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Anti-citrullinated protein antibodies have a low avidity compared with antibodies against recall antigens

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ABSTRACT

Objectives Anti-citrullinated protein antibodies (ACPA) are highly specific for rheumatoid arthritis (RA) and have been implicated in disease pathogenesis. Recent ongoing evidence indicates that the ACPA response broadens before precipitation of full-blown RA, as indicated by a more extensive isotype usage and an increased citrullinated epitope recognition profile. Nonetheless, the evolution of the ACPA response is still poorly understood and might intrinsically differ from the protective responses against pathogens.

Methods The avidity and the avidity maturation of ACPA in relation to the avidity of antibodies against recall antigens were analysed.

Results The avidity of ACPA was significantly lower than the avidity of antibodies to the recall antigens tetanus toxoid and diphtheria toxoid. Moreover, ACPA did not show avidity maturation during longitudinal follow-up and ACPA avidity was also relatively low in patients who displayed extensive isotype switching.

Conclusions These observations indicate that the natural evolution of ACPA differs from the development of antibodies against recall antigens. These data also indicate that ACPA avidity maturation and isotype switching are disconnected, whereby extensive isotype switching occurs in the setting of restricted avidity maturation. Intrinsic differences between the RA-specific autoantibody system and protective antibody responses against pathogens could be of relevance for designing novel B cell-targeted therapies for RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that mainly affects the joints. The identification of anti-citrullinated protein antibodies (ACPA) represents an important breakthrough in the field of RA.^{1,2} ACPA recognise post-translationally modified proteins in which arginines have been modified into citrullinated residues by peptidylarginine deiminase enzyme during inflammation. It is therefore hypothesised that citrullination of proteins in the joint may create epitopes that can serve as targets of ACPA, ultimately leading to inflammation and arthritis. Indeed, several observations implicate ACPA in disease pathogenesis as it has been shown that the presence of ACPA predicts the emergence and outcome of RA.^{3–5} Moreover, ACPA have been implicated in disease pathogenesis by the observations that ACPA can induce and aggravate arthritis in mice^{6,7} and can activate human immune effector mechanisms, such as triggering of cellular Fc receptors⁸ and activation of the complement system.⁹

During a B cell response, isotype switching and affinity maturation typically occurs in the germinal centre. Following somatic hypermutation, different B cell clones will compete for antigen on follicular dendritic cells. The B cells expressing surface immunoglobulins with a higher avidity will acquire the signals necessary for survival and proliferation. As a result, the total avidity of the immune response increases because low avidity B cells will not be stimulated and will eventually disappear from the population.

By definition, antibody affinity is the strength of interaction between a single antigen binding site and soluble monovalent antigens in solutions. However, in reality, antibodies are multivalent and contain 2 (IgG) to 10 (IgM) antigen binding sites. In addition, the antigens are also often multivalent and/or non-soluble. Antibody avidity, which is defined as the overall binding strength of polyclonal antibodies to a multivalent antigen, therefore provides a better measure of the strength of antibody responses.

Extensive information has been obtained regarding the avidity maturation of antibody responses against recall antigens, mostly following vaccination.^{10–12} Interestingly, in the mouse it was recently reported that B cells producing arthritogenic antibodies are relatively short-lived plasmablasts that are different from long-lived plasma cells typically producing protective antibodies.¹³

Given the implicated role of ACPA in RA, we investigated ACPA avidity and avidity maturation in patients with arthritis in relation to avidity of recall antigens.

MATERIALS AND METHODS

Patients

Ninety-two ACPA-positive patients with early arthritis were selected from the early arthritis clinic, an inception cohort of recent onset arthritis previously described.¹⁴ Serum samples for longitudinal analysis were selected from available samples at baseline, 1- and 5-year follow-up. The characteristics of the patients were as follows: 65.2% female, age at inclusion 51.7 years (range 17.0–82.3), disease duration 7.71 months (range 0.2–36.13), 80.4% rheumatoid factor (RF)-positive and 66.3% with erosions at baseline. These individuals were not vaccinated with tetanus toxoid (TT) and diphtheria toxoid (DT) for the purpose of this study. ACPA avidity at baseline during active disease and at remission was also studied in five

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patients with RA with complete drug-free remission. Paired serum and synovial fluid samples from 15 randomly selected patients with RA were analysed. The disease duration of these patients was 8.27 years (range 1–21).

