Avidity Maturation of Anti–Citrullinated Protein Antibodies in Rheumatoid Arthritis

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ABSTRACT

Objective
Anti–citrullinated protein antibodies (ACPs) are highly specific for rheumatoid arthritis (RA) and are present years before the onset of symptoms. The avidity of autoantibodies can have a strong impact on their effector potency. This study was undertaken to analyze the avidity of ACPs in serum samples obtained from ACPA-positive healthy individuals (predisease), patients with early disease, and patients with established RA as well as the avidity maturation over time in samples from healthy subjects who later developed RA.

Methods
We measured ACPA avidity in serum samples from ACPA-positive healthy individuals, symptomatic individuals, and patients with established RA in 5 collections from The Netherlands, Canada, and Austria. We determined the dynamics of avidity maturation of ACPAs from the predisease stage to established disease in 1 case from the native North American population and in 10 cases from a Dutch blood donor cohort.

Results
The overall ACPA response was characterized by low-avidity antibodies. Higher-avidity ACPAs were observed in symptomatic patients only, while low-avidity ACPAs were observed in both healthy subjects and patients. In longitudinal samples obtained from subjects prior to disease onset, ACPA avidity increased over time until disease onset. No further avidity maturation was observed after disease onset.

Conclusion
Our findings indicate that avidity maturation of the ACPA response takes place prior to disease onset.
INTRODUCTION

Anti–citrullinated protein antibodies (ACPAs) are highly specific for rheumatoid arthritis (RA). These autoantibodies can be detected several years before the onset of clinical symptoms, with titers increasing as disease onset approaches. ACPAs have been shown to initiate and enhance arthritis in murine models and they activate both Fc receptor–positive cells and the complement system, indicating that they may play a role in disease pathogenesis.

Several HLA alleles, particularly those encoding the shared epitope, are known to be associated with susceptibility to RA, especially ACPA–positive RA. These findings indicate that antigen presentation and T cell involvement are important in the induction of ACPAs. With T cell help, antigen–exposed B cells can undergo class switching and avidity maturation. This occurs in germinal centers, where B cells compete for a limited supply of antigens on follicular dendritic cells, under antigen–specific control of follicular T helper cells. It is known that ACPA–producing B cells undergo isotype switching, since ACPAs of all isotypes can be detected in the sera of RA patients. Relatively little is known about the avidity maturation of ACPAs before and during disease manifestation. Recently, we showed that the avidity of the ACPA response is relatively low compared with antibody responses against recall antigens in patients with established RA.

In the present study, using several sets of serum samples from different populations, we compared the ACPA avidity in ACPA–positive healthy (predisease) individuals, patients with arthralgia, patients with undifferentiated arthritis (UA), and patients with established RA. In addition, we performed longitudinal studies using samples that were collected before the onset of RA. Our data reveal a limited avidity maturation occurring before disease onset, thereby contributing to the growing body of evidence that the ACPA response matures before, but not after, disease onset.

PATIENTS AND METHODS

Patients and samples

Serum samples were obtained from 5 sets of ACPA–positive patients. The first set was from the Leiden Early Arthritis Clinic (EAC) cohort, an inception cohort of patients with recent–onset arthritis that was initiated at the Department of Rheumatology of the Leiden University Medical Center in 1993. The second set was from the North American Natives study, in which probands and their unaffected
relatives were recruited from rheumatology clinics in urban locations (Winnipeg and Saskatoon) and rural locations (Norway House and St. Theresa Point) in Canada. The unaffected populations consisted mainly of first-degree relatives (75.5%). The third set was an inception cohort of patients in the Austrian Early Arthritis Action registry. The fourth set was from the arthralgia cohort of the Jan van Breemen Research Institute | Reade, in which ACPA-positive and/or IgM rheumatoid factor-positive patients with arthralgia from rheumatology clinics in the Amsterdam area were enrolled between 2004 and 2007. The fifth set was a blood donor cohort from the Jan van Breemen Research Institute | Reade comprising patients with RA who had been blood donors before the onset of disease symptoms. The collection and use of patient samples were approved by the local medical ethics committee and were conducted in accordance with the Declaration of Helsinki.

