CHAPTER 2

Marked differences in fine specificity and isotype usage of the anti–citrullinated protein antibody in health and disease

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ABSTRACT

Objective

Anti–citrullinated protein antibodies (ACPAs) display high association with rheumatoid arthritis (RA) and are implicated in its pathogenesis. The presence of ACPAs is known to precede the onset of RA. In order to identify the features that could confer its pathogenicity, we extensively characterized this antibody response in a unique North American native population of patients with RA and their unaffected relatives.

Methods

The levels of IgA, IgM, and IgG ACPAs, as well as IgM and IgA rheumatoid factor (RF), were measured in serum samples obtained from 81 patients with RA and 195 of their unaffected relatives. The isotype distribution, the fine specificity of the ACPA response, and its association with RF were compared in health and disease.

Results

ACPA positivity was observed in 19% of the healthy relatives and ~91% of the patients with RA. ACPA isotype usage was strikingly lower in unaffected relatives than in patients with RA (1–2 versus 5–6 isotypes). Fine specificity studies showed that reactivity to citrullinated fibrinogen and vimentin was present in sera from patients with RA, while it was virtually absent in their unaffected relatives. Finally, the ACPA and RF responses were associated in patients with RA but were discordant in their healthy relatives. Extended analyses revealed that the presence of ACPAs was associated with RA irrespective of RF status, while the association of RF with disease relied on its interaction with ACPAs.

Conclusion

The fine specificity and isotype usage of the ACPA response are qualitatively different in health and disease. Epitope spreading and expansion of the isotype repertoire might be necessary for development of RA, and this could be facilitated by the presence of RF antibodies.

INTRODUCTION

Autoantibodies are characteristic of a large number of autoimmune diseases. Determining the pathogenicity of autoantibodies by showing their capacity to transfer disease, however, is complicated by ethical and practical difficulties. Therefore, only a small minority of autoantibodies, such as antiplatelet antibodies in idiopathic thrombocytopenia purpura or the antidesmoglein antibodies in pemphigus vulgaris, were convincingly shown to mediate a pathogenetic effect through placental transfer[1] or transfer into experimental animals[2], respectively.

Autoantibodies sometimes are found in healthy individuals as well and often are present years before the disease presentation. A detailed understanding of the characteristics of the autoantibody response that render it pathogenic is, therefore, of utmost importance, because such an understanding would allow targeted intervention in individuals at risk, before disease onset. Several characteristics of autoantibodies have been associated with pathogenicity. In pemphigus vulgaris, a skin disease, reactivity to different desmoglein epitopes is associated with different disease outcomes.[3] In antibody-mediated models of arthritis in mice, a combination of antibodies with different fine specificities and isotypes was found to be more efficient than a single antibody in inducing disease.[4, 5] Moreover, in these models, soluble immune complexes were shown to facilitate the access of pathogenic antibodies to the joint.[6]

Rheumatoid Arthritis (RA) is strongly associated with the presence of autoantibodies. Although the involvement of autoantibodies in RA is poorly understood, studies in several mouse models of arthritis indicate that autoantibodies are crucial to disease induction and progression.[7, 8] Among the different autoantibodies described in patients with RA, the anti-citrullinated peptide antibodies (ACPA) display the strongest RA specificity for RA (95-98%), with 70–80% sensitivity.[9] The presence of ACPA in patients with recent-onset RA is predictive for progression to erosive RA[10, 11] and their presence in patients diagnosed with undifferentiated arthritis is highly predictive of progression to RA.[12] Likewise, ACPAs were shown to contribute to disease progression in a mouse model of arthritis.[13] Taken together, data from human and mouse studies indicate that ACPAs are a possible pathogenetic mechanism in RA.

The prevalence of the ACPA response in healthy Caucasians is estimated to be 1-2% of the population.[14] Information about ACPAs in unaffected individuals is therefore scarce in the literature, and originates from retrospective studies that involve serum samples collected from patients with RA before disease onset.[15, 16] These studies, however, did not investigate the characteristics of the ACPA response in health versus disease. Characterization of the ACPA response, including isotype usage and fine specificity, in healthy individuals and patients with RA would provide valuable information about features of the ACPA response that could be

associated with pathogenicity and could indicate the biological pathways involved in disease development.

