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**Title:** Immune regulation by CD49b+ CD4+ T cells & modulation of B cell responses  
**Issue Date:** 2016-11-29
Anti-cyclic citrullinated peptide antibodies are a collection of anti-citrullinated protein antibodies and contain overlapping and non-overlapping reactivities

Chapter 4

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Ann Rheum Dis. 2011; 70(1):188-93

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Abstract

Objective Anti-citrullinated protein antibodies (ACPA) and anti-cyclic citrullinated peptide (anti-CCP) antibodies are a hallmark of rheumatoid arthritis and are believed to play a role in disease pathogenesis. These antibodies are typically detected in ELISA with citrullinated peptides [e.g., CCP2] or proteins as antigens. The absolute concentration of anti-CCP antibodies in serum is unknown. Although antibodies to several citrullinated proteins can mainly be detected within anti-CCP-positive sera, it is currently unknown whether anti-CCP antibodies are in fact ACPA. Likewise, it is unknown to what extent antibody responses to different citrullinated antigens are crossreactive.

Methods An affinity purification method was established in which citrullinated antigen-specific antibodies were eluted from ELISA plates and then used for detection of other citrullinated antigens in ELISA or western blot. For additional crossreactivity studies, ELISA-based inhibition assays were performed with citrullinated or control peptides as inhibitors.

Results The concentration of anti-CCP IgG antibodies was estimated to be at least 30 μg/ml in patients with high anti-CCP levels (>1600 μg/ml). Affinity-purified anti-CCP antibodies were able to recognise citrullinated fibrinogen (cit-fib) and citrullinated myelin basic protein (cit-MBP) on western blot. Furthermore, antibodies specific for cit-fib and cit-MBP were crossreactive. However, additional crossreactivity studies indicated that non-overlapping antibody responses to citrullinated peptides can also exist in patients.

Conclusions This report shows for the first time that anti-CCP antibodies recognise multiple citrullinated proteins and are thus a collection of ACPA. More importantly, the data indicate that different ACPA responses are crossreactive, but that crossreactivity is not complete, as distinct non-crossreactive responses can also be detected in patients with RA.

Introduction

Auto-antibodies are a hallmark of rheumatoid arthritis (RA). Anti-citrullinated protein antibodies (ACPA) and anti-cyclic citrullinated peptide (anti-CCP) antibodies are among the most specific auto-antibody systems described in RA [1] and are believed to play a role in the pathogenesis of the disease. This hypothesis is best supported by several studies in animal models in which transfer of monoclonal antibodies recognizing citrullinated fibrinogen (cit-fib) [2] or collagen [3] into mice could exacerbate inflammatory arthritis. Moreover, several studies have indicated that the presence of anti-CCP/ACPA antibodies in patients with early arthritis predicts progression to RA, [4] while anti-CCP/ACPA positive RA is more severe and progressive than seronegative RA [5, 6]. Additionally, anti-
CCP/ACPA antibodies were shown to be able to engage/activate effector mechanisms (Fc receptors/complement system) in vitro [7, 8].

Anti-CCP antibodies are typically detected in clinical practice using a commercially available ELISA kit employing CCPs with unknown sequence as antigen (CCP2 ELISA). Although this technique is able to detect the presence of anti-CCP antibodies in RA sera with high sensitivity, the biological relevance of the findings derived from studies employing the CCP2 ELISA is debated. A smaller percentage of studies employ other tests, in which in vitro citrullinated proteins such as fibrinogen [9], mutated vimentin [10] and others are used to detect ACPA. Positivity for these antigens is usually paralleled by positivity for CCP2 [11, 12]. Despite this association with anti-CCP, it is currently unclear whether the antibodies recognizing the CCP2 peptide are the same antibodies that recognize citrullinated proteins. Moreover, it is presently unknown to what extent ACPA detected with different citrullinated antigens reflect truly distinct antibody responses that could have a differential contribution to the pathogenesis of the disease.