In detail, we determined the avidity of ACPAs in cross-sectional serum samples obtained from patients with UA (n = 93), patients with RA (n = 133), and healthy individuals (n = 2) in the Leiden sample set. Of the 133 RA samples, 67 had already been analyzed for avidity and have been described previously. We replicated our findings using samples from the North American Native population, which consisted of RA patients (n = 50) and ACPA-positive healthy first-degree family members (n = 15); the Austrian population, which consisted of healthy individuals (n = 8) and RA patients (n = 32); and a separate Dutch population from the Jan van Breemen Research Institute in Amsterdam, which comprised healthy blood bank donors who ultimately developed RA (n = 10), patients with arthralgia (n = 54), and patients with RA (n = 156).

To determine the avidity maturation of ACPAs before disease onset, we studied the dynamics of ACPA avidity from the predisease period to the onset of arthritis in 1 instructive case from the North American Natives study and in 10 cases from a blood donor cohort from the Jan van Breemen Research Institute. The longitudinally analyzed individual from the North American Native cohort was a young woman from a family with multiple RA cases. She had no symptoms initially but developed arthritis within 6 months of followup. Her disease was controlled with hydroxychloroquine (HCQ) and naproxen. However, during pregnancy she received no medication for 9 months and had no synovitis. After delivery she developed synovitis once again, and her symptoms improved 2 months after restarting treatment with HCQ and naproxen. In this case, sera from 7 visits over 3 years of followup were available for avidity testing.
To investigate the avidity maturation of ACPAs after disease onset, we determined ACPA avidity in serum samples obtained at followup visits from patients with a diagnosis of RA according to the American College of Rheumatology (ACR) 1987 criteria. These were patients from the Leiden population who were treated with conventional disease-modifying antirheumatic drugs or with a biologic agent, anti-CD20 monoclonal antibody (rituximab; n = 14). In addition, serum samples from patients with UA who were treated early with high-dose methotrexate (MTX; 15–25 mg/week) or placebo in the Probable Rheumatoid Arthritis: Methotrexate versus Placebo Treatment (PROMPT) study (n = 17) were analyzed for ACPA avidity.

**Assays for the detection and avidity measurement of ACPAs**

ACPA positivity was determined by using a second–generation anti-cyclic citrullinated peptide 2 (anti–CCP–2) enzyme–linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Euro–Diagnostica) according to the instructions of the manufacturer, with minor modifications, i.e., we used ABTS as a substrate. Absorbance was determined at 415 nm. ACPA–positive samples were subsequently investigated in the avidity assay.

ACPA avidity was determined by elution assays on CCP–2 ELISA plates, using sodium thiocyanate (NaSCN) as a chaotropic agent as previously described. Avidity was presented as the relative avidity index, which was defined as the ratio of the amount of residual antibodies bound to the antigen–coated plate after NaSCN (1M) elution to the amount of bound antibodies in the absence of NaSCN, expressed as a percentage. All avidity measurements were performed at least twice, on different occasions, with similar results ($r^2 = 0.9, P < 0.001$).

**Statistical analysis**

Differences between groups were analyzed using a paired t-test, and correlation was determined by Spearman's correlation coefficient, using GraphPad Prism software, version 4.0, or SPSS for Windows, release 16.0. P values less than 0.05 were considered significant.
RESULTS

Low ACPA avidity in asymptomatic individuals

To study the dynamics of ACPA avidity from health to disease, we first analyzed the avidity of ACPAs in samples from healthy, asymptomatic, ACPA-positive individuals. In the Dutch sample sets, we observed a low ACPA avidity in healthy subjects, with an avidity index that never exceeded 40% (Figures 1A and B). We confirmed this observation in 2 independent international sets from North America (Figure 1C) and Austria (Figure 1D). Taken together, these data suggest that the range of avidity values that we previously demonstrated in patients with established RA is not yet reflected in ACPA-positive healthy individuals.

ACPA avidity in pre-RA samples.

Next, we analyzed ACPA avidity in patients in 2 Dutch cohorts, the Leiden EAC and the Amsterdam arthralgia cohort, who presented with early joint symptoms. The ACPA avidity index at the onset of arthralgia ranged from 1.6% to 62%. This range.
of avidity values is similar to the range that was observed in the sera of patients with established RA in this study (Figure 1B) and previously. Similar results were found for patients with UA who displayed an avidity index of 0–81.93% (Figure 1A). Comparing the ACPA avidity of patients with UA or arthralgia who developed RA to those who did not develop RA after 1 year of followup did not reveal significant differences (data not shown). Taken together, these data indicate that the ACPA avidity in ACPA–positive asymptomatic individuals is lower than the ACPA avidity in patients with joint symptoms, which resembles the ACPA avidity observed in patients with established RA.