Avidity assays for ACPA and recall antigens

Elution ELISA assays were used to determine the avidity of ACPA IgG and IgG antibodies against recall antigens.^{15 16}

For ACPA the appropriate serum dilution was first determined by performing a titration using a cyclic citrullinated peptide-2 (CCP2) ELISA (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands) with minor modifications, now using 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic-acid substrate. The serum dilution at which the response was 50% of maximum was considered 'optimal' and the minimal dilution used was 1:25. In addition, after developing the plate for 1 h, the absorbance at 415 nm had to be between 0.5 and 2.0 to allow optimal detection (figure 1A).

To determine the avidity of the anti-CCP2 antibodies, plates were incubated with the appropriate serum dilutions in phosphate buffered saline/Tween 1% bovine serum albumin for 1 h at 37°C. After washing, the wells were incubated with increasing concentrations of the chaotropic agent sodium thiocyanide (NaSCN) at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4 and 5 M for 15 min at room temperature. The wells were washed and bound antibodies were detected using horseradish peroxidase-labelled goat-anti-human IgG (DAKO, Glostrup, Denmark). The amount of antibody bound to the plate without elution and the amount that resisted elution by NaSCN were determined relative to a standard curve.

No difference in avidity was detected between ACPA avidities measured on commercial plates or in-house plates coated with CCP peptide (data not shown).

The relative avidity index (AI) was calculated.¹⁷ The AI is defined as the ratio of the amount of residual antibodies bound to the coated antigen after NaSCN (1 M) elution to the amount of binding antibodies in the absence of NaSCN expressed as a percentage (figure 1B):

$$AI = \frac{\text{remaining antibodies at 1M NaSCN (AU/ml)}}{\text{binding antibodies at 0M NaSCN (AU/ml)}} \times 100$$

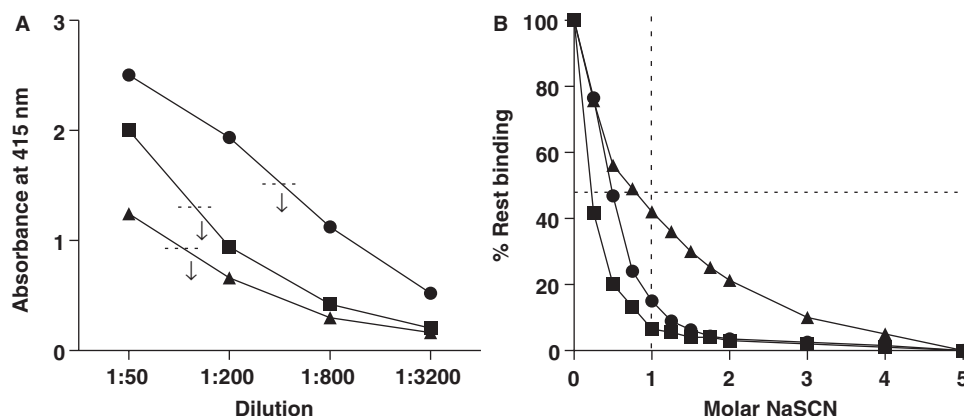


Figure 1 Characteristics of the avidity assay. (A) The appropriate serum dilution for the avidity assay was determined by performing a dose response study. The serum dilution was chosen in order to obtain 50% maximal binding (arrows). Three representative samples for anti-CCP2 reactivity are presented. (B) The relative avidity index is defined as the ratio of the amount of residual antibodies bound to the coated antigen after NaSCN elution (indicated by the vertical dotted line) relative to the amount of binding antibodies in the absence of NaSCN, expressed as a percentage. The horizontal line indicates the 50% elution profile which is used to calculate the concentration NaSCN that is necessary to elute 50% of the antibodies. Three representative samples for anti-CCP2 are presented. CCP2, cyclic citrullinated peptide-2; NaSCN, sodium thiocyanide.

To determine the avidity and levels of antibodies against citrullinated human fibrinogen (cit Fib) we used a plate-bound assay as described previously¹⁸ with minor modifications.

To determine the avidity of antibodies against modified citrullinated vimentin (anti-MCV) we used a commercial ELISA (ORGENTEC Diagnostika GmbH, Mainz, Germany).