North American Natives have previously been reported to have a younger age at disease onset and an increased prevalence and severity of RA. Genetic studies have also revealed a higher prevalence of shared epitope (SE) alleles in North American Natives and increased frequency of rheumatoid factor (RF) positivity in patients with RA in several North American native populations.[17] The high predisposition to RA prompted us to investigate the presence of the ACPA response in a unique population of patients with RA and their unaffected relatives from a North American native population in Central Canada.

Surprisingly, our results show a high prevalence of ACPAs in unaffected relatives of patients with RA in this population. This remarkable feature of this population allowed us, for the first time, to extensively study and compare the main characteristics of the ACPA response in health and disease and to identify the features that could confer its pathogenicity.

PATIENTS AND METHODS

Patients, relatives and control subjects

Patients with RA were selected from a Cree and Ojibway population in Central Canada. Probands visiting rheumatology clinics in urban (Winnipeg, Saskatoon) and rural (Norway House, St Theresa's Point) locations were recruited, and each patient was asked to bring along unaffected relatives who were willing to participate in this study. The unaffected population consisted mainly of first-degree relatives (75.5%), with the remainder being second-degree relatives. Additionally, 91 unrelated healthy controls were recruited from the same populations. Eighty-five families were included, comprising 76 probands and 208 relatives. Serum samples from 9 probands are missing. The largest family consisted of 1 proband and 13 relatives.

The characteristics of the patients with RA and their healthy relatives who were included in this study are shown in Table 1. RA was diagnosed according to the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria.[18] Clinical assessment of swollen, stiff and painful joints of all patients and relatives was performed by a rheumatologist at the time of inclusion in the study. The number of swollen joints is available for 62 probands (76.3%). Of these patients, 9 had no swollen joints at the first visit. Among the relatives, 5 were found to have RA, and 3 of these patients were also currently receiving disease modifying antirheumatic drugs. These 5 individuals were analyzed as part of the RA population. Of the remaining group of relatives, 8 presented with >1 swollen joint at the time inclusion but did not meet the criteria for RA or any other rheumatic disease and were classified as having undifferentiated arthritis.

The 91 unrelated controls had no swollen joints at the time of inclusion and no first-degree relative with RA. Their median age was 30 years (range 18-88 years) and 39.6% were women. Written consent was obtained from all participants, and the protocol was approved by the Biomedical Research Ethics Boards of the University of Manitoba and by the Band Councils of each rural community.

ACPA isotypes

ACPA isotypes (IgA, IgG1, IgG2, IgG3, IgG4 and IgM) were measured in baseline serum samples obtained from patients with RA, their healthy relatives and unrelated healthy control subjects, using CCP2 plates (Euro-Diagnostica, Arnhem, The Netherlands) and a previously described enzyme-linked immunosorbent assay (ELISA).[19] A successive dilution of one reference standard, consisting of a pool of 20 ACPA-positive samples, was used in all plates. Distinct dilutions of this standard (for IgA and IgM, 1:12.5; for IgG1, 1:400; for IgG2, 1:6.25; for IgG3 and IgG4, 1:12.5) were defined as containing 1,000 arbitrary units (AU) per milliliter.

Fine-specificity measurements

Antibodies against the Sa antigen were detected by ELISA as anti-citrullinated myelin basic protein and confirmed by Western blot analysis as anti-citrullinated vimentin, as previously described.[20] The screening dilutions ensuring 99% RA specificity were 1:300 in ELISA and 1:100 in Western blot analysis. The anti-Sa ELISA cutoff is based on 2 SD of the value for non-RA Caucasian control subjects with connective tissue diseases. Human fibrinogen (Sigma-Aldrich, Zwijndrecht, The Netherlands) was also citrullinated as previously described[21], under nondenaturing conditions. For detection of antibodies against citrullinated fibrinogen, MaxiSorp microtiter plates (Nunc, Sanbio, The Netherlands) were coated with 1 µg/well citrullinated fibrinogen or native fibrinogen in phosphate buffered saline (PBS)(pH 9.0) and were incubated overnight at 4°C. Plates were washed 4 times with PBS/0.05% Tween-20 after this and all other incubation steps. After blocking with 2% Bovine serum albumin (BSA)/PBS (pH 9.0) for 2 hours at room temperature, plates were incubated with serum samples diluted 1:50 in radioimmunoassay (RIA) buffer/5% normal sheep serum (Sigma-Aldrich), for 1 hour at room temperature. Antibodies to citrullinated fibrinogen/fibrinogen were detected using rabbit anti-human IgG-horseradish peroxidise (DAKO, Glostrup, Denmark), diluted 1:10,000 in RIA buffer, by incubating the plate for 1 hour at room temperature. Bound antibodies were visualized using avidin-biotinylated enzyme complex.

