Anti-homocitrullinated protein antibody isotype usage in rheumatoid arthritis and their unaffected first-degree relatives

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Abstract

Objective

The majority of rheumatoid arthritis (RA) patients express anti-citrullinated protein antibodies (ACPA). Unaffected first-degree relatives of RA patients (FDR) also express ACPA, commonly of the IgA isotype. IgG anti-homocitrullinated/carbamylated protein antibodies (AHCPA) have been detected in both RA and FDR. It is unknown whether other isotypes are expressed. We aim to investigate the AHCPA isotype profile in unaffected FDR of RA patients.

Methods

The enrolled subjects were examined by a rheumatologist. FDR and healthy controls (HC) were excluded if they had swollen joints. Serum AHCPA targeting homocitrullinated fibrinogen was determined using enzyme linked immunoabsorbant assay (ELISA). FDR were genotyped for HLA-DR4 alleles encoding the shared epitope (SE).

Results

125 RA (35 probands), 61 FDR and 40 HC were included. 20% of FDR expressed IgG AHCPA, compared to 30% in RA patients and 5% in HC (p=0.0010 for RA vs. HC). Levels of IgG AHCPA in FDR were similar to RA. FDR rarely expressed IgM (8%) and did not express IgA AHCPA. 20% of RA and 13% of HC subjects expressed IgM, but very few expressed IgA AHCPA (<7% in both groups). AHCPA expression in FDR was not significantly associated with joint symptoms, smoking or SE.

Conclusion

IgG AHCPA is the most commonly expressed isotype in RA and FDR. The significance of IgG AHCPA in FDR is unclear as it was not associated with joint symptoms or other risk factors for RA. Longitudinal studies are needed to determine whether AHCPA is meaningful in populations at risk for RA.

Key words

*r*heumatoid arthritis, autoantibodies, immunology, anti-citrullinated protein antibodies, rheumatoid factor

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Introduction

Rheumatoid arthritis (RA) is an autoimmune condition of unknown etiology. Evidence supports the role of antibodies to citrullinated proteins (ACPA) in the pathogenesis of RA (1-4). Citrullination is a post-translational modification that involves the conversion of arginine residues to citrulline through an enzymatic reaction catalysed by peptidyl arginine deiminase (PAD) (5). Although citrullination is ubiquitous, the production of ACPA is highly specific for RA (6) and can precede the clinical manifestation of RA by several years (7).

More recently, antibodies to homocitrullinated proteins (AHCPA) (also referred to as anti-carbamylated antibodies) have been described in RA (8-9). Homocitrullination is another post-translational modification in which lysine residues are converted to homocitrulline in the presence of cyanate (10). With the exception of one carbon atom, homocitrulline has an identical chemical structure to citrulline (10). Given this structural similarity, it is not surprising that ACPA and AHCPA are frequently cross-reactive (8, 11-14).

Similar to ACPA, AHCPA can also be detected in the serum several years before the onset of clinical disease (15) and appear to be specific to RA (8). Both families of antibodies have been detected in unaffected first-degree relatives (FDR) of RA patients (16-21). The isotype profile of ACPA-positive first-degree relatives (FDR) of RA patients has recently been described and consists frequently of the IgA isotype (16, 17, 19). In this study, we aimed to determine the AHCPA isotype profile in unaffected FDR of RA patients.

Methods

Study population

Patients with RA were recruited from a tertiary care rheumatology clinic, St. Joseph's Health Care (SJHC) (London, Ontario, Canada). All patients with RA were diagnosed according to the 2010 American College of Rheumatology/European League Against Rheumatism criteria (22). Patients with RA were asked to put research staff in contact with any FDR that would be interested in participating in the study. FDR were then contacted and screened for eligibility. Healthy control subjects (HC) were non-related subjects accompanying patients at SJHC. Inclusion criteria for FDR and HC consisted of no prior diagnosis of RA or other inflammatory arthritis and residing in Southwestern Ontario. All study participants completed a questionnaire regarding smoking history and joint symptoms. Swollen joint counts (SJC) were performed by one or more rheumatologists who assessed 64 joints in each subject; joints were counted if the swelling was felt to represent active disease attributable to RA. FDR and controls were excluded if they had any swollen joints. Informed consent was obtained from all enrolled study participants. The study protocol was approved by the Health Sciences Research Ethics Board at the University of Western Ontario (approval no. 105594 and 1087).