To determine the avidity of anti-TT IgG and anti-DT IgG we used an in-house ELISA.^{19 20} In brief, plates were coated with 100 µl/well TT (1.5 Lf/ml in 0.05 M carbonate buffer, pH 9.6) or DT (0.75 Lf/ml in carbonate buffer, pH 9.6) (both from RIVM, Bilthoven, The Netherlands).

The avidities of anti-cit Fib, anti-MCV, anti-TT IgG and anti-DT IgG antibodies were determined as for anti-CCP2 antibodies.

In all cases, the conditions used for the assays were optimised to allow maximal antibody binding. The buffers used for coating antigens are washed away before measurement of the avidity and thus will not impact on the avidity measurement. After the antibodies have bound to their different antigens, the plates were washed with the same buffer, eluted with elution buffer and subjected to a similar detection of the residual antibody binding to the plate. In this way, this assay allows the comparison of avidities of antibodies binding to different antigens.¹²

Since the presence of RF could potentially influence our observations, we analysed the occurrence of RF in the low and high avidity groups. No correlation between RF positive status and ACPA avidity was observed (data not shown). In addition, we measured the avidity of RF IgM and IgG in three patients and observed a low avidity for RF compared with the avidity of anti-TT and anti-DT (data not shown).

Isotype measurements were performed as described elsewhere.²¹

Statistical analysis

Differences between groups were analysed with the Mann-Whitney test or analysis of variance, and correlation was determined by the Spearman correlation coefficient with GraphPad Prism 4.0 software (GraphPad, San Diego, California, USA) or SPSS for Windows Release 16.0 (SPSS, Chicago, Illinois, USA). In all tests, $p < 0.05$ was considered significant.

RESULTS

The avidity of ACPA is low compared with the avidity of antibodies against recall antigens

As the avidity of antibodies towards antigens can have important implications for the biological effects mediated, we determined the avidity of the ACPA response in patients with RA and compared the avidity of ACPA IgG with the avidity of IgG against recall antigens.

In an initial group of eight patients, we analysed the avidity of antibodies directed against three citrullinated antigens—the CCP2 peptide and two citrullinated proteins (cit-Fib and MCV)—as well as the avidity of antibodies against the T cell-dependent recall protein antigens TT and DT. As shown in figure 2A, we observed within the same patients a low avidity for antibodies directed against all three different citrullinated antigens and a high avidity for antibodies against recall antigens TT and DT. To study this phenomenon in a larger cohort we focused on CCP2 and cit-Fib versus TT and DT. As shown in figure 2B, in the cohort as a whole the avidity of anti-CCP2 antibodies is generally low (median 19%), with most patients displaying an avidity with an AI <40% (figure 2B). We also analysed the avidity of antibodies directed against cit-Fib, an entire citrullinated protein, and similar results were obtained. In sharp contrast, the antibodies against the recall antigens TT and DT (medians 74% and 61%, respectively) had high avidity (figure 2B), with most individuals displaying an AI of >40%. This is significantly higher than the avidity of ACPA ($p<0.0001$). Our data therefore show that these ACPA are of low avidity compared with antibodies against recall antigens.

ACPA IgG avidity in serum represents overall avidity of ACPA

To control for the possibility that the overall avidity of ACPA as detected in serum is influenced by retention of high avidity ACPA in the inflamed joint, we next analysed ACPA avidity of patients during active disease and compared it with the avidity of ACPA of the same patients during complete drug-free remission ($n=5$). No increase in the overall avidity of ACPA was observed, indicating that ACPA avidity is not influenced by disease activity and that there is no evidence for the retention of high avidity antibodies in the inflamed joint (figure 3A). Likewise, we determined whether there was a difference in the avidity of ACPA IgG between patients who are only IgG positive and those who are also positive for IgA and/or IgM ACPA. No difference was seen in the IgG ACPA avidity depending on the presence of either IgM or IgA ACPA (data not shown). Furthermore, we purified IgG from serum that was positive for IgG, IgA and IgM ACPA and compared the ACPA IgG avidity from serum with that of the purified IgG; similar avidity was observed (data not shown). Together these data indicate that the other isotypes had no impact on the avidity measurement of ACPA IgG.

We also compared the avidity of anti-CCP2 in synovial fluid with the avidity of anti-CCP2 in paired serum samples. The avidity of anti-CCP2 in synovial fluid was comparable to that of anti-CCP2 in serum (figure 3B,C). These data indicate that the avidity of ACPA measured in the serum provides a good representation of the overall ACPA avidity.

