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Chapter 8

Epitope spreading of the ACPA response occurs before disease onset and is associated with the disease course of early arthritis

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ABSTRACT

**Objective** Antibodies against citrullinated proteins (ACPA) are the most predictive factor for the development of rheumatoid arthritis (RA). To investigate if the recognition of citrullinated epitopes changes during disease onset or progression, we studied the fine specificity of ACPA in serum samples collected throughout the disease course, from before the onset of arthritis to long-standing RA.

**Methods** Antibodies recognizing five distinct citrullinated antigens were determined by enzyme-linked immunosorbent assay. Sera from 36 individuals who had donated blood before and after disease manifestation were used to investigate the development of citrullinated antigen recognition before disease onset. The association of ACPA reactivities with disease outcome was studied using sera of anti-cyclic citrullinated peptide 2 (CCP)-positive patients with undifferentiated arthritis (UA) who did or did not progress to RA (UA-RA n=81, or UA-UA n=35). To investigate the ACPA recognition profile in RA patients over a prolonged period of time, baseline serum samples from 68 RA patients were compared to samples obtained 7 years later.

**Results** The number of recognized citrullinated peptides increased in the period preceding disease onset. At the time of disease manifestation, UA patients who later developed RA recognized significantly more peptides than UA-UA patients. At later stages of the disease course, the ACPA fine specificity did not change.

**Conclusion** Epitope spreading with an increase in the recognition of citrullinated antigens occurs before onset of RA. Immunological differences in ACPA fine specificity are present at baseline between UA-UA patients and UA-RA patients and are associated with the future disease course.
INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) are a very distinctive feature of rheumatoid arthritis (RA) patients. The presence of these antibodies, which is most commonly assessed by reactivity against cyclic citrullinated peptide-2 (CCP), has been shown to be highly predictive for both the development of RA and the extent of associated joint destruction \(^1\), \(^2\). Recent evidence indicates that well-known genetic risk factors for RA: the HLA-DRB1 shared epitope (SE) alleles and the PTPN22 T-allele are predominantly associated with ACPA-positive RA \(^3\), \(^4\). These reports, together with the finding that ACPA can exacerbate arthritis in mice \(^5\), \(^7\), suggest that anti-peptidylcitrulline immunity plays an important role in the pathogenesis of the disease. To elucidate the biological mechanisms underlying RA, and possibly identify targets for intervention, it is therefore very important to gain more insight into the development of ACPA.

Studies investigating at which point in time ACPA first appear, have revealed that these antibodies can often be detected several years before disease onset \(^6\)–\(^10\). The mere presence of ACPA therefore does not appear to be sufficient to precipitate disease. An explanation for this observation could be that the anti-citrullinated protein immune response first needs to mature more fully, in the course of which, ACPA could acquire distinct characteristics which are instrumental in mediating tissue damage.

An antibody characteristic which has been shown to be crucial for the pathogenicity of autoantibodies, is the fine specificity of antigen recognition \(^11\). An increase or shift in antigen recognition during the course of an immune response (a phenomenon known as epitope spreading), can have very important pathophysiological consequences as has been described in, for example, systemic lupus erythematosus (SLE) \(^12\).

Taking into consideration that ACPA can be detected before the clinical diagnosis of RA, and that the presence of ACPA is strongly associated with disease progression, we hypothesized that epitope spreading of the ACPA response may play a role in the evolution of the disease. In the present study we therefore investigated the reactivity pattern of ACPA before disease onset and during disease progression. In order to cover the entire spectrum of the disease course ranging from ACPA-positive healthy individuals to patients with long-standing RA, we made use of several different cohorts, which together provide an overview of the ACPA fine specificity development over time.
MATERIALS AND METHODS

Study population
In a previous study, individuals who had donated blood before the onset of arthritis were identified among RA patients treated at the Department of Rheumatology of the Umeå University Hospital. The time period between collection of the pre-disease sera and disease onset ranged from 43 days to 10.8 years with a median of 2.5 years.