Previous publications have indicated that the pattern of reactivity against different citrullinated antigens, at the population level, varies between patients [11, 13, 14]. This could indicate that ACPA responses against different citrullinated antigens are non-overlapping and simply coexist in some patients. However, other studies suggested that these responses are at least partially crossreactive in a subpopulation of patients with RA [12]. These results were further supported by a recent publication in which murine monoclonal antibodies generated against citrullinated collagen could crossreact with other citrullinated antigens [3]. There is, however, little data investigating the degree of crossreactivity in humans [15]. We have, therefore, set out to investigate for the first time, whether nonoverlapping ACPA responses could coexist in the serum of individual patients with RA. Furthermore, we investigated whether anti-CCP antibodies (detected by CCP2 ELISA) are ACPA (ie, antibodies recognizing citrullinated proteins) and we estimated the amount of IgG ACPA in serum. Our findings show that: (1) approximately 1 in every 500–1000 IgG molecules is directed against a citrullinated antigen, (2) anti-CCP antibodies do recognize citrullinated proteins and are thus, at least partially, ACPA, (3) there is a large degree of crossreactivity between ACPA recognizing various citrullinated antigens, expanding and confirming previous results and (4) non-overlapping ACPA responses exist in sera of at least some patients with RA.
Materials and methods

Patients and sera

Blood was collected from patients participating in the Leiden Early Arthritis Clinic cohort [16] at baseline. Serum was isolated and stored at −20°C until use. The study was approved by the local ethical committee and written informed consent was obtained from all participants.

ACPA, CCP2 and total IgG measurements

Antibodies recognizing CCP2 were determined in 1:50 diluted sera or undiluted eluates using CCP2 plates (Euro-Diagnostica, Nijmegen, The Netherlands), according to the manufacturer’s instructions. Citrullination of fibrinogen (Sigma-Aldrich, Zwijndrecht, The Netherlands) and of MBP (Sigma-Aldrich) and the specific ELISAs were performed as previously described [17, 18]. We elected to study citrullinated myelin basic protein (cit-MBP) as a citrullinated protein antigen as its ELISA has been previously standardised to be the clinical equivalent (positive vs negative) of the original anti-Sa (cit-vimentin) western blot assay [18]. Antibodies reactive with cit-fib or cit-MBP were determined in 1:50 or 1:100 diluted sera, respectively. Total IgG ELISA (Bethyl, Montgomery, Texas, USA) was performed according to the manufacturer’s instructions, using 1:40 000 diluted sera and undiluted eluates.

Antigen-specific affinity purification of antibodies

Antibodies recognizing CCP2, cit-fib or cit-MBP were allowed to adhere to CCP2, cit-fib-coated or cit-MBP-coated ELISA plates as described above. Next, antibodies bound to the plates were eluted with 100 μl/well 5 M NaSCN, 0.1 M citric acid pH 4 or 0.1 M glycine/0.3 M NaCl pH 2.8 for 15 min at room temperature, then neutralised with 2 M Tris-HCl pH 7.6 solution (only citric acid and glycine eluates). The eluates were dialysed against phosphate buffered saline (PBS) at 4°C overnight and then stored at -20°C until use. Eluates from multiple wells were usually pooled and used for further studies.

Elution using glycine buffer was used in all experiments except where indicated otherwise. For estimating the total amount of ACPA present in serum, CCP2-binding antibodies were eluted from 100 μl of 1:50 diluted serum by sequential incubations on CCP2 ELISA plates. Briefly, antibodies were allowed to bind to one well of a CCP2 plate for 1 h at 37°C, followed by the transfer of unbound antibodies present in solution to a new well. These sequences were repeated until no detectable antibodies were left in solution. Duplicate wells were used for elution of plate-bound antibodies from the positive wells using glycine buffer. Eluted antibodies were pooled, neutralized as described above, dialyzed against PBS overnight at 4°C and then stored at -20°C until use.
Western blot for detection of citrullinated antigens

A total of 5 μg of citrullinated/native antigens were run on 10% (fib and cit-fib) or 15% (cit-MBP and MBP) sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham, Diegem, Belgium). Blots were then incubated in blocking buffer (5% casein/PBS/0.05% Tween) overnight at 4°C, followed by incubation with 3 μl serum 1:1000 diluted in blocking buffer or undiluted eluates overnight at 4°C. After three washes with PBS/0.05% Tween, blots were incubated with 3 μl horseradish peroxidase conjugated rabbit anti-human IgG (DAKO, Heverlee, Belgium) 1:10 000 diluted in blocking buffer for 45 min at room temperature. Next, blots were washed and bound antibodies were visualised using enhanced chemiluminescence (ECL; Amersham). Equal protein loading was verified using Coomassie Brilliant Blue (Bio-Rad, Veenendaal, The Netherlands) or anti-MBP antibodies. Briefly, blots were stripped with 1% β-mercaptoethanol/2% SDS (in 50 mM Tris, pH 6.8) for 30 min at 50°C, followed by blocking with blocking buffer containing 5% bovine serum albumin and staining with horseradish peroxidase conjugated anti-MBP antibody (Abcam, Cambridge, UK) 1:10 000 diluted in blocking buffer, for 45 min at room temperature. All incubation steps were separated by three washes. Bound antibodies were visualised with ECL (Amersham).