Avidity diversity of ACPA differs from the avidity diversity of recall antigens

So far we have depicted the avidity as AI, a measure of the percentage of antibody still bound after elution with 1 M

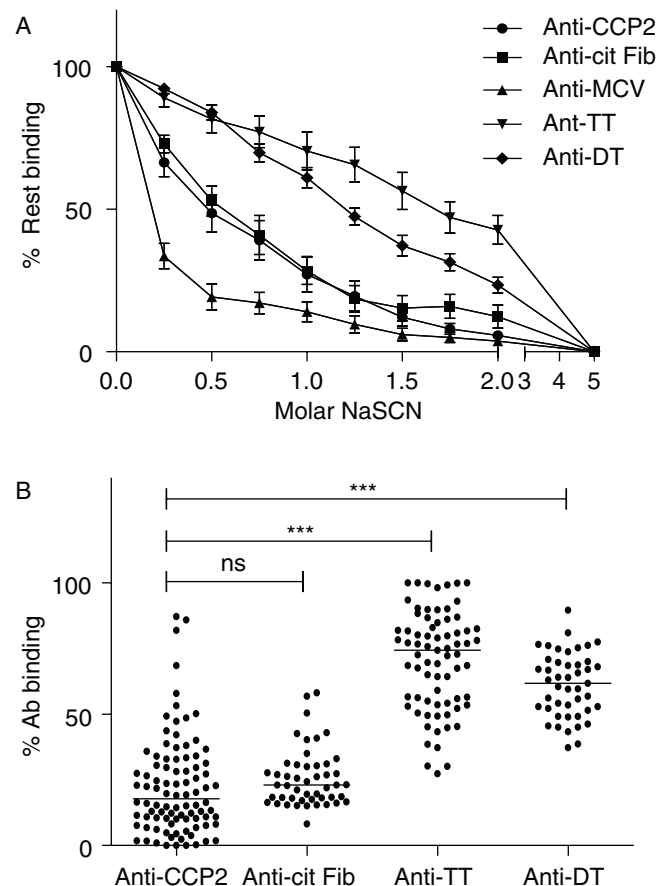


Figure 2 The avidity of ACPA IgG is lower than the avidity of IgG against recall antigens. (A) Avidities of ACPA IgG, anti-CCP2, anti-cit Fib and anti-MCV were compared with the avidity of IgG against recall antigens TT and DT in eight patients. The data of the elution profiles are presented as mean $\pm$ SEM. (B) Avidities of ACPA IgG, anti-CCP2 and anti-cit Fib were compared with the avidity of IgG against the recall antigens TT and DT in 67 patients and expressed as the relative avidity index. The avidity of ACPA IgG is significantly lower than the avidity of IgG to recall antigens ($p<0.0001$). ACPA, anti-citrullinated protein antibodies; CCP2, cyclic citrullinated peptide-2; cit Fib, citrullinated human fibrinogen; DT, diphtheria toxoid; MCV, modified citrullinated vimentin; TT, tetanus toxoid.

NaSCN. However, since it is possible that elution profiles differ while having a similar AI, we also analysed the elution profiles. Different antibody elution patterns for ACPA in comparison with IgG against recall antigens within one subject were observed (figure 4). For example, as shown in figure 4, the avidity of anti-CCP2 IgG present in the sample analysed displayed a rather homogenous response with >80% of the antibodies eluting in the first three elution steps (figure 4A). In contrast, a wide distribution of avidities was observed for IgG against TT (figure 4B). Interestingly, the antibody response to recall antigens displays a wider distribution of avidity whereas the avidity distribution of the ACPA response is, in general, narrow (figure 4C). The absence of heterogeneity within the avidity profile of ACPA further indicates limited avidity maturation of the ACPA response.