Patients with UA or RA were selected from the Leiden Early Arthritis Clinic (EAC), an inception cohort of patients with recent-onset arthritis (less than 2 years of complaints) that was initiated at the Department of Rheumatology of the Leiden University Medical Center in 1993. Diagnoses were recorded for all patients at annual follow-up visits and RA was diagnosed according to the 1987 revised ACR criteria. Patients who did not fulfill the diagnosis criteria and whose clinical presentation was not compatible with any other well-defined rheumatologic disease entity, were classified as having UA. For the present study we analyzed 116 anti-CCP positive UA patients and 68 anti-CCP-positive RA patients.

Informed consent was obtained and the study was approved by the local medical ethics review board.

Anti-CCP2 assays
Total IgG anti-CCP2 was measured by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions.

Anti-citrullinated peptide assays
ELISA assays were developed against peptides derived from vimentin, fibrinogen and alpha-enolase. Although there are also other targets for ACPA, we chose to primarily investigate epitopes from these proteins because they have been most consistently identified as citrullinated autoantigens.

Antibodies against both the citrullinated (Cit) and the uncitrullinated form of 2 linear peptides derived from vimentin (Vim 1-16: STCitS VSSS SYCitCit MFGG and Vim 59-74: VYAT CitSSA VCitLCit SSVP), 2 linear peptides derived from fibrinogen (Fibα 27-43: FLAE GGGV CitGPR VVER H and Fibβ 36-52: NEEG FFSA CitGHR PLDK K) and 1 linear peptide derived from alpha-enolase (Eno 5-20: KIHA CitEIF DSCitG NPTV) were determined by ELISA. All peptides were synthesized with a C-terminal spacer and biotin tag. The vimentin and fibrinogen epitopes which were used for the present study were selected based on the fact that they were most frequently recognized by sera from ACPA-positive patients with long-standing RA. A linear alpha-enolase peptide was used with a small difference in sequence compared with the peptide used by Lundberg et al. Because antibodies from
different RA patients have been shown to recognize distinct epitopes of the same protein, an approach using citrullinated peptides rather than whole proteins was used in order to attain optimal discriminative ability 20.

Fine specificity ELISA assays were performed as described previously 21. Briefly, streptavidin-coated pre-blocked microtiter plates were coated with the different peptides, followed by incubation with the serum samples. After washing, antibodies were detected with a rabbit anti-human IgG HRP-conjugated antibody and tetramethylbenzidine (TMB) as the coloring substrate. Baseline and follow-up samples were always analyzed on the same day and on the same plate. The inter-assay variability of the assays was very low (≤ 2% of samples with conflicting positive/negative results).

Definition of cut-off values and citrulline-specificity

Cut-off values on the citrullinated and arginine-containing peptides were defined as the mean plus two times the standard deviation of the values of 30 control subjects. A patient sample was considered to recognize a particular peptide in a citrulline-specific manner when it fulfilled all three of the following requirements: 1) an OD value on the citrullinated peptide above the citrulline cut-off and 2) an OD value on the arginine-variant below the arginine cut-off and 3) an OD difference (=OD for citrullinated peptide – OD for arginine-containing peptide) of at least 0.1. The number of patients that recognized both the citrullinated and the arginine-containing peptide above cut-off levels and thus did not bind to the peptide in a citrulline-specific way was small (approximately 3%).

Statistical analysis

To assess changes in the frequency of recognition of the citrullinated peptides between baseline and follow-up samples, McNemar’s test was used. Differences in the number of epitopes recognized, and in the levels of antibody reactivity between baseline and follow-up samples were evaluated using Wilcoxon signed ranks test. When two independent patient groups were compared (UA-UA versus UA-RA patients), Mann Whitney U tests were used to analyze differences between median optical density (OD-)values and the median number of recognized peptides. Relative risks (RR) with 95% confidence intervals were calculated for the development of RA from UA, based on the presence of a certain fine specificity. Logistic regression analysis was performed to calculate the risk associated with recognizing one additional citrullinated peptide for the outcome UA or RA after one year of follow-up. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) 14.0 and StatCalc 2.0.
RESULTS

Epitope spreading occurs before disease onset

In order to assess the recognition of citrullinated epitopes before disease onset, we made use of a unique serum collection of 36 individuals who had donated blood before the onset of RA and shortly after disease manifestation (on average 7.5 months post-disease onset) \(^8\). As shown in Figure 1A, the sera collected before disease onset (pre-RA) had a
significantly lower reactivity against all of the peptides, except for vimentin 1-16, than the sera collected after disease manifestation. For individual patients, the changes in reactivity to the different peptides over time were often correlated, but this correlation was not absolute.