Antibody assays

Serum from study subjects was obtained at the time of the clinical assessment. AHCPA was measured using an in-house enzyme-linked immunoabsorbant assay (ELISA) as previously described (8). Human fibrinogen (Millipore, Etobicoke, Canada) was homocitrullinated by incubating with 0.125 M potassium cyanate (KOCN) in 0.188 M sodium phosphate buffer at 37°C for 24 h. Excess KOCN was removed by washing with PBS in 100 kDa MacrosepTM centrifugal filters (Pall Laboratory, St. Laurent, Canada). Homocitrullination of fibrinogen was verified via western blot analysis using a mouse monocolonal (clone F95) anti-peptidyl citrulline antibody (Millipore). Additionally, in a prior study, homocitrullination using this method was confirmed by mass spectrometry (8). Nunc MaxiSorp ELISA plates (Thermo Scientific, Waltham, USA) were coated with 10 μg/ml of homocitrullinated fibrinogen diluted in carbonate buffer. Plates were then washed (PBS/0.05% Tween-20) and blocked (PBS/0.1% with bovine serum albumin (BSA)) for 30 min at room temperature. Wells were incubated with human sera diluted at 1:100 in diluent containing PBS, 0.1% BSA, and 0.05% Tween-20 for 30 min at room temperature. After washing, wells were

incubated with a biotin-conjugated goat anti-human immunoglobulin (Jackson ImmunoResearch, West Grove, USA) of one of the following isotypes: IgG (1:5000), IgM (1:2500) or IgA (1:5000) and streptavidin horseradish peroxidase (HRP) polymer (1:4000; Sigma-Aldrich, Oakville, Canada) diluted in PBS, 0.1% BSA, and 0.05% Tween-20 for 30 min at room temperature. After thorough washing, wells were incubated for 10 min with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) and the reaction stopped with the addition of 1 M H_2SO_4. Absorbance was read at 450 nm.

Serum samples were tested at least in triplicate. Inter-assay coefficients of variation were <20% for all samples tested. AHCPA were quantified in arbitrary units/ml (AU/ml) by comparison to a reference serum sample for each antibody isotype. Cut-off values for positive antibody reactivity to homocitrullinated fibrinogen were calculated using the mean value for healthy control sera + 2 standard deviations (SD). The positive cut-off values were: 121.2 AU/ml for IgG AHCPA, 51.9 AU/ml for IgM AHCPA, and 0.8 AU/ml for IgA AHCPA. Healthy control sera samples that tested positive for any AHCPA isotype were not included in the cut-off calculation, however were used for other analyses.

Rheumatoid factor (RF) was measured by nephelometry at the immunology laboratory in London Health Sciences Centre, London, Ontario, Canada. For samples in which nephelometry testing was not available, RF IgM was tested using a commercial ELISA assay from Inova Diagnostics (San Diego, USA). For RF assays, a positive result was defined as >21 international units (IU)/ml (nephelometry) or >6 IU/ml (ELISA). Values for RF were comparable for the two assays used (data not shown). IgG anti-cyclic citrullinated peptide 2 (anti-CCP2) was measured using a commercial ELISA (Euroimmun US, Mountain Lakes, USA). Positive values were defined as per manufacturer's recommendation (≥ 5 relative units (RU)/ml).

Genetic testing

FDR were HLA typed to detect the presence of the SE. Testing was done by the Immunogenetics and Transplantation Laboratory of the London Health Sciences Centre (London, Ontario, Canada). Polymerase chain reaction sequence-specific priming using commercial typing kits (Qiagen) was performed as previously described (23). HLA typing of the following SE alleles was performed: DRB1*0101, 0102, 0401, 0404, 1001, and 1402.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software v. 6.0 (La Jolla, USA). Fisher's exact test was used to compare categorical variables. Associations were reported using odds ratios with 95% confidence intervals. For continuous variables, values were reported as medians and inter-quartile ranges (IQR). For statistical comparisons of continuous variables between groups, Kruskal-Wallis with Tukey's test for multiple comparisons was used. Statistical significance was considered $p<0.01$.

