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Chapter 3

Avidity maturation of ACPA in rheumatoid arthritis

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ABSTRACT

Objectives
Anti-citrullinated protein antibodies (ACPA) are highly specific for rheumatoid arthritis (RA) and are present years before onset of symptoms. Avidity of (auto) antibodies can have a strong impact on their effector potency. Here, we analyzed the avidity of ACPA in pre-disease, early-disease and established RA samples as well as avidity maturation in time in samples of healthy subjects that later developed RA.

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

Results
The overall ACPA response was characterized by low avidity antibodies. Individuals with higher avidity ACPA were confined to symptomatic patients, while low avidity ACPA were observed in both healthy subjects and patients. In longitudinal samples obtained from subjects prior to disease onset, the ACPA avidity increased over time until disease onset. Following onset of disease no further avidity maturation was observed.

Conclusions
Avidity maturation of the ACPA response takes place prior to disease onset.
INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) are highly specific for rheumatoid arthritis (RA) (1). These autoantibodies can be detected several years before the onset of clinical symptoms, with increasing titers as patients approach disease onset (2-4). ACPA have been shown to be able to initiate and enhance arthritis in murine models of arthritis (5;6) and they are able to activate both FcR-positive cells (7;8) and the complement system (9), arguing that they could play a role in disease pathogenesis.

Several human leukocyte antigen (HLA) alleles, particularly those encoding the Shared Epitope (SE), are known to be associated with RA susceptibility (10), especially with ACPA-positive RA (11). These data indicate that antigen presentation and T-cell involvement is important in the induction of ACPA. 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 source of antigens on follicular dendritic cells (12), under antigen-specific control of follicular helper T cells (13). It is known that ACPA producing B cells undergo isotype switching since ACPA of all isotypes can be detected in sera of RA patients (8;14). Relatively little is known about the avidity maturation of ACPA before and during disease manifestation. Recently, we have shown that the avidity of the ACPA response is relatively low as compared to antibody-responses against recall antigens in patients with established RA (15).

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

PATIENTS AND METHODS

Sera were selected from five sets of ACPA-positive patients. The first set was derived from the Leiden Early Arthritis Clinic (EAC), 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 (16). The second set was from the North American Natives (NAN) study, in which probands and unaffected
relatives, visiting rheumatology clinics in urban (Winnipeg, Saskatoon) and rural (Norway House, St. Theresa Point) locations of Canada were recruited (17). The unaffected populations consisted mainly of first-degree relatives (75.5%). The third set was an inception cohort of patients recruited within the Austrian Early Arthritis Action (18). The fourth set was from the arthralgia cohort of the Jan van Breemen Research institute (part of Reade) (19), in which ACPA-positive and/or IgM-RF-positive patients with arthralgia from rheumatology clinics in the Amsterdam area were enrolled between 2004 and 2007. In addition, the fifth set was a blood donor cohort from the Jan van Breemen Research institute | Reade (2), comprised patients with RA who had been blood donors before the onset of disease symptoms. The collection and use of patient samples was approved by the local medical ethics committee in compliance with the Helsinki declaration.

In detail, we cross-sectionally determined the avidity of ACPA in UA (N=93), RA (N=133) and healthy individuals (N=2), in the Leiden sample set. From these 133 RA samples 67 were already analyzed for avidity and have been depicted in a previous study (15). We replicated our findings using samples from the North American Natives (NAN) population consisting of healthy first degree ACPA-positive family members (N=15) and RA patients (N=50), from the Austrian population containing healthy individuals (N=8) and RA patients (N=32) and another Dutch population from the Jan van Breemen Research Institute in Amsterdam comprising healthy blood bank donors that ultimately developed RA (N=10), arthralgia patients (N=54) and RA patients (N=156)

To determine avidity maturation of ACPA before disease onset, we studied the dynamics of ACPA avidity from the pre-disease period to onset of arthritis in one instructive case of the NAN study and 10 cases of a blood donor cohort from the Jan van Breemen Research Institute.

The longitudinally analyzed individual from the NAN cohort was a young woman from a multi-cases RA family (17). Initially, she had no symptoms and then developed arthritis within 6 months of follow up, which was controlled with hydroxychloroquine (HCQ) and naproxen. However during her pregnancy, no medication was taken for 9 months without synovitis. After delivery, she developed synovitis once again and her symptoms were improved in 2 months after retaking HCQ and naproxen. In this case, sera from 7 visits in 3 years follow-up were available for avidity testing.