ELISA-based inhibition assays

For inhibition studies, previously described specificity measurements were performed, with some modifications. 17 Briefly, 1:100 diluted sera were preincubated with the indicated concentrations of inhibitors for 1 h at room temperature, with continuous mixing, followed by a 15 min centrifugation for removal of insoluble immune complexes. Biotinylated peptides were used for coating and non-biotinylated counterparts as inhibitors. The following linear citrullinated peptides and their native counterparts were used: C3 (VYA TCitS SAV CitLCit S SV P) derived from human vimentin, C4 (NEE GFF SACit GHR PLD KK) and C5 (FLA EGG GVCit GPR VVE RH) derived from human fibrinogen and C6 (KIH ACiE IFD SCitG NPT V) derived from human non-neuronal enolase.

Results

Affinity purification of anti-CCP antibodies

To estimate the amount of anti-CCP antibodies present in serum and to analyze whether citrullinated proteins can be recognized by anti-CCP antibodies, we have isolated these antibodies by eluting them from CCP2 plates. Three different elution buffers were tested and the functionality of the affinity-purified antibodies was compared in a CCP2 ELISA (Figure 1A). Our results showed that the antibodies eluted using glycine were the most functional. Next, we wished to assess the amount of anti-CCP antibodies that can be eluted by this method. We show that IgG was readily detectable in eluates from
anti-CCP positive sera, while no IgG could be detected in eluates from anti-CCP negative sera (Figure 1B).

Because the binding capacity of the CCP2 ELISA plate is limited and there is still a significant amount of anti-CCP antibodies left unbound (data not shown), we estimated the amount of anti-CCP antibodies present in serum by transferring unbound antibodies to subsequent wells until no binding of anti-CCP antibodies could be detected. The amount of IgG in the eluates from the positive wells was measured (Figure 1C). By using 2 sera with high levels of anti-CCP antibodies and 1 serum with intermediate levels, we estimated that the amount of anti-CCP IgG antibodies in serum can reach 30 μg/ml in sera with high anti-CCP titres, which amounts to more than 1/1000 of the total IgG measured in those sera. No IgG could be detected in eluates from anti-CCP negative sera (data not shown).

**Anti-CCP antibodies are a collection of ACPA**

Anti-CCP antibodies isolated by this method were subsequently tested for recognition of two citrullinated antigens previously shown to be recognized by ACPA-positive sera: cit-fib and cit-MBP on western blot. The results depicted in figure 2A,B show that affinity purified anti-CCP antibodies are able to recognize cit-fib and cit-MBP on western blot, but not their non-citrullinated counterparts. Similar results were obtained with three different anti-CCP+/anti-cit-fib+ or anti-MBP+ sera. Together, these data show that anti-CCP antibodies are able to detect citrullinated proteins and are, at least partially, ACPA.

**ACPA are crossreactive towards different citrullinated antigens**

Previous studies have used inhibition assays to show that ACPA responses are crossreactive [15]. Our data indicate that several ACPA could exist within the anti-CCP antibodies, as shown in figure 2A,B. Therefore, we wished to investigate, employing a more direct method than previously used, whether different ACPA within the anti-CCP antibodies are distinct antibody responses or whether they are crossreactive to multiple citrullinated antigens.