Table I. Demographic and clinical features of RA patients (n=125), unaffected FDR (n=61) and healthy controls (n=40).

	RA	n	FDR	n	HC	n	*p*-value
Female, n (%)	92 (74)	125	42 (69)	61	26 (65)	40	0.5388
Age, median (IQR)	59 (21)	125	54 (18)	60	52 (16)	39	0.0007
Smoking history, n (%)	78 (64)	121	30 (50)	53	13 (36)	36	0.0070
Joint pain, n (%)	67 (55)	122	23 (37)	61	9 (25)	37	0.0041
Presence of swollen joints, n (%)	60 (49)	122	0 (0)	61	0 (0)	40	<0.0001
SJC, median (IQR)[a]	5 (9)	60	N/A		N/A		
Morning joint stiffness, median (IQR) minutes	20 (60)	119	0 (0)	58	0 (0)	37	<0.0001
Disease duration, median (IQR) years	4.0 (15)	123	N/A		N/A		
Periodontal disease, n (%)[b]	15 (15)	101	6 (11)	57	6 (17)	36	0.6545

[a]Value calculated for patients with ≥1 SJC as determined by the treating rheumatologist.
[b]Periodontal disease was self-reported based on a diagnosis made by a dentist.
RA: rheumatoid arthritis; FDR: first-degree relative; HC: healthy controls; IQR: inter-quartile range; SJC: swollen joint count.

Results

Characteristics of study population

There were 125 RA patients and 40 healthy controls (HC) enrolled in the study. Of the 125 RA patients, 35 had 61 FDR that were willing to participate in this study and met criteria for enrolment. The demographics and clinical features of the study participants are outlined in Table I. Study participants were gender-matched across all three groups. The demographics of the 35 RA probands were similar to that of the other RA subjects (data not shown). RA patients were older compared to FDR and HC: median age 59 (IQR 21) *vs*. 54 (IQR 18) and 52 (IQR 16) years, respectively; $p=0.0007$. A history of smoking cigarettes was most common in RA patients (64%) compared to FDR (50%) and HC (36%); $p=0.0070$. RA patients had long-standing disease (median disease duration of 4.0 (IQR 15) years) and all patients were treated with disease-modifying anti-rheumatic drugs (DMARDs). At the time of the study, 51% of patients were in remission (defined as SJC of 0) and the rest had significant disease activity with a median swollen joint count of 5.0 (IQR 9). As expected, RA patients had more joint pain and longer morning stiffness than the other groups. Although FDRs were more likely to complain of joint pain (43%) compared to healthy controls (25%), this was not statistically significant.

Presence of AHCPA in RA patients, FDR and healthy controls

IgG, IgM, and IgA isotypes of AHCPA targeting homocitrullinated fibrinogen were measured in the study populations (Fig. 1). As expected, RA patients had significantly higher IgG AHCPA antibody levels compared to HC, median 69.5 (IQR 99.6) AU/ml *vs*. 41.1 (IQR 35.5) AU/ml, $p=0.0043$. The median IgG AHCPA levels for FDR of 71.3 (IQR 75.9) AU/mL were not statistically significantly different than the levels for RA or HC subjects. The observed IgM AHCPA antibody levels were