For avidity maturation of ACPA after disease onset, we determined ACPA avidity in follow up sera of patients with RA according to the 1987-ACR criteria (20) from the Leiden population who were treated with conventional DMARDs (16), or with a biological agent, anti-CD20 monoclonal antibody (Rituximab) (N=14) (21). In addition, sera of patients with undifferentiated arthritis (UA) who were
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Avidity assays for the detection and avidity of ACPA

ACPA-positivity was determined by using an anti-cyclic citrullinated peptide ELISA of the second generation (CCP2) (Immunoscan RA Mark 2, Euro-Diagnostics, Arnhem, The Netherlands) following the instructions from the manufacturer with minor modifications, i.e. now using 2,2’-azino-bis-3-ethylbenzothiazoline-6-sulphonic-acid (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 CCP2 ELISA plates, using sodiumthiocyanate (NaSCN) as chaotropic agent as described before (15). Avidity was presented as relative avidity index (AI) that 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 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 the Spearman’s correlation coefficient with GraphPad Prism 4.0 software (GraphPad Inc., San Diego, CA, USA) or SPSS for Windows (release 16.0, SPSS, Chicago, IL, USA). In all tests, $P < 0.05$ was considered significant.

RESULTS

The avidity of ACPA in non-symptomatic individuals is low

To study the dynamics in ACPA avidity from health to disease, we first set out to analyze the avidity of ACPA in samples from healthy, non-symptomatic, individuals that were positive for ACPA. In the Dutch sample sets we observed that the ACPA response displayed a low avidity, never exceeding an AI of 40% (Fig 1A, B). We confirmed this observation in two independent international sets of North American Natives (Fig 1C) and Austrian (Fig 1D) samples. Together these data suggest that the range of avidities as observed in established RA as we published before (15) is not yet reflected in ACPA-positive healthy individuals.
ACPA avidity in pre-RAD samples

Next, we wished to analyze ACPA avidity in patients that present with early joint complaints. For this purpose we studied two sets of Dutch patients; the Leiden EAC and the Amsterdam Arthralgia cohort.

We observed that the avidity of ACPA derived from patients at the onset of arthralgia ranged in AI from 1.6% to 62%. This range of avidities is similar to the range of avidities observed in sera of patients with established RA (Figure 1B) and as reported before (15). The same observation was found for patients suffering from UA, as they also display an avidity range with an AI from 0% to 81.93% (Figure 1A). Comparing the ACPA avidity of UA or arthralgia patients that develop RA to those that do not develop RA after one year follow-up did not reveal significant differences (data not shown). Together, these data indicate that the avidity of ACPA of ACPA-positive asymptomatic individuals is lower than the ACPA avidity present in patients with joint complaints that resembles the ACPA avidity observed for patients with established RA.

Avidity maturation occurs before onset of symptoms

The data described above indicate that the ACPA response of healthy ACPA-positive individuals displays a low avidity and that the ACPA response of ACPA-positive symptomatic individuals as well as RA patients displays a range of 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 wished to study longitudinal samples of healthy subjects that later developed RA to obtain an impression when such avidity maturation occurred. First we examined the change in ACPA avidity in pre-disease samples of a North
American Native individual (17) of 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 (Fig 2A). The increment of avidity and levels of ACPA continued also during the period of pregnancy when the patient had no symptoms of arthritis.

To confirm these observations we next analyzed samples derived from Dutch, healthy blood donors that later developed RA. A total of 10 donors who developed RA, after 6-7 years of follow up, were analyzed. We again observed an increase in the avidity of ACPA during the transition from pre-disease to disease onset. This is exemplified in two representative cases (Fig.2B,C) and summarized for all

![Graphs showing ACPA avidity maturation](image)

**Figure 2. ACPA avidity maturation takes place during the pre-symptomatic phase**

The avidity of ACPA in sera derived from ACPA-positive healthy individuals that ultimately developed RA is depicted as AI (%). A) Follow up samples of an instructive case of NAN study. Time points of sampling are depicted on the horizontal axis and AI on the vertical axis. (B,C) 2 representative cases from the blood donor cohort from the Jan van Breemen Research institute | Reade. Time points of sampling (1-2 year intervals) are depicted on the horizontal axis and AI on the vertical axis. (D) ACPA avidity maturation in all 10 individuals of the blood donor cohort. The horizontal line represents the baseline avidity and the vertical axis shows the ratio of avidity index compared to baseline avidity index.
individuals in Fig 2D. Interestingly, we observed one individual who displayed a decline in ACPA avidity when there was an abrupt increase of ACPA levels from the third to the fourth visit as this individual approached disease onset (Fig 2D). In some individuals the increase in avidity was very limited, whereas in other individuals the increase was much more pronounced (Fig 2D). We compared ACPA avidity at baseline and at disease onset by avidity index (AI) as well as the fold change of the AI. This analysis revealed that both the AI and the fold change were statistically significantly different between these two time points (i.e. p=0.027 and 0.019 respectively). Towards the point of RA diagnosis the avidity distribution in this population was similar as observed in established RA (Fig 1) with most patients displaying a low avidity whereas some displaying a considerably higher avidity (AI range 7.7% to 67.7%). Although there is an increase in both the levels of ACPA and the avidity of ACPA we did not observe a correlation between ACPA avidity and levels ($r^2 0.03$, $P = 0.2$).