Dynamics of ACPA IgG avidity

To evaluate whether there is affinity maturation of the ACPA IgG response over time following onset of arthritis, we next determined the avidity of ACPA in a longitudinal fashion by

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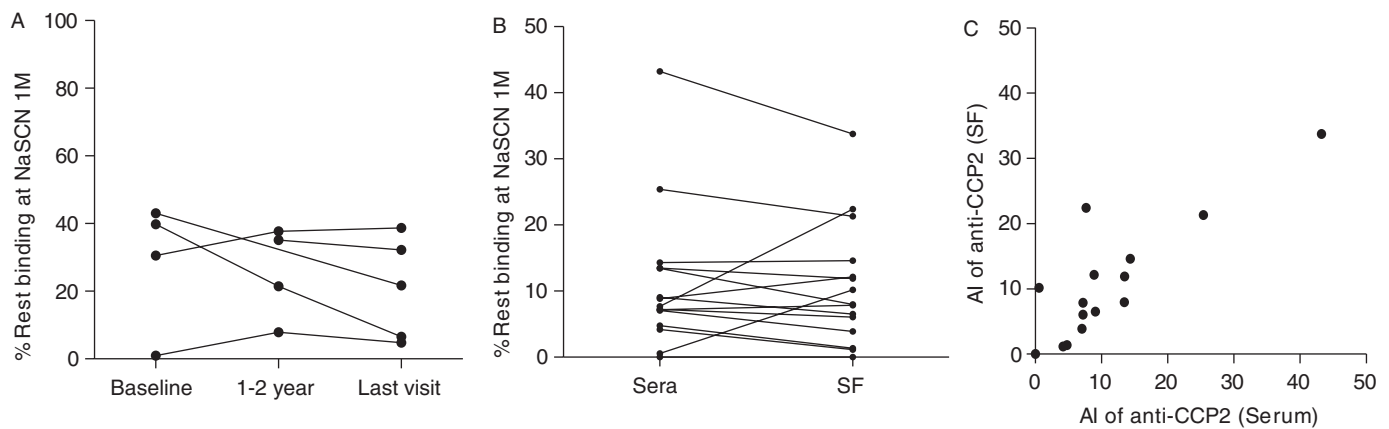


Figure 3 Avidity of ACPA IgG in serum is not influenced by disease activity and is similar to ACPA IgG in synovial fluid. (A) Comparison of the avidity of ACPA IgG in serum measured in patients with rheumatoid arthritis during active disease at baseline and in those with complete absence of disease activity at drug-free remission. The avidity of ACPA measured in serum is not influenced by disease activity. (B) Comparison of avidity of ACPA in serum and in paired samples of synovial fluid (SF). (C) Correlation between avidity of anti-CCP2 in sera (x axis) and in paired synovial fluid (y axis). ACPA, anti-citrullinated protein antibodies; CCP2, cyclic citrullinated peptide-2; NaSCN, sodium thiocyanide.