The same reference standard as that used for measurements of ACPA isotypes was used in the citrullinated fibrinogen ELISA. The 1:12.5 dilution was defined as containing 1,000 AU/ml of antibody. For the anti-Sa ELISA, reference standards are based on optical density (OD) values given by low, medium and high serum titers at a 1:300 dilution in 1% BSA.[20]

The following linear citrullinated peptides and their native counterparts were used for fine-specificity studies: C2 (vim) (STCit SVS SSS YCitCit MFG G)[22] and C3 (vim) (VYA TCitS SAV CitLCit SSV P)[23] derived from human vimentin, C4 (fib) (NEE GFF SACit GHR PLD KK)[23] and C5 (fib) (FLA EGG GVCit GPR VVE RH) (unpublished data) derived from human fibrinogen, and C6 (enolase) (KIH ACitE IFD SCitG NPT V)[24] derived from human non-neuronal enolase. All synthetic peptides were coated on streptavidin-coated plates (Fisher, Winnipeg, Manitoba, Canada) via a C-terminal long-chain biotin. Detection of antibodies recognizing these peptides was performed as described.[23]

A successive dilution of one serum sample reactive with all vimentin and fibrinogen peptides was used as reference standard for these peptides. The 1:25 dilution of the reference sera was defined as containing 1,000 AU/ml of antibody.

Determination of cut-off values for ACPA ELISA assays

The level of specific antibodies in each serum sample was determined using the reference standard curve. Cutoff values for the citrulline-specific responses were calculated in each assay and were defined as 2 SDs above the mean concentration of serum samples obtained from 30 healthy Caucasian control subjects. In case the concentrations of several control samples were below the detection limit of the ELISA, the cutoff was calculated as the lowest concentration situated on the ascending region of the standard curve. Borderline samples (with concentrations between 10 AU above and 10 AU below the cut-off value) were tested at least twice for IgA ACPA and all IgG isotypes. All samples that reacted with the citrullinated peptides in fine specificity assays were retested. Only samples that were positive every time tested were considered positive.

In fine-specificity studies, citrulline-specific signals were defined as a positive signal against the citrullinated antigen and a negative signal against the uncitrullinated antigen, with a minimum difference of 0.1 in OD values. For uncitrullinated control antigens, a signal higher than 2 SDs above the average OD value for at least 4 Caucasian control subjects tested on the same plate was considered positive. Samples were tested on the same plate for citrullinated and control antigens to minimize experimental variations in OD measurements.

Rheumatoid Factor measurements

IgM RF values reported throughout the study were determined by nephelometry. IgA-RF and IgM-RF were also measured by ELISA, using human IgG1 as the capture antigen and F(ab')2 fragments of peroxidise-conjugated anti-human IgA or IgM, as previously described.[25] The cutoff values used to assign positivity were based on 60 Caucasian controls. The IgM-RF values as determined by ELISA were comparable to those obtained by nephelometry.

Statistical analysis

Statistical analyses were performed using SPSS version 14.0 software (SPSS, Chicago, IL). Differences in the distribution of the ACPA isotypes between patients with RA and their healthy relatives were calculated using the chi-square test. The chi square test was also used for calculating odds ratios (ORs) with 95% confidence intervals (95% CIs), and P values were used for the association of RF and ACPA with RA. When a cell contained fewer than 5 individuals, the P value was calculated with Fisher's 2-tailed exact test. P values less than 0.05 and 95% CIs which excluded the value of 1 were considered significant. Multivariate logistic regression analyses with IgM-RF and ACPAs or IgA RF and ACPAs as covariates were performed for testing the interaction between RF and ACPAs.

Moreover, the individual variables significantly associated with RA were also tested in a multivariate and conditional logistic regression with age and sex as possible confounders (data not shown). These analyses were performed using the Stata 9.0 software (StataCorp, College Station, TX).

