) using a commercial kit (Promega). One microliter of IVTT product was mixed with 1 μl of serum and incubated 1 h at 4°C in NP-40 lysis buffer containing 0.2% BSA and protease inhibitors. Protein A beads (Thermo Scientific) were added and incubated 30 min at 4°C. The beads were washed by resuspension and pelleting in NP-40 lysis buffer and then boiled in sodium dodecyl sulfate sample buffer. Samples were separated by polyacrylamide gel electrophoresis (PAGE) and immunoprecipitated proteins were visualized by radiography. Densitometry was performed, values were normalized to a known high-titer anti-PAD4-positive serum, and antibody positivity was defined as a normalized densitometry value of > 0.01. A semiquantitative scale (0, 1, 2, and 3+) based on densitometry of scanned immunoprecipitation autoradiographs was used to assign a value to each serum sample, as described[11,21].

HLA testing. HLA-DRB1 typing was performed by PCR using sequence-specific oligonucleotide primers and sequence-based typing. Study participants were classified according to the presence or absence of shared-epitope alleles. The following alleles were included as shared-epitope alleles: DRB1*0101, 0102, 0401, 0404, 0405, 0408, 0410, 1001, and 1402, as described[22,23].

Statistical analysis. Continuous variables were analyzed using t tests, ANOVA, or nonparametric alternative tests as appropriate. Categorical variables were analyzed with chi-square or Fisher's exact tests, as appropriate. A 2-sided p value < 0.05 was considered significant. Data analysis was done using Stata/IC (version 11.2; Stata LP) and GraphPad Prism (version 5.03; GraphPad Software, Inc.).

RESULTS

The characteristics of the study population by group are shown in Table 1. The FDR and controls were similar with respect to age, sex distribution, and prevalence of smoking, and were younger than the RA probands. Smoking prevalence was high in all study groups. Shared-epitope prevalence and number of copies were tested in the RA probands and FDR, but not in the controls. For the probands, the mean RA disease duration at the time of the study visit was 14.2 years.

The prevalence of autoantibodies in the RA probands and the FDR are shown in Table 2. All controls were negative for anti-CCP, RF, and anti-PAD4 (data not shown). All autoantibodies were more common in probands than in relatives ($p < 0.0001$ for all comparisons). Anti-PAD4 antibodies were present in 24 of 82 probands (29.3%) and in only 2 of 147 relatives (1.4%), despite a high prevalence of anti-CCP antibodies in the relatives (27.1%). As shown in Figure 1, the median titer of anti-CCP antibodies was lowest in the relatives, intermediate in the RA probands who were anti-PAD4-negative, and highest in the probands who were anti-PAD4-positive (33, 205, and 353 units, respectively; $p < 0.0001$). Anti-PAD4 and anti-CCP positivity overlapped in all 24 probands with anti-PAD4, and the overlap between anti-PAD4 and RF was slightly less frequent (22 of 24

Table 1. Characteristics of study participants.

Characteristic	RA Probands, n = 82	First-degree Relatives, n = 147	Indigenous North American Controls, n = 44	White Controls, n = 20
Age at study visit, yrs, mean (SD)	53.4 (14.3)	39.3 (13.0)	37.6 (11.7)	38.8 (10.6)
RA disease duration at study visit, yrs, mean (SD)	14.2 (10.9)			
Sex, n (%) female	71 (86.6)	99 (67.3)	31 (70.4)	13 (65.0)
Smoking				
Ever, n (%)	57 (69.5)	105 (71.4)	26 (59.1)	14 (70)
Current, n (%)	31 (39.2)	67 (47.5)	15 (34.1)	9 (45)
Shared epitope				
Any copy, n (%)	60/65 (92.3)	88/103 (85.4)	NA	NA
2 copies, n (%)	30/65 (46.2)	28/103 (27.2)		

RA: rheumatoid arthritis; NA: not available.

Table 2. Autoantibody results.

