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The concentration of anticitrullinated protein antibodies in serum and synovial fluid in relation to total immunoglobulin concentrations


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ABSTRACT

Background
Anticitrullinated protein antibodies (ACPA) are one of the best predictors for the development of rheumatoid arthritis. Nonetheless, relatively little information is present on the absolute concentration of ACPA in relation to total immunoglobulin (Ig) concentrations. Such information would be of relevance to compare ACPA levels to other antibody levels. Here, we estimated the relative abundance of ACPA immunoglobulins in serum and synovial fluid using a quantitative approach.

Methods
ACPA were purified using HiTrap Streptavidin columns coupled with biotinylated cyclic citrullinated peptide (CCP2). Total Ig and anti-CCP2 isotype reactivities were measured by ELISA.

Results
ACPA were successfully isolated as substantial antibody amounts were eluted from sera of ACPA positive patients and neglectable antibody amounts were eluted from sera of ACPA negative patients. Up to 1 in 80 IgG-molecules were estimated to be ACPA. Strikingly, IgM-ACPA was most abundant in synovial fluid (with the highest enrichment in the range of 1 IgM-ACPA for every 8 IgM-antibodies).

Conclusion
ACPA-IgG levels are estimated to be within the range of peak levels of protective antibody responses against recall antigens. IgM-ACPA is abundantly present in synovial fluid, suggesting the presence of a continuous ongoing autoimmune response in the synovial compartment.
INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) have been shown to initiate and enhance disease in murine models of arthritis\(^1\)\(^-\)\(^2\) and to activate both FcR-positive cells\(^3\)\(^-\)\(^4\) and the complement system,\(^5\) arguing that they could play a role in disease pathogenesis. ACPA are highly specific for RA and can be detected years before clinical manifestations.\(^6\)\(^-\)\(^7\) Activation of naïve B-cells upon the first antigen encounter results in proliferation and differentiation in IgM-secreting cells. During their differentiation upon further contact with T cells, some B cells undergo isotype switching and affinity maturation. The ACPA response utilize all isotypes.\(^4\)\(^-\)\(^10\) Intriguingly, an expanded ACPA-isotype profile associates with more severe radiographic damage, indicating that the extent of the ACPA-response impacts on disease progression.\(^11\)

It has been shown that anti-CCP2-antibody levels are higher in synovial-fluid than serum.\(^12\)\(^-\)\(^14\) However, limited information on absolute ACPA levels in either synovial fluid or serum is present as the levels are generally expressed as arbitrary units. Nonetheless, information on the absolute concentration of ACPA is of interest as it allows the comparison of the ACPA response to other antibody responses in quantitative terms. To quantitate ACPA levels it is required to isolate ACPA. Here we present data on the estimation of ACPA quantities in serum and synovial-fluid.

MATERIALS AND METHODS

Patient population

Patients analyzed were derived from the Leiden Early Arthritis clinic (EAC) cohort.\(^15\) All patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA.\(^16\) Serum samples of 10 ACPA-positive and 2 ACPA-negative RA patients and 2 healthy controls were obtained for ACPA purification. Knee synovial-fluid was obtained at the time of therapeutic arthrocentesis. Samples were obtained from 5 ACPA-positive (including 2 paired synovial-fluid-serum samples selected for high IgM-ACPA levels) and 1 ACPA-negative RA patient and 1 patient with osteoarthritis, attending the outpatient clinic of the rheumatology department in Leiden. The protocols were approved by the relevant local ethics committee and all participants provided informed consent.

Affinity-purification of ACPA

Synovial-fluid samples were treated with hyaluronidase type IV from bovine testes (Sigma Aldrich; 100 µg/ml) and protease-inhibitor (Sigma Aldrich; 1:50). ACPA from synovial-fluid and serum were purified using HiTrap-streptavidin HP 1 ml columns (GE-Healthcare) coupled with biotinylated CCP2 peptides as described previously.\(^17\) The CCP2 peptide was obtained from Dr Drijfhout, Department of
IHB, LUMC, The Netherlands. Antibodies were eluted with 0.1M Glycine Hydrogen Chloride pH2.5 and neutralized with 2 M Tris. A control column coated with CCP2 arginine was attached before the CCP2 citrulline column. This was necessary to control for non-specific adherence of antibodies to the bead material. We observed that, indeed, a considerable amount of IgG (mean: 10μg/ml) adhered to the control column (data not shown). After running the sample the columns were disconnected and eluted separately to guarantee the citrulline specificity of the purified antibodies.

**Anti-CCP2 assays**

Anti-CCP2 IgG was measured by ELISA (Immunoscan RA Mark2; Eurodiagnostica, Arnhem, The Netherlands). Samples with values > 25 units/ml were considered positive according to the manufacturer’s instructions. Anti-CCP-positive individuals were considered ACPA-positive.