These data indicate that next to an increase in ACPA levels also an increase in ACPA avidity takes place during the asymptomatic phase before arthritis becomes apparent.

**No further avidity maturation after disease onset**

Next, we wished to address the question, whether additional avidity maturation would take place following onset of arthritis. Previously, we have obtained indications that such avidity maturation does not occur as we observed recently that ACPA-avidity was similar at baseline and after 5 years of follow-up (15) (Fig 3A). Here, we extended this study by analyzing samples from sets of patients with different disease durations and, importantly, different protocolized treatment.

First, we analyzed undifferentiated arthritis patients that were treated with placebo or methotrexate 15-25 mg/week according to protocol (PROMPT study) be-
fore they fulfilled the ACR-1987 criteria for RA. In this group we did not observed additional avidity maturation during follow-up (Fig 3B). Stratifying for treatment regiments, i.e. analyzing the placebo and methotrexate treated individuals separately, revealed no pronounced differences. Likewise, in patients with longstand-ing RA who were treated with anti-CD20 monoclonal antibody (Rituximab) (Fig 3C), no change in the overall avidity during the course of established disease was observed either. Collectively, these data indicate that following the onset of arthritis no additional avidity maturation of ACPA takes place.

**DISCUSSIONS**

Recently several studies have shown that during the pre-disease stage of RA the ACPA response recognizes more epitopes (22;23), uses more isotypes (14) and increases in levels (4;19). Our data add to these observations that also avidity maturation takes place during this asymptomatic phase. Together, these data indicate that the ‘maturation’ of the ACPA response takes place before the manifestation of disease.

The extent of avidity maturation during the pre-disease stage displayed considerable variation between persons. By far most individuals displayed limited avidity maturation whereas only a subgroup underwent substantial avidity maturation. The reason why the majority of individuals display 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 limiting source of antigen 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 (24). Nonetheless, also other possibilities could explain these observations as it has been shown that somatic hypermutation still takes place in the bone marrow following vaccination of mice with NP-ficoll, arguing for antigens independent effects as well (25;26).

The increase in avidity stabilizes at the pre-disease stage and we observed no additional avidity maturation after the patients fulfilled the ACR 1987 RA criteria (20) or, as in the PROMPT-study, fulfilled the 2010-ACR-EULAR criteria for RA (27). It is not feasible to follow the natural course of ACPA avidity in untreated RA patients and it cannot be excluded that the lack of additional avidity maturation is induced by treatment. However, comparing avidity maturation in patients suffering from arthritis that were treated with three different treatment strategies (Fig 3) revealed that there was no additional avidity maturation following disease
onset. The time period that patients received placebo in the PROMPT-study was quite short (6 months) and the number of patients receiving placebo was low. Therefore, it cannot be excluded that medication affects affinity maturation. Nevertheless, we feel that the current dataset suggests that the lack of avidity maturation following the onset of arthritis is presumably not the result of treatment and is conceivably reflecting the natural course of the ACPA response.

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

To analyze the avidity of ACPA we have used CCP2 plates. Although the antigen used to coat CCP2 plates is unknown, we consider the use of CCP2 plates appropriate as anti-CCP2 antibodies are a collection of ACPA recognizing various ‘natural’ antigens (28). More importantly, the avidity of ACPA directed against CCP2, MCV or citrullinated fibrinogen is similar (15) indicating that the antigen used to measure ACPA avidity is not affecting the outcome.

The combination of cross-sectional studies with follow up data from several international cohorts now allows the conclusion that avidity maturation of the ACPA response is taking place during the asymptomatic or at the least the very early phases of the disease. The marked inter-individual difference in the avidity of ACPA is intriguing. Differences in the amount of citrullinated antigen, immune activation status, genetic make up 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 be operational on other antibody responses as within the same individuals the responses against recall antigens like tetanus toxoid are of a normally high avidity (15).

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

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