Using the same technique as used for elution of anti-CCP antibodies (Figure 1B), we eluted anti-cit-fib antibodies or anti-MBP antibodies and studied their crossreactivity to cit-MBP and cit-fib, respectively (Figure 2C). Our results indicate that the affinity purified anti-cit-fib and anti-cit-MBP antibodies recognize both citrullinated antigens. These data were confirmed with three different sera that were positive for anti-cit-fib and anti-cit-MBP. In conclusion, our results confirm and expand previous data demonstrating that ACPA recognizing different citrullinated proteins, present in an individual serum, are crossreactive.
Figure 1. Affinity purification of anti-cyclic citrullinated peptide (anti-CCP) antibodies. (A) Levels of functional anti-CCP antibodies eluted from CCP2 ELISA plates using different elution buffers were determined in CCP2 ELISA. Concentrations are indicated in arbitrary units/ml. (B) Levels of anti-CCP or IgG antibodies in four different sera and eluates from these sera are depicted. (C) Total IgG and anti-CCP levels in three different sera are indicated. The estimated concentrations of anti-CCP antibodies in these sera are indicated in the last column.
Figure 2. Anti-cyclic citrullinated peptide (anti-CCP) antibodies recognise citrullinated proteins. Citrullinated fibrinogen (cit-fib), myelin basic protein (MBP) or their non-citrullinated counterparts (fib, MBP) were detected on western blot using anti-CCP-positive sera that are positive for anti-cit-fib (anti-CCP+ anti-cit-fib+) (A) anti-MBP (anti-CCP+ anti-MBP+) (B) or both (serum) (C) and antibodies eluted from these sera using CCP2 ELISA plates (eluate) (A, B). Additionally, antibodies eluted from cit-fib-coated (eluate cit-fib) or cit-MBP-coated (eluate cit-MBP) ELISA plates were used for detection (C). Additionally, anti-CCP-positive sera that were negative for anti-cit-fib (anti-CCP+ anti-cit-fib-) (A) or anti-cit-MBP (anti-CCP+ anti-MBP-) (B) were used. Equal protein loading was determined using Coomassie blue or anti-MBP antibodies, as indicated. Indicated molecular weights are in kDa.

One representative experiment out of three performed is presented.
Anti-CCP2 are a collection of ACPA

Figure 3. ELISA-based inhibition assays. Antibody responses to the indicated citrullinated peptides were measured in the presence of the indicated concentrations of inhibitors (A–C). Representative example of crossreactive antibody responses (A and C) and non-crossreactive antibody responses (B). The citrullinated peptides (peptide-cit) and their non-citrullinated counterparts (peptide-Arg) were used in these experiments. (D) Reciprocal (upper panels) or self-inhibition (lower panels) of C5 and C6 in four sera positive for anti-C5 and anti-C6 antibodies is depicted.
Distinct ACPA responses exist in RA serum

The data presented in figure 2 suggest that crossreactivity is present but not complete. To more directly study whether noncrossover reactive ACPA can exist within an individual, we assessed the extent of crossreactivity between different ACPA by using four citrullinated peptides containing a restricted number of citrullinated epitopes, in ELISA-based inhibition assays. The capacity of each citrullinated peptide to inhibit binding of ACPA to ELISA plates coated with the same peptide or one of the other citrullinated peptides was studied in these assays (Figure 3). The non-citrullinated counterparts of the peptides were used as controls. Two sera that contained detectable levels of antibodies to all four peptides were used for the initial studies in which all possible pairs of peptides were tested. Representative results are depicted in figure 3A-C.

Our results show different degrees of inhibition between different pairs of peptides: from almost complete inhibition (Figure 3A, C) to no inhibition (Figure 3B). All citrullinated peptides were able to inhibit binding to the plate-bound counterpart of the same peptide (Figure 3A-C, lower panels figure 3D and data not shown). Remarkably, one pair of peptides, C5 and C6, showed very limited crossreactivity in these two sera (Figure 3B and data not shown). Therefore, we wished to study whether this observation can be generalized to a larger number of patients with RA. From 116 RA sera tested, only 4 (3.5%) contained anti-C5 and anti-C6 responses that could be inhibited by C5 and C6, respectively (data not shown), which further supports the hypothesis that C5-specific and C6-specific responses display very limited crossreactivity. This is also the case for sera that do contain both reactivities, as inhibition studies performed with all sera positive for anti-C5 and anti-C6 responses show that only limited crossreactivity could be detected in these sera (Figure 3D) with no crossreactivity in two sera (serum 1 and 2, Figure 3D).