RESULTS

The characteristics of the patients with RA and their healthy relatives included in this study are shown in Table 1. The patients were significantly older than the healthy relatives (median 52 years versus 35 years; $p<10^{-8}$ by Mann-Whitney-test) and were more likely to be females (p=0.001 by Chi-square test). Correction for these variables, as described in Materials and Methods, did not result in significant changes in ORs and P values (data not shown).

We first determined the prevalence of ACPA in the patients with RA and their relatives (Table 2). The prevalence of ACPAs was 91.4% in the patients with RA. Intriguingly, an unusually high prevalence of ACPAs (19.0%) was also found in the healthy relatives. Of the unrelated healthy control subjects, 8.8% were positive for ACPAs; this prevalence was significantly lower than the prevalence in relatives (P=0.03 by chi-square test), although it was still higher than the prevalence reported in healthy Caucasians. These data suggest that there is a high background frequency of ACPAs in this healthy North American native population, which is higher in the relatives of patients with RA compared with unrelated healthy individuals. Of note, the small subset of relatives who presented with undifferentiated arthritis at baseline, the frequency of ACPAs was intermediate (37.5%). This group was excluded from further analyses, due do its small size.

The most prevalent isotypes were IgG1 and IgA, with a higher prevalence in patients with RA than in control subjects (Table 2) (for IgG1, 98.6% versus 62.2% [P<10-6]; for IgA, 82.4% versus 32.4% [P<10-6] for ACPA-positive patients with RA versus their healthy relatives). In contrast, IgM, IgG2, IgG3, and IgG4 were

Characteristic	RA patients (n = 81)	Healthy Relatives (n = 195)
Age, median (range) years	52 (19-76)	35 (18-78)
Female sex	90.1	71.8
Location		
Winnipeg	51.9	51.3
Sasketoon	16	7.7
Norway House	23.5	35.4
St. Theresa point	8.6	5.6
Total HAQ score		
Mean	0.74	0.13
Median	0.75	0
No. of DMARDS, mean (range)	3.1 (0-8)	0 (0-0)
Erosions	40.3	Not tested
No. of swollen joints, median (range)	3.5 (0-36)	0 (0-0)
Diagnosis		
RA	100	0
Undifferentiated arthritis	0	0
Healthy	0	100

Table 1. Clinical characteristics of the study populations. Except where indicated otherwise, values are the percent of patients with rheumatoid arthritis (RA) and their healthy relatives. Health Assessment Questionnaire (HAQ) scores were determined in 70 patients with RA. The use of disease-modifying antirheumatic drugs (DMARDs) was assessed in 71 patients. The presence of erosions was determined in 67 patients with RA. The number of swollen joints was determined in 62 patients with RA and in 187 healthy relatives.

detectable in a significant percentage of patients with RA, but were virtually undetectable in the healthy relatives (Table 2). As a consequence, the total number of isotypes used (Table 3) was also markedly different in patients with RA compared with their healthy relatives. Although many serum samples from patients with RA (n=20) were positive for all 6 ACPA isotypes, sear from healthy ACPA-positive relatives showed usage of mostly 1 isotype (n=30) or a maximum of 2 (n=7). These data indicate the presence of a broader ACPA response in patients with RA compared with their healthy relatives, suggesting that the immune response to citrullinated antigens in the healthy relatives is relatively limited compared with that in the patients.

	Healthy relatives (n = 195)	Relatives with undifferentiated arthritis (n = 8)	RA patients (n = 81)	Unrelated healthy subjects (n = 91)
ACPA positive	19.0	37.5	91.4	8.8
Isotype				
IgM	2.1	0.0	49.4	ND
IgA	6.2	0.0	75.3	3.3
IgG1	11.8	25.0	90.1	6.6
IgG2	0.5	0.0	49.4	ND
IgG3	1.5	12.5	43.2	ND
IgG4	0.5	0.0	38.3	ND
IgM-RF	15.4	37.5	81.5	29.7
IgA-RF	21.6	14.3	48.1	19.8

Table 2. Percentage of individuals positive for ACPAs and for isotypes of ACPAs and RF in the healthy and affected population. IgA rheumatoid factor (IgA-RF) positivity was determined in 185 healthy relatives, 7 relatives with undifferentiated arthritis, and 79 patients with rheumatoid arthritis (RA). ACPA = anti–citrullinated protein antibody; ND = not determined.