Autoantibody	RA Probands, n = 82	First-degree Relatives, n = 147
Anti-PAD4 positive, n (%)	24 (29.3)	2 (1.4)
CCP2-positive (≥ 40 AU), n (%)	68/81 (84.0)	39/144 (27.1)
Rheumatoid factor (RF)-positive (≥ 50 IU), n (%)	64/74 (86.5)	24/126 (19.1)
CCP+ and RF-positive, n (%)	59/73 (80.8)	9/123 (7.3)
Anti-PAD4+ and CCP-positive, n (%)	24/81 (29.6)	0/144 (0)
Anti-PAD4+ and RF-positive, n (%)	22/74 (29.7)	1/126 (0.8)

$p < 0.001$ for all comparisons of prevalence in probands vs relatives. All controls were negative for anti-PAD4, CCP, and RF.

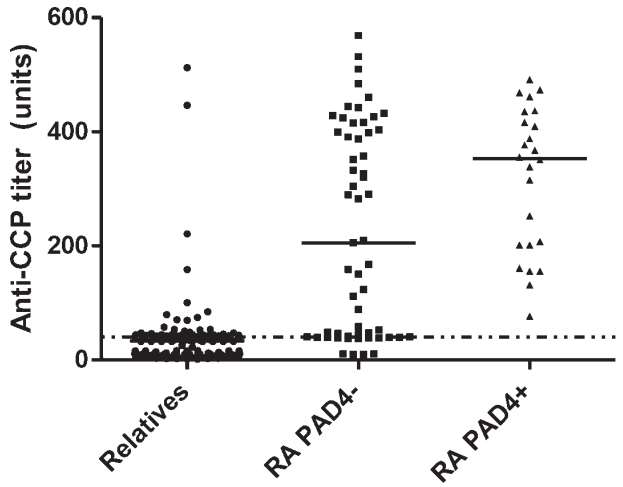

Figure 1. Anti-CCP antibody titers by group. Anti-CCP titers in units by group [relatives, probands without anti-PAD4 antibodies (RA PAD4–), and probands with anti-PAD4 antibodies (RA PAD4+)]. Median titer is noted on plot for each group, and dotted line at cutoff for positive anti-CCP antibodies (40 units).

probands). None of the relatives with anti-PAD4 antibodies had anti-CCP antibodies, and only one of the 2 had RF detected. None of the relatives had inflammatory arthritis at the time of the study visit, and neither of the relatives with anti-PAD4 antibodies present have had a followup visit to determine whether they have developed joint symptoms since the visit. Serial serum specimens were not available for these 2 relatives to evaluate for the persistence of anti-PAD4 antibodies over time. Anti-PAD4 antibody densitometry values were assigned as described above. Both relatives were strongly positive for anti-PAD4 antibodies that had densitometry readings of 3+.

Anti-PAD4 associations were evaluated in the RA probands, as shown in Table 3. RA probands with anti-PAD4 antibodies had significantly longer disease duration than those without PAD4 antibodies ($p = 0.0082$) and were more commonly anti-CCP antibody-positive ($p = 0.008$). There was a nonstatistically significant trend toward lower frequency of ever smoking in anti-PAD4-positive probands ($p = 0.067$). There was no association of anti-PAD4 with sex or shared-epitope presence, although at least one shared-epitope allele was present in a high proportion of the probands (92% overall). Because our study design is primarily focused on the characteristics of the FDR

Table 3. Anti-PAD4 associations in probands.