Discussion

Anti-CCP antibodies and ACPA can usually be detected in the same sera. However, it was currently unknown to what extent these antibody responses are overlapping. Our study shows, for the first time, that anti-CCP antibodies recognize citrullinated proteins and are thus, at least partially, ACPA. Moreover, we show that ACPA responses present within one patient are crossreactive, confirming previously published data. Importantly, however, crossreactivity is not complete, as in some sera distinct and non-overlapping reactivities against certain citrullinated epitopes could be detected.

To be able to obtain insight into the amount of anti-CCP antibodies present in serum and to study crossreactivity of ACPA/anti-CCP antibodies, it is crucial to affinity purify these antibodies in an antigen-specific fashion. Therefore, we first optimized the affinity purification method in order to
maintain the integrity of the eluted antibodies as much as possible. With this rather mild method, however, a significant amount of ACPA remained bound to the CCP2 plate.

This amount varies per serum but is estimated to represent between 25% and 50% of the total serum ACPA, based on CCP2 ELISA performed on the eluted wells (data not shown). Moreover, it is conceivable that part of the eluted antibodies will be destroyed during the elution procedure. Our estimation of serum ACPA levels is, therefore, most probably an underestimation. However, the calculated levels fall in the range of 10 μg/ml, which is of the same order of magnitude with previously published data on IgG antibody responses [19-21].

For anti-CCP antibodies to have a biological function in vivo, recognition of a citrullinated protein would be required. As the antigen used in the CCP2 ELISA is unknown, it is difficult to assess the possible biological relevance of the antibodies detected by this ELISA. Our data formally show that anti-CCP antibodies are in fact a collection of various ACPA, suggesting that the characteristics of the anti-CCP antibody response in a serum reflect a summary of ACPA responses in that serum.

Several groups have studied the ACPA response against a particular antigen in a population of patients with RA [15, 17], providing relevant insight into the ACPA response against that particular antigen. We now confirm and expand previous data showing that there can be considerable crossreactivity between ACPA recognising different antigens within one patient. This suggests that caution should be taken when drawing conclusions about the in vivo involvement of a citrullinated antigen in the pathophysiology of RA by extensively characterising the ACPA response against that antigen as it might be a proxy for recognition of other citrullinated antigens.

Recognition of several citrullinated antigens by ACPA complicates the identification of the antigen(s) responsible for the induction of this antibody response. Longitudinal analyses of ACPA responses in cohorts of patients prior to onset of RA [22] and extensive fine specificity analyses of ACPA responses in samples from unaffected relatives of patients with RA [17] suggest that the ACPA response changes over time. Because ACPA seems to reflect an ongoing immune response at least in some patients with RA [23], a conceivable explanation for this phenomenon is that continuous activation of naive B cells takes place that introduces new reactivities in the ACPA response. Alternatively, one could imagine that part of pre-existing activated B cells could preferentially be selected upon encounter of a different (more abundant) citrullinated antigen, resulting in a change in reactivity pattern against citrullinated epitopes, as previously shown for other hapten responses [24].

Although crossreactivity against different citrullinated epitopes has been studied at population level before [13], our study is, to our best knowledge, the first to show the presence of distinct, noncrossreactive ACPA responses against two citrullinated epitopes in at least some of the tested sera. Our results formally indicate that distinct antibody-producing B cells can exist in the blood of
patients with RA. More extensive analyses and a larger number of citrullinated epitopes are necessary to determine the extent to which distinct ACPA responses exist in patients with RA. Moreover, although this suggests that these responses evolved from two different naive B cell progenitors, a definite proof for this can only be obtained following a careful analysis of ACPA-producing B cells.

In summary, we have shown that anti-CCP antibodies are in fact a collection of ACPA and are largely crossreactive, but that crossreactivity is incomplete. This indicates that distinct responses to citrullinated antigens can occur during the course of RA development, which underlies the importance of further research aimed at identifying the citrullinated antigen(s) relevant for the induction of ACPA, as well as for the biological effects mediated by ACPA in vivo.

Funding

This work was financially supported by EU FP6 programme Autocure and FP7 programme Masterswitch, a grant from Centre for Medical Systems Biology (CMSB) within the framework of The Netherlands Genomics Initiative (NGI), the Dutch Arthritis Association and the Dutch Organisation for Scientific Research (VENI grant 916.46.079 to AIF, VENI grant to LAT and VICI grant to REMT) and the Canadian Institutes for Health Research (HAM).
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