Most peptides, with the exception of vimentin 1-16, were recognized substantially more frequently by the RA samples than by the pre-RA sera as listed in Table 1. The presence of (one or more isotypes of) RF also increased in the period before disease onset (data not shown). While many of the sera demonstrating reactivity to one of the fine specificity peptides also contained IgA- or IgM-RF, this was not always the case. Recognition of the fine specificity peptides was limited to the 29 individuals who were anti-CCP2-positive after disease onset.

The overall gain in peptide recognition resulted in a significant increase in the number of patients who recognized 1 or more peptides (38% versus 66%, p= 0.01) (Figure 1B). The median number of recognized peptides was 0 before disease development compared to 1 in the RA samples (p=0.08). As the period between pre-RA sample collection and disease manifestation varied among patients, the expansion of epitope recognition over time is shown in more detail in Figure 1C. This figure illustrates that the recognition of citrullinated epitopes increases before disease onset.

Table 1. Recognition of individual citrullinated peptides before and after disease onset.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Pre-RA n=36</th>
<th>RA n=36</th>
<th>lost reactivity</th>
<th>gained reactivity</th>
<th>p-value McNemar</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCP2</td>
<td>21 (58%)</td>
<td>29 (81%)</td>
<td>2</td>
<td>10</td>
<td>0.039</td>
</tr>
<tr>
<td>Vimentin 1-16</td>
<td>3 (8.1%)</td>
<td>1 (2.7%)</td>
<td>3</td>
<td>1</td>
<td>0.63</td>
</tr>
<tr>
<td>Vimentin 59-74</td>
<td>10 (27%)</td>
<td>14 (38%)</td>
<td>4</td>
<td>8</td>
<td>0.39</td>
</tr>
<tr>
<td>Fibrinogen α 27-43</td>
<td>2 (5.6%)</td>
<td>7 (19%)</td>
<td>0</td>
<td>5</td>
<td>0.062</td>
</tr>
<tr>
<td>Fibrinogen β 36-52</td>
<td>9 (25%)</td>
<td>15 (42%)</td>
<td>4</td>
<td>10</td>
<td>0.18</td>
</tr>
<tr>
<td>Enolase 5-20</td>
<td>4 (11%)</td>
<td>9 (25%)</td>
<td>1</td>
<td>6</td>
<td>0.13</td>
</tr>
<tr>
<td>Recognition of ≥1 peptide</td>
<td>14 (38%)</td>
<td>24 (66%)</td>
<td>2</td>
<td>12</td>
<td>0.013</td>
</tr>
</tbody>
</table>

The overall gain in peptide recognition resulted in a significant increase in the number of patients who recognized 1 or more peptides (38% versus 66%, p= 0.01) (Figure 1B). The median number of recognized peptides was 0 before disease development compared to 1 in the RA samples (p=0.08). As the period between pre-RA sample collection and disease manifestation varied among patients, the expansion of epitope recognition over time is shown in more detail in Figure 1C. This figure illustrates that the recognition of citrullinated epitopes increases before disease onset.

Taken as a whole, the analysis of the ACPA fine specificity profile before and after disease onset reveals that there is epitope spreading prior to the onset of RA.

The baseline ACPA reactivity profile differs between UA patients who progress to RA (UA-RA) and those who do not (UA-UA).