No. ACPA isotypes used	Healthy relatives (n = 195)	RA patients (n = 81)
0	158	7
1	30	12
2	7	16
3	0	4
4	0	7
5	0	15
6	0	20

Table 3. ACPA isotype usage in probands and relatives. For differences between the distribution of the number of isotypes used in healthy relatives and patients with rheumatoid arthritis (RA), $P < 10^{-35}$; for differences among patients with RA, P= 0.20, by chi-square test. ACPA = anti–citrullinated protein antibody.

Expansion and maturation of antibody responses include not only extended isotype usage but also intramolecular and intermolecular epitope spreading.[3] We therefore studied the fine specificity of the IgG ACPA response in paitents with RA and their healthy relatives. We specifically examined the recognition of citrullinated

fibrinogen and vimentin (the Sa-antigen), autoantigens expressed in the inflamed joint that have been shown to be recognized by IgG ACPAs from patients with RA.[26] [27] The results depicted in Table 4 show that although citrullinated fibrinogen and Sa were recognized by IgG ACPAs from more than half of the patients with RA, virtually none of the IgG ACPA-positive sera from healthy relatives recognized these antigens. (The results described in Table 4 were obtained with a linear α-enolase peptide containing a small difference in sequence compared with the peptide used by Lundberg et al, whose report appears elsewhere in this issue28). Interestingly, most of the RA sera that reacted with citrullinated antigens recognized both citrullinated fibrinogen and Sa.

	Healthy relatives (n = 27)	**RA (n = 27)**
Proteins		
Anti-Sa	0.0	61.1
Anti-Citrullinated fibrinogen	3.7	59.7
Anti-Sa and anti-citrullinated fibrinogen	0.0	47.2
Peptides		
C2 (vim)	0.0	2.8
C3 (vim)	0.0	20.8
C4 (fib)	0.0	26.4
C5 (fib)	0.0	8.3
C6 (enolase)	3.7	13.9

Table 4. Percentage of IgG ACPA-positive individuals whose sera recognized citrullinated vimentin/SA and/or fibrinogen or citrullinated peptides derived from these antigens. Sera from individuals positive for IgG anti–citrullinated protein antibodies (ACPAs) were tested for recognition of citrullinated vimentin (anti-Sa) or anti–citrullinated fibrinogen or peptides derived from citrullinated vimentin (C2 [vim] and C3 [vim]), citrullinated fibrinogen (C4 [fib] and C5 [fib]), or citrullinated enolase (C6 [enolase]). RA= rheumatoid arthritis.

Furthermore, a spectrum of citrullinated epitopes previously shown to be recognized by sera from patients with RA, were also tested for recognition by the IgG ACPA-positive sera in this study. The vimentin and fibrinogen epitopes were selected from larger sets of peptides that were derived from the amino acid sequences of human vimentin and the α- and β-chains of human fibrinogen. The peptides were selected from these sets based on the fact that they allowed adequate differentiation of ACPA fine specificities. The α-enolase peptide was synthesized

based on a study demonstrating that this is the most frequently recognized epitope of that protein.[24] We observed the same striking difference between reactivities, with a significant proportion of RA sera recognizing 1 or more citrullinated epitopes (41.1% of IgG ACPA-positive patients), while virtually none of the sera from healthy relatives reacted against the citrullinated peptides tested. Taken together, these data indicate a markedly different recognition of citrullinated antigens by ACPAs in health and disease.

Previous retrospective studies have shown that the specificity and positive predictive value of ACPAs for RA are increased when ACPAs are combined with RF antibodies, especially of the IgM and IgA isotypes.[14-16] Therefore, we also studied the association of IgM-RF and IgA-RF with the ACPA response in health and disease. The data shown in Table 5 indicate that having both IgM-RF and ACPAs is a characteristic feature of RA (present in 65 of 81 patients with RA), although positivity for both IgM-RF and ACPAs was rare in healthy relatives (7 of 195 healthy relatives) (OR 209, 95% CI 68–647 [$P< 10^{-35}$] for IgM-RF-negative ACPA-negative versus IgM-RF-positive ACPA-positive). Similar data were obtained for IgA-RF and ACPAs (OR 63, 95% CI 22–179 [$P<10^{-22}$] for IgA-RF-negative ACPA-negative versus IgA-RF-positive ACPA-positive) (Table 6).