Occurrence of avidity maturation before symptom onset.
The data described above indicate that the ACPA response in ACPA–positive healthy individuals displays a low avidity and that the ACPA responses in ACPA–positive symptomatic individuals and RA patients display low to moderately high avidity. These data indicate that avidity maturation has occurred to a certain degree in a proportion of ACPA–positive subjects. Therefore, we next studied longitudinal samples from healthy subjects who later developed RA, in order to obtain an idea of when such avidity maturation occurred. First, we examined the change in ACPA avidity in a subject in the North American Natives study17 for whom 7 serum samples were available over a period of 3 years. We observed an increase in ACPA avidity before disease onset. This increase was paralleled by a similar increase in ACPA levels (Figure 2A). The increase in ACPA levels continued during pregnancy, when the patient had no symptoms of arthritis.

To confirm these observations, we next analyzed samples obtained from healthy Dutch blood donors who later developed RA. A total of 10 donors who developed RA after 6–7 years of followup were analyzed. We again observed an increase in the avidity of ACPAs during the transition from predisease to disease onset. This was exemplified in 2 representative cases (Figures 2B and C). Figure 2D shows the changes in the avidity index in all 10 individuals. Interestingly, 1 individual displayed a decline in ACPA avidity and an abrupt increase in ACPA levels from the third to the fourth visit, as disease onset approached (Figure 2D). In some individuals the increase in avidity was very limited, whereas in other individuals the increase was much more pronounced (Figure 2D).
Figure 2. Occurrence of ACPA avidity maturation during the asymptomatic phase. The avidity of ACPAs in sera derived from ACPA-positive healthy individuals who ultimately developed RA is shown. A, Avidity index and levels of ACPAs in followup samples from an instructive case in the North American Natives study. Arthralgia was treated with hydroxychloroquine (HCQ) at onset and at recurrence after pregnancy. Values on the x-axis are the month and year that the sample was obtained. B and C, Avidity index and levels of ACPAs in 2 representative cases from the blood donor cohort from the Jan van Breemen Research Institute/Reade. The x-axis shows the time points of sampling (sequence of visits before the onset of RA, with 1–2 years between visits). D, ACPA avidity maturation in all 10 individuals from the blood donor cohort. The horizontal line represents baseline avidity. The y-axis shows the ratio of the avidity index to the baseline avidity index, and the x-axis shows the time points of sampling (sequence of visits before the onset of RA, with 1–2 years between visits). Anti–CCP-2 = anti–cyclic citrullinated peptide 2 (see Figure 1 for other definitions).

We compared ACPA avidity at baseline and at disease onset by avidity index as well as by the fold change in the avidity index. Both the avidity index and the fold change were significantly different between these 2 time points (P = 0.027 and P = 0.019, respectively). Near the time of RA diagnosis, the avidity distribution in this population was similar to that observed in established RA (Figure 1), with most patients displaying a low avidity and some displaying a considerably higher avidity.
Avidity maturation of ACPA in RA

(avidity index range 7.7–67.7%). Although there was an increase in both the levels of ACPAs and the avidity of ACPAs, we did not observe a correlation between ACPA avidity and level ($r^2 = 0.03$, $P = 0.2$). These data indicate that in addition to an increase in ACPA levels, an increase in ACPA avidity takes place during the asymptomatic phase before arthritis becomes apparent.

Figure 3. No further ACPA avidity maturation following disease onset. The avidity index of ACPAs in sera obtained from patients treated according to different strategies is shown. Time point 0 indicates the initiation of treatment. A, Avidity index in patients with early RA from the Early Arthritis Clinic (EAC) cohort who were treated with conventional disease-modifying antirheumatic drugs. B, Avidity index in patients with UA who were treated early with methotrexate or placebo in the Probable Rheumatoid Arthritis: Methotrexate versus Placebo Treatment (PROMPT) study. C, Avidity index in patients with RA who were treated with anti-CD20 monoclonal antibody (rituximab). Horizontal lines connect the data points for individual patients. See Figure 1 for other definitions.

No further avidity maturation after disease onset.