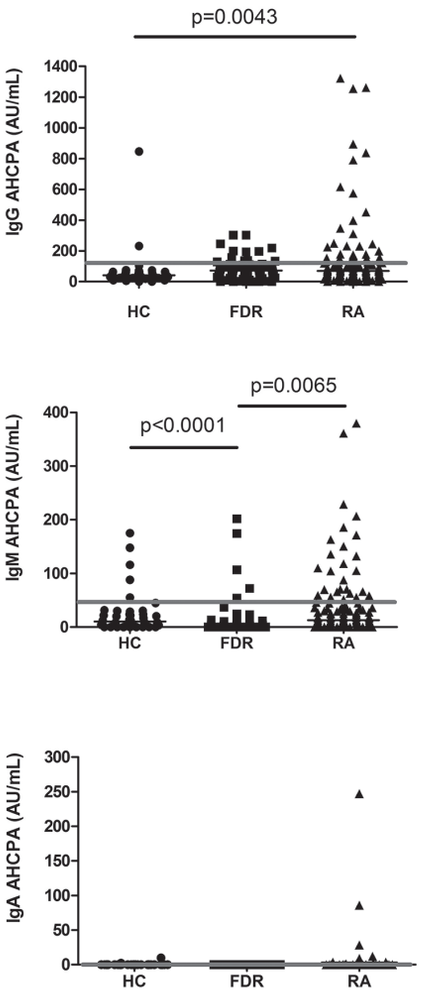

Fig. 1. Serum levels of different isotypes of AHCPA.
Serum levels in AU/ml of antibodies of (**A**) IgG, (**B**) IgM, and (**C**) IgA isotypes targeting homocitrullinated fibrinogen (AHCPA) in RA, unaffected FDR, and HC. Grey line represents cut-off value for AHCPA positivity. Black line represents the mean level for the group. Differences between groups were analysed by Kruskal-Wallis.
RA: rheumatoid arthritis; FDR: first-degree relative; HC: healthy control.

higher in RA patients and HC compared to FDR: median levels of 12.9 (IQR 36.6), 10.4 (IQR 25.5) and 0 (IQR 7.1) AU/ml, respectively ($p \leq 0.0001$ for RA *vs*. FDR and $p=0.0065$ for HC *vs*. FDR). Antibody levels of IgA AHCPA were low across all three groups and not significantly different.

The frequency of AHCPA-positive subjects is shown in Table II. IgG AHCPA was the most frequent isotype detected in both RA patients (30%) and FDR (20%). Healthy controls infrequently expressed IgG AHCPA (5%); $p=0.001$ for RA *vs*. HC and $p=0.0425$ for FDR *vs*. HC. Expression of IgM AHCPA was highest in RA patients (20%), but also present in FDR (8%) and HC (13%). IgA AHCPA positivity was uncommon in the sera samples tested: only 7% of RA patients and 5% of healthy controls had positive IgA AHCPA reactivity. None of the FDRs were positive for IgA AHCPA. RA patients were more likely to be positive for at least one AHCPA isotype (42%), compared to FDR (25%) and HC (23%), $p<0.0001$ for RA *vs*. FDR and HC. RA patients were also more likely to express at least two isotypes (13%) than were the FDR (3%); none of the HC expressed more than one AHCPA isotype. Only 2% of RA patients and none of the other two groups were positive for all three isotypes. Figure 2 illustrates the overlapping reactivity for the different isotypes of antibodies targeting homocitrullinated fibrinogen in the three study groups. The characteristics of FDR who were positive for at least one isotype of AHCPA did not differ significantly compared to FDR that did not express any AHCPA: median age 56 (IQR 12) *vs*. 51 (IQR 15) years, 6/10 (60%) *vs*. 19/34 (56%) were current or past smokers, 1/7 (14%) *vs*. 3/31 (10%) expressed the shared epitope and 7/11 (64%) *vs*. 22/38 (58%) had joint pain and/or morning joint stiffness ≥30 minutes. Similarly, the characteristics of AHCPA-positive did not differ to those of AHCPA-negative HC. Probands of AHCPA-positive FDR were commonly anti-CCP2-positive (7/9), but not AHCPA-positive (3/9), which did not differ significantly compared to the probands of AHCPA-negative FDR. In RA patients, the presence of AHCPA of any isotype was not significantly associated with disease activity (defined as SJC ≥1 attributable to RA)) and levels of AHCPA did not correlate significantly with active joint count (data not shown).

Table II. RA-associated antibodies in RA patients, unaffected FDR, and healthy controls.