	Healthy relatives	RA patients	OR (95% CI)	P-value
IgM RF-				
ACPA-	135	6		
ACPA+	30	9	6.8 (2.23-20.40)	$p < 0.0002$
IgM RF+				
ACPA-	23	1		
ACPA+	7	65	213.6 (24.91-1830.86)	$p < 10^{-14}$
ACPA-				
IgM RF-	135	6		
IgM RF+	23	1	0.98 (0.11-8.51)	$p = 1.000$
ACPA+				
IgM RF-	30	9		
IgM RF+	7	65	30.9 (10.53-90.99)	$p < 10^{-12}$

Table 5. Association of ACPAs and IgM-RF with RA. Values are the number of healthy relatives and patients with rheumatoid arthritis (RA) positive for IgM rheumatoid factor (IgM-RF) and/or anti–citrullinated protein antibodies (ACPAs). P values were calculated by chi-square test or, when a cell contained fewer than 5 events, by Fisher's exact test. OR = odds ratio; 95% CI = 95% confidence interval.

	Healthy relatives	RA patients	OR (95% CI)	p-value
IgA RF-				
ACPA-	122	6		
ACPA+	23	5	30.9 (11.68-81.94)	$p < 10^{-16}$
IgA RF+				
ACPA-	28	1		
ACPA+	12	37	86.3 (10.59-703.77)	$p < 10^{-9}$
ACPA-				
IgA RF-	122	6		
IgA RF+	28	1	0.7 (0.08-6.28)	p = 1.000
ACPA+				
IgA RF-	23	35		
IgA RF+	12	37	2.0 (0.88-4.68)	p = 0.096

Table 6. Association of ACPAs and IgA RF with RA. Values are the number of healthy relatives and patients with rheumatoid arthritis (RA) positive for IgA rheumatoid factor (IgA-RF) and/or anti–citrullinated protein antibodies (ACPAs). P values were calculated by chi-square test or, when a cell contained fewer than 5 events, by Fisher's exact test. OR= odds ratio; 95% CI= 95% confidence interval.

Furthermore, after stratification for the presence of either IgM-RF or IgA-RF and ACPAs, we observed that the presence of ACPAs was significantly associated with RA, irrespective of IgM-RF. However, this association became stronger in the presence of IgM-RF. Intriguingly, IgM-RF was not associated with RA in the absence of ACPAs, but the association became significant when ACPAs were also present. These results suggest that the association of IgM-RF with RA is attributable to an interaction with ACPAs. This was further supported by a multiple logistic regression analysis indicating that the odds for having RA when both antibodies were present was 31.64 times higher than expected based on the ORs for the individual antibodies (P=0.005).

When we stratified for the presence of IgA-RF and ACPAs, we observed that ACPAs were associated with RA irrespective of positivity for IgA-RF, but this association is stronger in the presence of IgA-RF. Furthermore, an association between IgA-RF and RA was present only in the presence of ACPAs, but it did not reach the level of significance. Likewise, a logistic regression analysis indicated a trend toward an interaction between IgA-RF and ACPAs (exp(B)=2.79; P=0.276); however, this trend did not reach the level of significance.

Taken together, these data confirm the previously recognized association of IgM/IgA-RF with RA, but this study is the first to show that this association is attributable to an interaction with ACPAs.

DISCUSSION

In this study, we have characterized the ACPA response in a unique population of patients with RA and their unaffected relatives. The cohort was derived from the North American native populations in Central Canada, characterized by a high prevalence of RA and frequent multicase families ([29] and Peschken C et al, unpublished data). We detected a surprisingly high frequency of ACPAs in the unaffected relatives. This offered us the opportunity to study and compare the characteristics of the ACPA response in health and disease. Our data indicate the presence of a qualitatively different ACPA response in the 2 populations, characterized by more extensive isotype usage and recognition of citrullinated antigens present in the inflamed joints exclusively by sera from patients with RA. Moreover, by studying ACPAs in relation to IgM-RF and IgA-RF, we found that ACPAs are associated with RA independently of RF, while RF is associated with RA only in the presence of ACPAs. Our data indicate the presence of an interaction between these risk factors, with high odds for having RA when both antibodies are present.