Characteristic	Anti-PAD4+, n = 24	Anti-PAD4–, n = 58	p
Disease duration, yrs, median	19.3	9.8	0.0082
Sex, n (%) female	22 (91.7)	49 (84.5)	0.495
Ever-smoker, n (%)	13 (54.2)	44 (75.9)	0.067
Current smoker, n (%)	5/22 (22.7)	26/57 (45.6)	0.157
CCP2-positive, (≥ 40 AU), n (%)	24 (100)	44/57 (77.2)	0.008
Rheumatoid factor-positive (≥ 50 IU), n (%)	22/23 (95.7)	42/51 (82.4)	0.158
Any shared-epitope allele, n (%)	20/21 (95.2)	40/44 (90.9)	1.000

with possible preclinical RA, radiographic data were not available on all probands and we were not able to assess the association of anti-PAD4 with radiographic damage. Of the 24 probands with anti-PAD4 antibodies, 19 (79.2%) had densitometry values of 3+, while the remaining 5 (20.8%) had densitometry values of 1+. In Figure 2, disease duration of RA probands is plotted against level of anti-PAD4 antibodies. In those with 3+ densitometry readings, disease duration was longest (median 24.6 yrs, as compared to 9.8 yrs in the group without anti-PAD4 antibodies and 8.6 yrs in those with 1+ densitometry; p = 0.0028).

DISCUSSION

In our study, we tested for anti-PAD4 antibodies in the sera of patients with RA and their FDR in a population with high rates of RA and anti-CCP positivity. We hypothesized that anti-PAD4 antibodies would be detectable in a significant proportion of FDR with anti-CCP antibodies but without RA, given the high frequency of anti-CCP antibodies and previous evidence that anti-PAD4 antibodies can precede clinical RA[21]. However, despite the high frequency of anti-CCP antibodies in the FDR, we found that anti-PAD4 antibodies were almost exclusively found in established RA patients and not in FDR.

Anti-PAD4 antibodies were found in 29% of RA patients, which is consistent with the prevalence in other published reports (ranging from 23%–50%)[11,12,13,14,21,25,26]. We examined associations with anti-PAD4 antibodies in RA and found that anti-PAD4 positivity was associated with disease duration and anti-CCP positivity. There was a nonsignificant trend toward lower prevalence of smoking among RA probands with anti-PAD4 antibodies compared to those without anti-PAD4 antibodies and no association with shared-epitope alleles. These findings are consistent with previous reports, which have shown an association of anti-PAD4 with disease duration[11] and anti-CCP antibodies[11,12,13], but not smoking[13] or shared epitope[11,12].

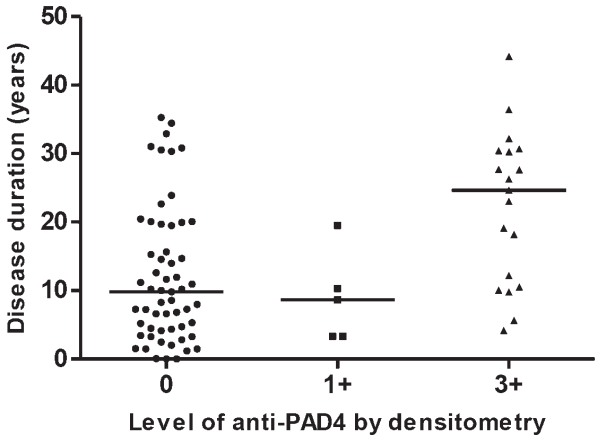

Figure 2. RA disease duration by level of anti-PAD4 by densitometry in probands. Disease duration in years, with median noted on plot. Densitometry labeled as 0, 1+, 2+, or 3+ as defined. No individuals had densitometry readings of 2+.

Based on the findings of this study and Kolfenbach, *et al*[21], it appears likely that in the majority of cases of RA, anti-CCP antibodies appear in serum prior to the development of anti-PAD4 antibodies. Although much remains to be determined about the timing of anti-PAD4 development in the setting of preclinical and very early RA, it appears that antibodies directed against PAD4 develop either just prior to onset of RA (but after anti-CCP antibody development) or after RA onset. Consistent with this finding, only 1.4% of the FDR tested had high-titer PAD4 antibodies, while 27.1% had anti-CCP antibodies. Since our study was designed to prospectively evaluate relatives who may or may not develop RA, and the time to potential RA diagnosis is unknown, the percentage of anti-PAD4 positives in this group is likely lower than would be expected in a retrospective evaluation of people who have already developed RA. The finding that the prevalence of anti-PAD4 antibodies increases with duration of RA in this study and others supports the hypothesis than anti-PAD4 development occurs later[11].