To investigate if a distinct recognition pattern of citrullinated peptides is associated with disease progression, we made use of serum samples from a different cohort: the Leiden EAC. ACPA reactivities were determined in baseline serum samples from UA patients who had not been treated with disease modifying antirheumatic drugs (DMARDs). Of
116 anti-CCP2 positive UA patients, 81 fulfilled the ACR criteria for RA after one year of follow-up (UA-RA), whereas the remaining 35 patients did not develop RA (UA-UA). In Figure 2A, the baseline reactivity is depicted in the form of the raw optical density (OD-) values. At baseline, the reactivity of the UA-RA sera was significantly higher than that of the UA-UA sera against two of the five peptides. When the OD-values were translated into negative/positive recognition by applying cut-off values based on 30 control subjects,

**Figure 2: Baseline reactivity of UA-UA versus UA-RA sera.**

**Figure 2A:** Depicted are the raw optical density (OD-) values for each of the UA-UA (n=35) and UA-RA (n=81) patients. Lines indicate the median OD. Differences between the median OD-values of the UA-UA and UA-RA patients for each of the five peptides were investigated by Mann-Whitney U tests.

**Figure 2B:** Recognition of individual citrullinated peptides at baseline by UA-UA and UA-RA patients. Depicted are the percentages of patients per group that recognized the different peptides in a citrulline-specific manner. Relative risks (RR) with 95% confidence intervals were calculated for the development of RA from UA, based on the presence of a certain fine specificity.

**Figure 2C:** Number of peptides recognized by UA-UA and UA-RA patients. Error bars indicate the minimum and maximum number of peptides recognized per patient. Boxes designate the 25th and 75th percentile, while the lines in the boxes indicate the median number of recognized peptides.
the prevalence of recognition of both vimentin peptides and fibrinogen β 36-52 was significantly higher among UA-RA than among UA-UA patients (Figure 2B). As shown in Figure 2C, the median number of recognized peptides per patient was larger in UA-RA patients (2 peptides) than in UA-UA patients (1 peptide) (p=0.02). Regression analysis revealed that for every additional epitope which was recognized, the risk of developing RA increased by a factor of 1.55 (p=0.02).

In summary, these results indicate that ACPA-positive UA-UA and UA-RA patients are immunologically distinct at baseline with regards to their ACPA reactivity profile. The UA-RA patients displayed reactivity against a significantly larger number of citrullinated epitopes.

Recognition of citrullinated peptides does not change during disease progression
To investigate whether the reactivity against citrullinated antigens would further increase during disease development from UA to RA, we performed fine specificity assays with serum samples obtained at baseline and after approximately 1.5 year of follow-up of the same UA-RA patients as described above. All patients had progressed to RA at this point in time. Serum samples were available for 67 of the previously tested 81 patients. As shown in Figure 3A, the recognition of all five fine specificity peptides did not markedly change. At follow-up, a smaller number of patients recognized the individual peptides, but the difference was not statistically significant. The number of citrullinated peptides recognized per patient was also lower at follow-up as compared to baseline (Figure 3B).

On the basis of these results, we can conclude that the ACPA reactivity with regard to the five fine specificity peptides we measured, did not further increase during the progression of UA to RA.

Figure 3: Recognition of individual citrullinated peptides by UA-RA patients at baseline and after 1.5 years of follow-up. Figure 3A: Depicted are the percentages of patients (n=67) which recognized the peptides in a citrulline-specific manner at baseline (when all patients had UA), and after 1.5 years of follow-up when all patients had developed RA.
Figure 3B: Number of peptides recognized by UA-RA patients at baseline and after 1.5 years of follow-up. Error bars indicate the minimum and maximum number of peptides recognized per patient. Boxes designate the 25th and 75th percentile, while the lines in the boxes indicate the median number of recognized peptides.
No changes in recognition of anti-citrullinated peptides by RA patients after seven years of follow-up

To further substantiate the finding that the ACPA recognition pattern does not change during the course of the disease, we examined a group of 68 patients who presented with RA and of whom serum samples were available at baseline and after a median follow-up time of 7 years (interquartile range: 6.2-7.9 years). As depicted in Figure 4A, the percentage of patients that recognized the five peptides did not change after 7 years of follow-up. With regard to the absolute number of peptides that was recognized per patient, the findings were similar to the observations described above for UA-RA patients. The number of recognized peptides was slightly lower at the time of follow-up, as can be seen in Figure 4B, but this difference was not statistically significant (p=0.25).