Next, we addressed the question of whether additional avidity maturation takes place following the onset of arthritis. Previously, we obtained evidence that such avidity maturation does not occur; we recently observed that ACPA avidity was similar at baseline and after 5 years of followup (Figure 3A). In this study, we extended these observations by analyzing samples from patients with different disease durations and, importantly, different treatment protocols. First, we analyzed patients with UA who were treated with placebo or MTX 15–25 mg/week according to protocol (in the PROMPT study) before they fulfilled the ACR 1987 criteria for RA. We did not observe any additional avidity maturation during followup in this group (Figure 3B). Stratifying for treatment regimens, i.e., analyzing the placebo–treated and MTX–treated patients separately, revealed no pronounced differences. Likewise, in patients with RA of longstanding duration who were treated with anti–CD20 monoclonal antibody (rituximab) (Figure 3C), no change in the overall avidity during the course of established disease was observed. Collectively, these data indicate that no additional avidity maturation of ACPAs takes place after the onset of arthritis.
DISCUSSION

Recently, several studies have shown that prior to the onset of RA the ACPA response recognizes more epitopes,\textsuperscript{22,23} uses more isotypes,\textsuperscript{14} and increases in level.\textsuperscript{4,19} In this study, we demonstrated that avidity maturation also takes place during this asymptomatic phase. Taken together, these findings indicate that the “maturation” of the ACPA response takes place before the manifestation of disease. The extent of avidity maturation during the predisease stage varied considerably among individual subjects. Most individuals by far displayed limited avidity maturation, while only a small subgroup underwent substantial avidity maturation. The reason the majority of individuals undergo less pronounced avidity maturation is not clear, but we hypothesize that this may be partly related to the abundance of citrullinated antigens. Avidity maturation is thought to take place when B cells compete for a limited supply of antigens to receive signals to survive. It is tempting to speculate that, if sufficient antigen is available, there is no advantage for higher-avidity clones to overgrow the low-avidity populations.\textsuperscript{24} Nonetheless, there are other possible explanations for these findings, as it has been shown that somatic hypermutation still takes place in the bone marrow of mice vaccinated with NP-Ficoll, supporting the notion that antigens have independent effects as well.\textsuperscript{25,26}

The increase in avidity stabilized during the predisease stage, and no additional avidity maturation was observed after the patients fulfilled the ACR 1987 criteria for RA\textsuperscript{20} (or, in the case of the PROMPT study, fulfilled the 2010 ACR/European League Against Rheumatism Classification Criteria for RA).\textsuperscript{27} It is not feasible to follow up the natural course of ACPA avidity in untreated RA patients, and the possibility that the lack of additional avidity maturation is a result of treatment cannot be excluded. However, comparing avidity maturation in patients with arthritis on 3 different treatment regimens (Figure 3) revealed that there was no additional avidity maturation after disease onset. The time period during which patients received placebo in the PROMPT study was quite short (6 months), and the number of patients receiving placebo was low. Therefore, we cannot exclude the possibility that medication affects affinity maturation. Nevertheless, we believe that the findings of the present study suggest that the lack of avidity maturation following the onset of arthritis is not the result of treatment and may conceivably reflect the natural course of the ACPA response.

Our results also show that the magnitude of the ACPA avidity index is, most likely, not useful for the identification of individuals who will ultimately develop RA. Low-avidity ACPAs can be detected in healthy individuals, patients with UA, and patients
with arthralgia as well as in patients with established RA. High-avidity ACPAs can be observed in patients with UA, patients with arthralgia, and patients with established RA. Comparing the ACPA avidity in patients with UA and patients with arthralgia who developed RA within a year of followup with those who did not develop RA within a year of followup did not provide evidence to suggest that ACPA avidity measurements can be used to predict disease persistence (data not shown).

To analyze the avidity of ACPAs, we used CCP–2 plates. Although the antigen used to coat CCP–2 plates is unknown, we consider the use of CCP–2 plates appropriate since anti–CCP–2 antibodies are a collection of ACPAs recognizing various “natural” antigens. More importantly, the avidity of ACPAs directed against CCP–2, mutated and citrullinated vimentin (MCV), or citrullinated fibrinogen is similar, indicating that the antigen used to measure ACPA avidity does not affect the outcome.

The combination of cross–sectional studies with followup data from several international cohorts allows the conclusion that avidity maturation of the ACPA response takes place during the asymptomatic period, or at the least during the very early phases of the disease. The marked differences among individuals with regard to the avidity of ACPAs is intriguing. Differences in the amount of citrullinated antigen, immune activation status, genetic makeup, and environmental factors could all contribute to the avidity maturation process. The factors that prevent the avidity maturation of the ACPA response do not seem to affect other antibody responses, since within the same individuals the responses against recall antigens such as tetanus toxoid are of a normally high avidity.

In conclusion, the avidity of ACPAs is in general low, but when analyzing individual patients, marked differences in ACPA avidity can be observed. The avidity maturation of ACPAs takes place before disease onset and then stabilizes.

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