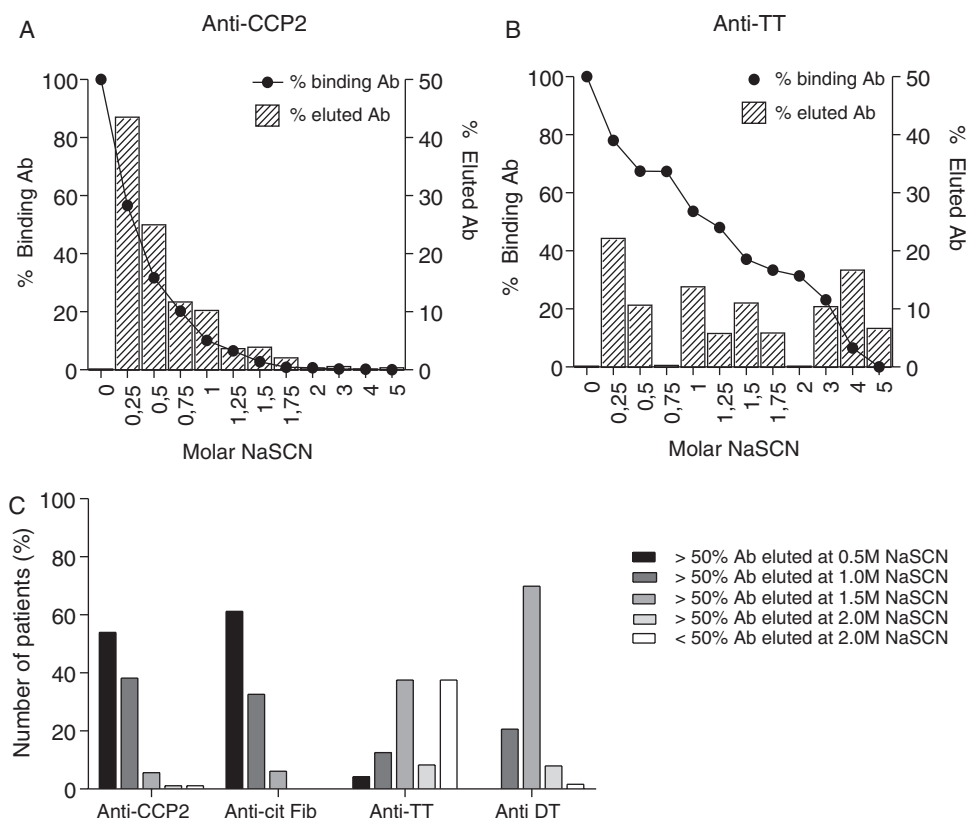


Figure 4 Different elution profiles of ACPA and IgG against recall antigens. Different patterns of elution between (A) anti-CCP2 IgG and (B) anti-TT IgG following NaSCN elution. The percentage of antibody eluted after each consecutive step of NaSCN elution is expressed in bars and the percentage of antibody still bound after NaSCN elution is presented as a linear graph. The elution profiles of anti-CCP2 IgG and anti-TT IgG of one representative patient are shown. (C) Proportion of patients with different elution profiles for the indicated antibody response. ACPA, anti-citrullinated protein antibodies; CCP2, cyclic citrullinated peptide-2; NaSCN, sodium thiocyanide; TT, tetanus toxoid.

comparing samples from the same patients at baseline and at 1- and 5-year follow-up. We did not observe a correlation between ACPA avidity and baseline clinical characteristics such as age of onset, sex, disease activity score or C-reactive protein (data not shown). No increase in ACPA IgG avidity over time was seen in the patients analysed (figure 5A). As expected, the result of avidity measurements of anti-cit Fib antibodies in

time confirms this observation (figure 5B). In addition, we did not observe a change in the high avidity (>2 M) population of ACPA during follow-up of patients (data not shown). These data indicate that the ACPA response does not undergo extensive avidity maturation during disease progression. However, these data do not exclude limited avidity maturation before disease onset.

ACPA avidity maturation, titres and isotypes

We determined the relation between antibody levels and avidity of both ACPA and IgG against recall antigens (figure 6A–D). The data indicate that the avidity of IgG against recall antigens can be high even if the titre is low, confirming previous results. More importantly, however, patients with a high titre of ACPA IgG still display only a relatively low avidity. This is in contrast to antibodies against recall antigens which have a high avidity if the titre is high.

Likewise, the isotype usage of ACPA was assessed in relation to the avidity of ACPA IgG. Unlike the distribution of the isotype usage of anti-TT antibodies which is dominated by IgG1 (data not shown),¹⁹ we observed that the ACPA response is characterised by a wide isotype usage, even in the eight patients with the lowest avidity (figure 6E). Together these data reveal poor avidity maturation of ACPA IgG despite high antibody titres and extensive isotype switching.

DISCUSSION

In this study the ACPA response is generally of a much lower avidity than the recall responses and, in our limited longitudinal study, we did not obtain evidence for avidity maturation during the course of established disease. These data indicate that regulation of the RA-specific autoimmune response against citrullinated antigens differs from the regulation of recall responses.

At present, only limited information is available on the endogenous citrullinated antigens recognised by ACPA. We therefore used three different antigens for the determination of ACPA-avidity—the CCP2 peptide and two citrullinated proteins. The CCP2 ELISA is the most commonly used for the detection of ACPA,²² whereas the proteins cit-Fib and MCV have been shown to be present in the serum, synovial fluid and synovium of patients with RA^{23 24} and are recognised by ACPA of most patients.²⁵ We made similar observations for all three systems analysed, providing internal confirmation of the results.

To exclude the possibility that the observed low avidity of ACPA detected in serum was a reflection of preferential retention of high avidity antibodies in the inflamed joint, we analysed ACPA avidity during active disease and complete drug-free remission when all clinical activity was absent. These studies indicate that there is no change in the avidity of ACPA detected in serum between active disease and remission. In addition, as we did not observe a difference in ACPA avidity in paired

samples of synovial fluid and serum, we consider it unlikely that the preferential retention of high avidity antibodies in the joint due to the presence of citrullinated epitopes explains the low avidity of ACPA, although this possibility cannot formally be ruled out.