To our knowledge, this is the first report of such a high prevalence of ACPAs in an unaffected population. Although the cause of this phenomenon is unknown, it has previously been shown that the Cree and Ojibway populations have a high frequency of disease predisposing SE alleles (59%).[30] Furthermore, we have shown in a Dutch population that SE alleles are a risk factor for the development of ACPAs rather than an independent risk factor for RA.[31] We are currently investigating whether this is also true for our North American native population of healthy relatives of patients with RA.

Previous retrospective studies have indicated that the presence of ACPA often precede disease onset. Considering that the unaffected relatives are, on average, younger than patients with RA, it is conceivable that disease will develop in the future in at least some of the ACPA-positive relatives. Follow-up studies are currently under way and will provide valuable information about changes in antibody characteristics that may be associated with disease development.

Although we detected a relatively high prevalence of ACPAs in the population of healthy relatives, our data indicate that the mere presence of ACPAs is not enough to induce disease. The limited ACPA isotype usage in the healthy relatives is consistent with a relatively immature autoantibody response. In patients with RA, chronic exposure to citrullinated antigens in the joint conceivably results in continuous (re)activation of antigen-specific B cells and favors isotype switching. This hypothesis is also supported by the low frequency of IgM ACPAs in healthy relatives (4 of 37 ACPA-positive individuals), as IgM is indicative of ongoing immune responses.

The fine-specificity data indicate that ACPAs recognize at least partially different

antigens in patients with RA and their healthy relatives, with responses against citrullinated fibrinogen and citrullinated vimentin/ Sa being present in more than half of the patients while being virtually undetectable in their healthy relatives. These citrullinated antigens are present in the inflamed joint and may serve as an important source of continuous antigen stimulation in RA synovium.[26, 27] In contrast, the antigens that stimulate the ACPA response in healthy individuals and are responsible for the initial loss of immune tolerance are currently not known. Based on observations made in several populations, it has been hypothesized that the association between smoking and ACPAs may be attributable to increased expression of citrullinated antigens in the lungs of smokers.[32-34] Alternatively, it has been proposed that the oral pathogen porphyromonas gingivalis, which is capable of citrullinating endogenous antigens, may be involved in the initial breaking of immune tolerance.[35] To address these hypotheses, a spectrum of environmental stimuli and their association with fine-specificity of ACPAs will need to be systematically examined in multiple populations, particularly genetically susceptible populations, such as North American natives.

An important observation made in this study is that the ACPA and RF responses are closely associated in the patients with RA while being discordant in their healthy relatives. Although IgG-RF was not tested in this study due to technical difficulties, these results are consistent with results from earlier studies indicating that IgM-RF and IgA-RF are risk factors for RA.[15, 16] Additionally, this study is the first to demonstrate that this association is confined to the ACPA-positive individuals, indicating that the contribution of RF antibodies to RA is dependent on their interaction with ACPAs.

The interaction between RF and ACPAs implies that these autoimmune responses may converge to precipitate disease. Although the biological mechanism underlying this observation is unknown, this association could be explained by a model proposed in a recent study, suggesting that that autoantibody-mediated articular inflammation in mice may be facilitated by soluble immune complexes that enable the access of pathogenic antibodies into the joint.6 Because RF antibodies recognize IgG molecules, they can form soluble immune complexes, which could facilitate access of ACPAs joints. An alternative explanation is that RF could amplify the effector mechanisms induced by ACPAs in the joint, thereby exacerbating joint inflammation.

In summary, our data indicate a diversification of the ACPA response in patients with RA when compared with that in healthy individuals who are at risk of disease. We speculate that the relatively limited ACPA response in healthy individuals will change over time, leading to disease manifestations. These changes likely involve broader isotype usage and/or epitope spreading and could be facilitated by RF antibodies that may allow access of ACPAs into the joint or amplify their effects. Un-

derstanding the pathways responsible for the diversification of the ACPA response in RA is important, because such an understanding could provide new treatment possibilities for targeting the pathological autoimmune response before disease becomes manifest.

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