Potential mechanisms of development of autoantibodies against PAD4 and the role of these autoantibodies in disease initiation and propagation have been investigated in several studies. PAD4 has been postulated to have a pathogenic role in RA due to its ability to citrullinate antigens, its localization in the synovium in the setting of inflammatory arthritis, and increased synovial expression in the setting of more profound inflammation[27]. It has been hypothesized that PAD4 may generate citrullinated antigens, which leads to the development of an anti-CCP autoantibody response, and subsequent generation of autoantibodies targeting PAD4 due to its close association with its cognate substrate[21,28]. Consistent with the hypothesis that PAD4 may contribute to disease initiation or propagation in RA, it has recently been shown that anti-PAD3/4 crossreactive antibodies increase the enzymatic activity of PAD4 by decreasing the enzyme's calcium requirement[29]. In contrast to those findings, Auger, *et al*[30] found that the majority of anti-PAD4 antibodies inhibit citrullination of fibrinogen and hypothesized that anti-PAD4 antibodies precede antibodies against citrullinated proteins in the development of RA. Additional research is needed to clarify the mechanism of anti-PAD4 antibody formation and the pathogenic role of these autoantibodies in RA.

The role of other PAD in RA also remains to be determined. Recently, human PAD2, PAD3, and PAD4 have all been shown to be expressed in neutrophils and to have different substrate specificities[31]. Further, PAD generated by the oral bacterium *Porphyromonas gingivalis* has been shown to citrullinate human fibrinogen and α-enolase, which could account for the observed association between

periodontal disease and RA[32]. In animal models, a pan-PAD inhibitor reduced synovial and serum citrullination, reactivity to citrullinated epitopes, and disease activity in a murine model of collagen-induced arthritis, suggesting that PAD is necessary for inflammatory arthritis in this model[33]. PAD inhibition has been proposed as a potential therapeutic option for RA[34].

Our study has a few limitations. First, the focus of our study is healthy FDR of patients with RA who do not yet have RA themselves. Because this is a prospective study, it is not known how many of these relatives will go on to develop RA. However, the high proportion of individuals with anti-CCP antibodies present and the high prevalence of RA in these populations makes it likely that a significant proportion will develop RA. We are continuing these longitudinal studies in order to be able to answer that question more definitively in the future, including retesting anti-PAD4 antibodies in the relatives over time and identifying possible predictors of imminent RA. Second, our focus on the relatives meant that we did not collect longitudinal radiographic data on all probands. Therefore, we were not able to confirm previous findings that anti-PAD4 antibodies are associated with radiographic damage in RA. However, assessing associations of anti-PAD4 in established RA was not our primary focus in this analysis, and several other studies[11,12,13] have already confirmed the radiographic associations. We did not include a non-autoimmune chronic inflammation control group in this study. However, given our generally negative results in the relatives, we feel that this is not a significant limitation. Our assay detected only antibodies directed against uncitrullinated PAD4, so we are unable to comment on the prevalence or significance of antibodies directed against citrullinated PAD4. Finally, the high prevalence of HLA shared-epitope alleles and smoking in the population likely affected our ability to detect any associations between anti-PAD4 antibodies and these variables, if present.

We found that the prevalence of anti-PAD4 antibodies in indigenous North American populations with established RA was similar to the prevalence in other populations. Anti-PAD4 antibodies were present almost exclusively in the setting of RA, and were uncommon in first-degree relatives without RA, even among relatives with anti-CCP antibodies. This suggests that development of anti-PAD4 antibody may occur more proximal to the onset of clinical RA than anti-CCP antibody. Further studies are needed to examine the role of anti-PAD4 antibodies in the development and progression of RA.

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