The difference in the ACPA response compared with ‘conventional’ B cell responses studied (ie, B cell responses against recall antigens) might be due to the nature of the antigen whereby citrullinated antigens are likely to be present continuously in the body at multiple sites whereas recall antigens are present for a short time at a localised site. This would lead to a relative abundance of citrullinated antigens in the body and, as a consequence, the absence of competition for antigens by different B cell clones in the germinal centre, as expected for recall antigens, absence of affinity maturation and hence low affinity antibodies. Unlike isotype switching, avidity maturation not only critically depends on the presence of antigen, but most likely also on proper amounts of antigen in the germinal centres.²⁶ This will result in an antibody response that undergoes isotype switching but does not display avidity maturation, as observed both at baseline and at follow-up. We favour this explanation over other possibilities such as lack of adequate T helper cells or a limited amount of time passed after antigen exposure. The ACPA response is characterised by extensive isotype switching, including IgA and IgE.^{18 21 27} The generation of these isotypes appears to be dependent on T helper cells as patients suffering from hyper-IgM syndrome (caused by a gene defect in CD40 ligand) do not develop IgA responses.^{28 29} Moreover, the fact that HLA alleles predisposing to RA only predispose to ACPA-positive RA and not to ACPA-negative disease³⁰ also indicates the involvement of CD4 T helper cells in the formation of ACPA. Likewise, avidity maturation of antibody responses against recall antigens takes place within weeks whereas the disease duration in most patients with RA is much longer.

Our data indicate that ACPA-producing B cells behave differently from ‘conventional’ B cells. It was recently shown in mice that rituximab specifically depletes B cells producing autoantibodies while sparing the ‘conventional’ plasma cells producing the protective antibodies.¹³ Treatments targeting the crucial biological mechanisms underlying ‘conventional’ B cell responses may therefore not work in the same way on ACPA-producing B cells. In this respect, a possible interaction that could be of relevance is a treatment targeting the survival factors for which B cells compete during the germinal centre reaction.³¹

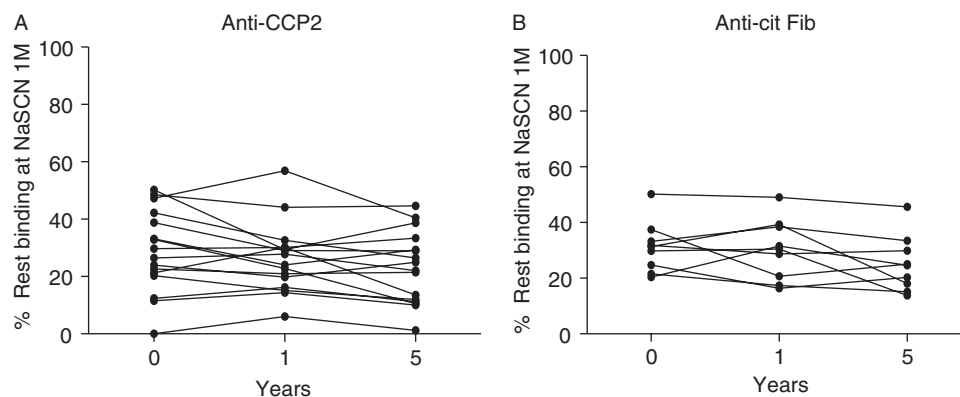


Figure 5 Absence of avidity maturation of ACPA IgG during 5-year follow-up. The avidity of ACPA in serial samples at baseline, 1- and 5-year follow-up were analysed. (A) Anti-CCP2 avidity was analysed in 16 patients with RA and (B) anti-cit Fib avidity was analysed in 9 patients with RA. The ACPA response did not show extensive avidity maturation. ACPA, anti-citrullinated protein antibodies; CCP2, cyclic citrullinated peptide-2; cit Fib, citrullinated human fibrinogen; NaSCN, sodium thiocyanide; RA, rheumatoid arthritis.