	RA (n=125)	FDR (n=61)	HC (n=40)
Antibody expression, n (%)			
IgM RF	88 (70)[a]	9 (15)	4 (10)
IgG anti-CCP2	90 (72)[b]	5 (8)	0 (0)
AHCPA:			
IgG isotype	37 (30)[c]	12 (20)	2 (5)
IgM isotype	25 (20)	5 (8)	5 (13)
IgA isotype	9 (7)	0 (0)	2 (5)
Any Isotype:			
≥ 1 isotype	52 (42)[d]	15 (25)	9 (23)
≥ 2 isotype	16 (13)	2 (3)	0 (0)
3 isotypes	3 (2)	0 (0)	0 (0)
None	73 (58)	46 (75)	31 (78)

[a]$p<0.0001$ for RA *vs*. FDR and HC. [b]$p<0.0001$ for RA *vs*. FDR. [c]$p=0.001$ for RA *vs*. HC. [d]$p<0.0001$ for RA *vs*. FDR and HC. All other p-values were non-significant ($p>0.01$, corrected for multiple comparisons. RA: rheumatoid arthritis; FDR: first-degree relative; HC: healthy controls.

Association of AHCPA reactivity with anti-CCP2 and RF

IgM RF positivity was detected in 70% of RA patients, 15% of FDR, and 10% of HC ($p<0.0001$ for RA compared to FDR and HC) (Table II). IgG anti-CCP2 was positive in 72% of RA patients and 8% of unaffected FDR ($p<0.0001$); HC did not express IgG anti-CCP2 antibodies (Table II). 83% of RA patients tested positive for at least one autoantibody (IgG, IgM or IgA AHCPA, anti-CCP2 or RF), compared to 41% of FDR and 27% of HC. AHCPA positivity was significantly associated with anti-CCP2 (OR 3.23, 95% CI: 1.33, 7.87) and RF (OR=4.29, 95% CI: 1.70, 10.79) in RA patients but not FDR. Only 1 FDR was positive for both AHCPA and RF and 3 were positive for AHCPA and anti-CCP2.

Discussion

This study examined the expression of various isotypes of AHCPA in an unaffected FDR population of RA patients. In our population, FDR predominantly expressed IgG AHCPA with a prevalence of 20%, which is consistent with a prior study in a population of indigenous North Americans (18%) (21) and higher than what was seen in an Italian population (9%) (20). We were underpowered

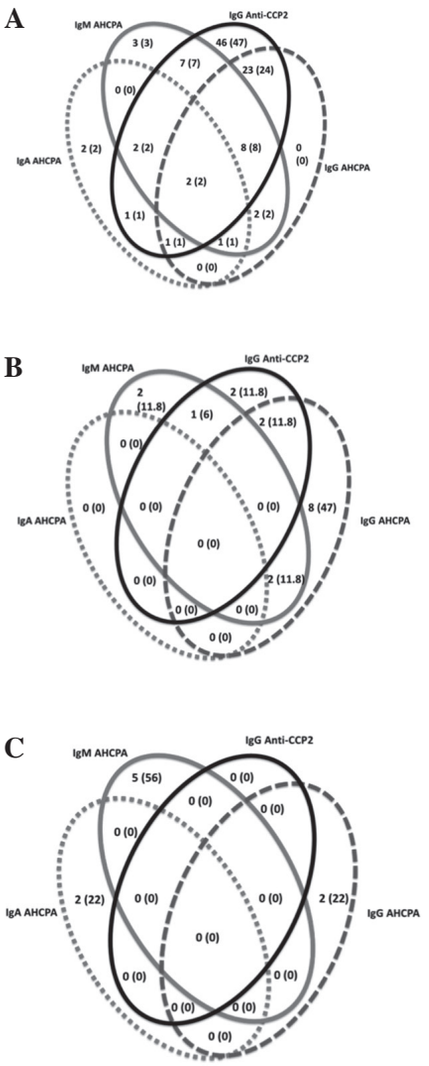

Fig. 2. Expression of different isotypes of AHCPA and IgG anti-CCP2.
(**A**) RA patients (**B**) unaffected FDR and (**C**) HC subjects. Values are n (%). The following were negative for all isotypes of AHCPA and IgG anti-CCP2 and were not shown in the figure: 27 (22%) of RA patients, 44 (72%) of FDR and 31 (78%) of HC.
AHCPA: anti-homocitrullinated protein antibodies; anti-CCP2: anti-cyclic citrullinated peptide antibody-2; RA: rheumatoid arthritis; FDR: first-degree relative; HC: healthy control.

to detect a difference in the expression and levels of IgG AHCPA in FDR (20%) compared to HC (5%). Although Alessandri *et al*. (20) and Koppejan *et al*. (21) found that levels of IgG AHCPA in FDR were higher than controls, we did not detect a significant difference in antibody levels between these two groups. Discrepancies between studies may be attributable to the characteristics of the study populations and the homocitrullinated antigen used. While previous studies have investigated the antibody response to homocitrullinated/carbamylated fetal calf serum (FCS), we studied reactivity to homocitrillinated fibrinogen, as it is found in the arthritic joint, and thus potentially relevant in the pathogenesis of RA (24).