**Quantitative IgA, IgM, IgG ELISA**

Quantitative Ig analyses were performed using the Human IgA-ELISA, IgG-ELISA and IgM-ELISA Quantitation Set (Bethyl Laboratories, Inc, USA) according to the manufacturer’s instructions.

**RESULTS**

**Affinity-purification of ACPA**

ACPA were successfully isolated using HiTrap Streptavidin columns loaded with CCP2 peptides as substantial amounts of antibodies were eluted from sera of ACPA positive RA patients and negligible amounts from sera of ACPA-negative patients or healthy controls (range: 0-2.1 μg/ml) (table 1). Two affinity-columns, one coated with biotinylated CCP2 arginine and one coated with biotinylated CCP2 citrulline, were used to purify ACPA (figure 1A). The CCP2-arginine coated column was essential in the purification procedure, as considerable amounts of, most likely, non-specific antibodies were eluted from this control column (mean: 10μg IgG/ml). The flow-through was collected and antibodies were eluted with glycine-HCl and directly neutralized. The flow-through was completely devoid of anti-CCP2-reactivity, except for 4 high-titer samples (figure 1B), probably due to overloading of the column. Recovery of ACPA-activity was not complete. In some samples only 20% of ACPA-activity could be detected. The recovery rate of ACPA of the different immunoglobulins (Ig) after purification was comparable. Together, these data indicate that ACPA can be purified from ACPA-positive sera and indicate that numbers of absolute ACPA levels are possibly an underestimation as not all ACPA activity originally present in the samples has been recovered.
ACPA concentration in serum and synovial fluid

Figure 1. Affinity-purification of ACPA. ACPA was purified using 2 affinity-columns. A control column coated with CCP2-arginine was attached to the column with CCP2-citrulline to guarantee the citrulline specificity of the purified antibodies (A). In the elution step the two columns were disconnected and the antibodies were eluted off separately. Start material (serum or synovial-fluid) (1), flow through (2), elution CCP2-arginine (3) and elution CCP2-citrulline (4) were measured for ACPA presence and total immunoglobulin content. ACPA were effectively purified using HiTrap Streptavidin columns (B). Hardly any ACPA remained in the flow-through (FL). However, not all ACPA-activity could be recovered as in some patients only 20% of the ACPA-activity present in the start material were present in the elution, indicating an underestimation of the amount of quantified ACPA. The elution of the arginin-column contained barely any ACPA. The percentage in the graph represents the amount of ACPA present in relation to the start material. The different fractions were measured with an anti-CCP2 ELISA and the amount was measured in AU/ml.

Quantification of ACPA immunoglobulins present in serum and synovial-fluid

After affinity purification, ACPA-IgG levels of ACPA-positive patients were quantified by measuring the amount of IgG present. In sera of ACPA-positive patients, we measured up to 60 μg/ml IgG-ACPA (mean: 28μg/ml). As hardly any IgG could be isolated and measured from sera of ACPA-negative RA patients (mean: 1.5 μg IgG/ml), we conclude that considerable amounts of ACPA are present in ACPA-positive sera (table 1). These levels were estimated to be on average almost 1 in 183 IgG-antibodies. Within patients with high levels even up to 1 in 80 antibodies were ACPA (figure 2). IgA-ACPA is less abundant, only 4 patients displayed a level higher than 1 μg/ml in serum. The abundance of IgA-ACPA appeared, therefore, limited.
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<th>IgG-ACPA AU/ml</th>
<th>Total IgG mg/ml</th>
<th>Total IgA mg/ml</th>
<th>Total IgM mg/ml</th>
<th>anti-CCP2 IgG μg/ml</th>
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<th>anti-CCP2 IgM μg/ml</th>
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Table 1. Relative abundance of ACPA-immunoglobulins in serum and synovial fluid in relation to total immunoglobulin levels.
IgG-ACPA (AU/ml), total IgG, total IgA and total IgM were measured in serum or synovial fluid before purification of ACPA. Purified ACPA was measured for the IgG, IgA, and IgM concentration (μg/ml). The paired samples (P1 and P2) were selected for their high IgM ACPA levels (~5000 AU/ml). ACPA= anti-citrullinated protein antibody. SF= synovial Fluid. RA=rheumatoid arthritis. OA=osteoarthritis. P= paired sample. HC= healthy control.
with a maximum of 0.36% of all IgA present in serum. IgM-ACPA was abundantly present in some subjects, in 2 patients around 10% of the serum IgM appeared to be ACPA.

Next we compared ACPA levels in paired serum- and synovial-fluid samples. A trend towards higher levels of ACPA-IgG in synovial fluid as compared to serum was observed, however this difference does not reach statistical significance (p=0.07). In contrast, a significant increase in IgM-ACPA was noted in synovial fluid as compared to serum (Fig 2a). To quantify these ACPA-levels we next purified ACPA from synovial-fluid to investigate the local presence of the ACPA isotypes in the joint. Surprisingly, IgM-ACPA was most abundantly present in synovial fluid, where up to 13% of IgM-antibodies were estimated to be ACPA (figure 2). These 2 patients displayed high IgM ACPA levels in synovial-fluid (~5000 AU/ml). Up to 101 μg/ml IgM-ACPA was present in synovial-fluid (range: 2.8-101 μg/ml).