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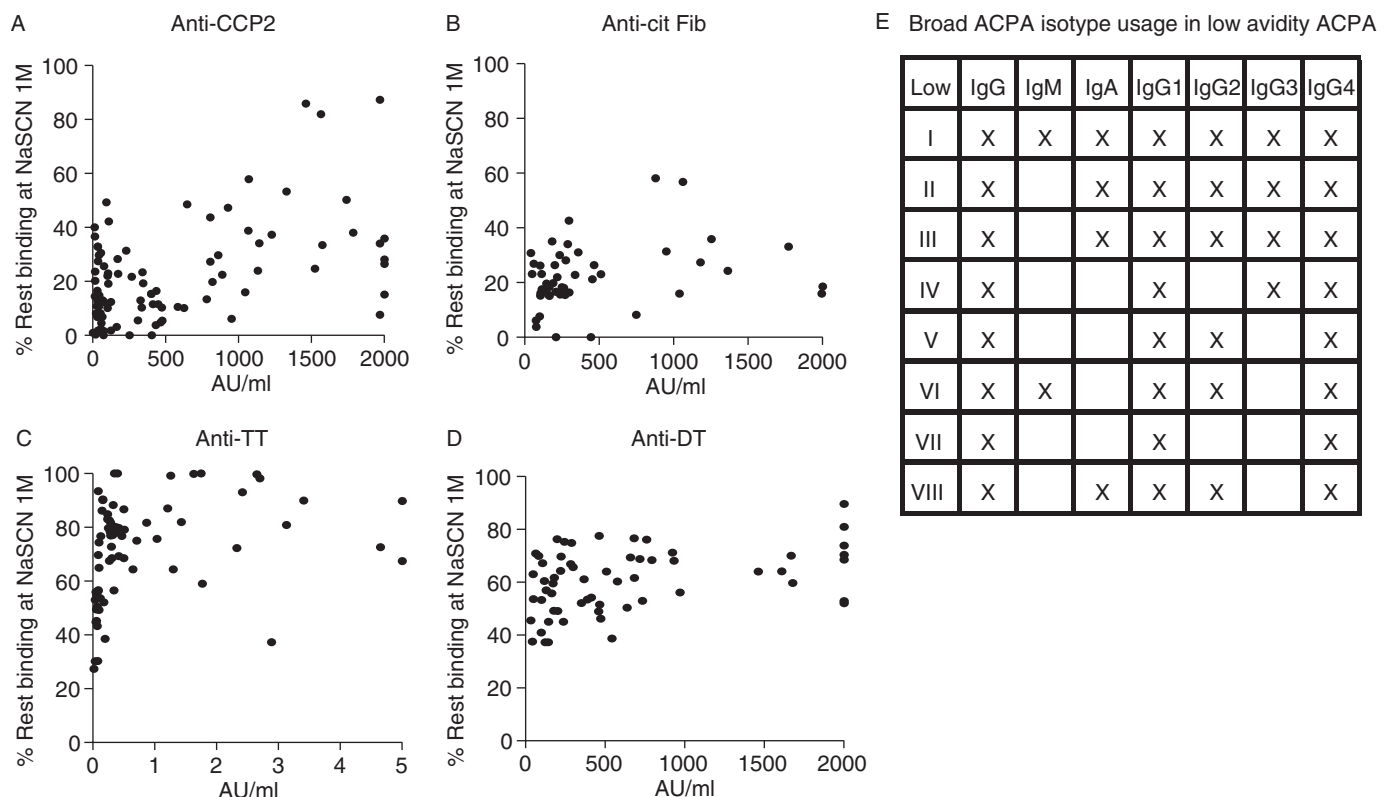


Figure 6 Avidity maturation, titres and isotypes of ACPA. Correlation between the levels and avidity of ACPA IgG (A) anti-CCP2 and (B) anti-cit Fib, and IgG against recall antigens (C) anti-TT and (D) anti-DT. (E) Broad ACPA isotype usage in patients with low avidity of ACPA IgG. Eight patients with the lowest avidity (numbered I–VIII) are shown with their use of isotypes in the ACPA response (× indicates positivity for an isotype). ACPA, anti-citrullinated protein antibodies; CCP2, cyclic citrullinated peptide-2; cit Fib, citrullinated human fibrinogen; DT, diphtheria toxoid; NaSCN, sodium thiocyanide; TT, tetanus toxoid.

In conclusion, our data indicate that the ACPA response is different from antibody responses against recall protein antigens. The ACPA response can be of high titre, can use all isotypes yet is of low avidity, in contrast to antibodies against recall antigens that are of high avidity. These and possibly other differences between ACPA and recall responses probably reflect differences in the underlying B cell response. Understanding these differences may be of relevance for the design of novel B cell-targeted therapies in RA.

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Ethical approval The collection and use of patient samples was approved by the local medical ethics committee in compliance with the Declaration of Helsinki.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed

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