The significance of AHCPA expression in FDR is unclear. In subjects with arthralgia, the presence of IgG AHCPA has been shown to predict the development of RA (25). In our study, AHCPA was not significantly associated with joint symptoms. Imaging of the joint to detect subclinical arthritis was not performed, but in another study, AHCPA did not predict ultrasound findings of early arthritis (20). Similar to Jiang *et al*., we also did not detect a significant association between AHCPA and risk factors for RA (cigarette smoking and the SE) (26). In FDR, ACPA expression has been found to be associated with arthralgias (18, 19, 27). Also, ACPA appears to be arthritogenic in animal models (1-4). Given that ACPA and AHCPA are concordantly expressed and cross-reactive (11-14), AHCPA may also be pathogenic. Future studies are needed to elucidate the pathogenic role of AHCPA in RA.

IgM AHCPA were infrequently expressed in our population (20% of RA patients and 8% of FDR). A recent study from a Latin American population reported a high prevalence of IgM AHCPA (80%); however, the measured antibodies targeted carbamylated vimentin rather than fibrinogen (28). Reported prevalence of IgM ACPA has also varied, 7–60% for RA and 1-22% for FDR depending on the target antigen (16-19, 29-31). IgM ACPA appears have a more restricted antigen recognition profile than IgG ACPA (29), but it is unknown whether the same is true for AHCPA. In addition, the pathologic role of IgM antibodies to citrullinated and homocitrullinated proteins is unclear. Generally, the humoral immune response starts with an IgM response, which is short-lived. Activation of T cells through the recognition of the antigen in the context of major histocompatibility complex II allows for B cell class switching, the differentiation to memory B cells and the production of other isotypes with longer half-lives. Our study is cross-sectional and therefore may not capture IgM AHCPA that is transient. The persistence of IgM in the serum may be a marker of ongoing inflammation by continued exposure to the antigen (30, 32); nevertheless, we were unable to detect a significant association between IgM AHCPA expression and disease activity in our RA population.

IgA is the predominant isotype involved in mucosal immunity and has been suggested to play a role in the development of RA (33, 34). There is an association between ACPA-positive RA and periodontal disease (PD) (35, 36). One of the major PD pathogens, *Porphyromonas gingivalis* expresses peptidyl arginine deiminase, providing a potential source of bacterial citrullinated antigens, which can induce autoimmunity through molecular mimicry (37, 38). We did not find that AHCPA-positivity was associated with PD; however, we had a small sample size and PD was self-reported, therefore it may be inaccurate. In addition, smoking is a major risk factor for RA and can induce inflammation and citrullination of proteins at the mucosal surface and may contribute to the increased presence of IgA ACPA seen in patients with preclinical and early stages of RA (38). IgA ACPA have also been shown to be the most common isotype expressed in Caucasian populations of FDR (17, 19). Isotypes other than IgG have not previously been studied for ACHPA in FDR. We found that IgG AHCPA was more common than IgA in our population of unaffected FDR. It is possible that the mechanisms underlying the break of tolerance to homocitrullinated proteins are different than those for citrullinated proteins, which can lead to different isotype usage.

In conclusion, we found that FDR express AHCPA, predominantly of the IgG isotype. IgM and IgA isotypes were uncommon in our FDR cohort. In FDR, the presence of AHCPA was not associated with ACPA, RF, arthritic symptoms or risk factors for RA. The AHCPA isotype profile of our FDR population was different from previously published ACPA data, whereby the IgA isotype was the most predominant. Prospective cohorts and mechanistic studies are warranted to better understand

the significance of the expression of different isotypes of AHCPA in populations at risk for RA.

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