**Figure 2. ACPA immunoglobulins in serum and synovial fluid of RA patients.** ACPA IgG in relation to total IgG (aU/mg total IgG) is not significantly higher in synovial fluid as compared to serum, only a trend was found (Wilcoxon signed-rank test p=0.09) (A). ACPA IgA (aU/mg total IgA) and ACPA IgM (aU/mg total IgM) are significantly enriched in synovial fluid as compared to serum (Wilcoxon signed-rank test: IgA, p=0.002, IgM, p=0.0003)
IgG-ACPA was found in synovial fluid up to 103 μg/ml (table 1), based on the total amount of IgG present, this indicates that in synovial-fluid up to 2.3% of total IgG is ACPA.

In line with the small amount IgA-ACPA in serum, also little IgA-ACPA was present in synovial fluid. We included synovial fluid of one ACPA-negative patient with RA and one patient with osteoarthritis patient as controls. In these samples, no IgA-ACPA and IgG-ACPA were detected. The purified sample of the ACPA-negative patient contained 0.5 μg/ml IgM.

DISCUSSION

In this study, we quantified the abundance of different ACPA-immunoglobulins in serum and synovial-fluid of RA patients. IgG-ACPA is present in relatively high concentrations in serum and synovial-fluid as up to 1 out of 80 IgG-antibodies can be ACPA. These findings extend a previous observation describing the estimation of IgG-ACPA amounts using 3 RA sera. Furthermore, we now report that IgM-ACPA can be abundantly present in synovial-fluid (up to 13% of total IgM). Next to IgM-ACPA, also IgG-ACPA was found in relatively high concentrations in synovial-fluid (up to 2.3% of total IgG). The estimations presented are conceivably an underestimation of the true quantity as not all ACPA-activity could be recovered after purification possibly as a result from the isolation procedure. Nonetheless, we feel that the estimations presented provide a good reflection of total Ig ACPA levels as also evidenced by the good correlation between ACPA-IgG levels in serum and purified ACPA-IgG concentrations (Spearman’s rank correlation coefficient: 0.931. p<0.01).

The amounts of IgG-ACPA (mean: 28 μg/ml) present in the sera are remarkably in line with the peak levels of IgG directed against tetanus following repetitive vaccinations (20-28 μg/ml). Protective antibody titers after vaccination have been described as titers above 1 μg/ml against for example Haemophilus influenzae type-b and group-B streptococci. Surprisingly, the ACPA-concentrations found exceed these protective antibody titers and are in the same range as the amount of antibody present shortly after vaccination. The relatively high ACPA antibody levels might be related to the continuous presence of citrullinated antigens in the joint, which could activate ACPA producing B cells.

Furthermore, the results indicate the abundant presence of IgM-ACPA in synovial
ACPA concentration in serum and synovial fluid

fluid, as up to 1 in 8 IgM antibodies can be ACPA in patients with high ACPA-levels. IgM responses against T cell dependent antigens are, in general, not continuously present. In the setting of vaccination, for example, levels of antigen specific IgM decrease within weeks after immunization against rabies. Therefore the high concentrations of IgM-ACPA in sustained disease is intriguing and suggest the continuous local production and conceivably persistence of autoreactive B cell clones at the site of the inflamed joint. Previously, we reported that some IgG-ACPA positive patients harbour IgM-ACPA 7 years after the initial presence of IgG-ACPA.10 Since IgM-antibodies have a half-life of days and long-lived plasma cells producing IgM against protein antigen have not been described in humans, the continuous presence of IgM against T cell dependent antigens, indicate the continuous triggering of newly generated B cells. This suggests also that novel IgM producing B-cells are continuously recruited into the ACPA-response, indicating that the ACPA-response is continuously reactivated during the course of arthritis.10

Nonetheless we feel that the data presented on IgM-ACPA levels should be taken with caution as we can formally not exclude that the measured IgM-ACPA levels are influenced by IgM-rheumatoid factor (RF) bound to ACPA. However, previously reported data by our group showed that the depletion of IgM-RF did not result in reduction of IgM-ACPA levels. Furthermore mixing sera of RF-positive with IgM-ACPA-negative patients did not change the reactivity.10 Likewise, not all IgM-ACPA positive patients included in this manuscript were IgM-RF-positive, excluding a contribution of IgM-RF, at least in these patients.

In conclusion, high concentrations of ACPA are present in serum and synovial fluid of ACPA-positive RA patients, exceeding protective antibody levels against recall-antigens. Furthermore, the abundance of IgM-ACPA in synovial-fluid indicates ongoing recruitment of new B cells into the ACPA response, reflecting a continuous (re)activation of the RA specific ACPA response during the course of arthritis.
REFERENCE LIST