Overall, these results reveal that with regard to these five fine specificity peptides, the ACPA recognition pattern does not change during the course of RA and that the reactivity profile does not expand after disease onset.

DISCUSSION

In this study, we analyzed the recognition pattern of ACPA throughout the disease course of RA. The results reveal that the recognition of citrullinated epitopes expands before disease onset. Furthermore, at the time of disease onset, an extensive recognition pattern of citrullinated peptides by patients who present with UA, is associated with rapid disease progression to RA in the first year of follow-up. After disease development, we could not detect a further increase in reactivity to the five fine specificity peptides used in this study.
The accumulation of autoantibody reactivities before disease manifestation which we observed in RA patients, has also been reported in other studies of human disease, such as SLE\(^{12}\) and pemphigus\(^{22}\), as well as in animal models such as the collagen-induced arthritis model (CIA)\(^{21}\). Together, these findings suggest that epitope spreading before disease onset may be a commonly occurring phenomenon which, as shown for the first time by the data presented here, may also take place before the onset of RA.

It is intriguing that at the time of disease onset, ACPA-positive UA patients who will subsequently develop RA and those who will not, are already immunologically distinct with regards to their ACPA response. The fact that none of the tested fine specificity peptides was exclusively recognized by UA-RA patients, indicates that the measurement of these ACPA reactivities may not have immediate clinical utility for individual patients. Rather, the results indicate that on the population level, the “footprint” of past epitope spreading (being an expanded epitope recognition repertoire) is associated with disease progression. These differences in the ACPA fine specificity repertoire are not an isolated immunological phenomenon, since UA patients who rapidly progress to RA also exhibit a broader use of ACPA isotypes\(^{24}\). The characteristics of the ACPA response at baseline thus reflect the progression of the disease in UA patients. These findings are consistent with the hypothesis that the nature and characteristics of the ACPA response may determine disease development and progression, but additional studies will be required to fully elucidate the role of ACPA in the pathogenesis of the disease.

Although baseline sera from UA-RA patients recognized more peptides than sera from UA-UA patients, we could not detect a further increase in reactivity after disease onset. A very similar observation has been reported in SLE, where the accrual of new types of autoantibodies gradually increased up to the time of diagnosis and then virtually stopped\(^{12}\). These findings are also in line with previous reports which have shown that anti-CCP2-titers and levels of ACPA isotypes decrease with time\(^{24, 25}\). A possible explanation for these observations is the effect of immunosuppressive treatment such as DMARDs, which are commonly prescribed once the diagnosis of RA has been established. This makes it difficult to discern if the observed decrease in ACPA reactivities over time is due to the natural course of the immune response or to the influence of medication.

The patients studied in the current investigation were generally treated with mono-therapy with conventional DMARDs. Consistent with the severe phenotype known to be associated with ACPA-positive disease, medication was frequently changed in all patients, which hampered a thorough analysis of clinical subgroups stratified for treatment. Further studies will be required to definitively answer the question whether specific therapies do or do not affect epitope recognition profiles.

Although we could not observe a further expansion of the ACPA reactivity profile after disease manifestation, it should be noted that the number of citrullinated epitopes investigated in the current study is limited. Therefore, one cannot rule out that epitope spreading
after disease onset was in fact present, but could not be detected. However, the data of
the cohort of individuals who had donated blood before disease onset, reveal that there
is clearly epitope spreading of the ACPA response before disease onset, suggesting that
the five peptides had enough discriminative potential to allow the detection of diversifica-
tion of epitope recognition. Nonetheless, further studies using a larger set of citrullinated
antigens will be required to fully explore the possibility of epitope spreading after disease
onset in RA.

When investigating epitope spreading, two other immunological phenomena must be
taken into account: antibody cross-reactivity and affinity maturation. With regard to the
peptides used in the current study, a previous report described that the HLA SE alleles
are strongly associated with the recognition of citrullinated vimentin 59-74, but not with
the recognition of citrullinated fibrinogen β 36-52, indicating that there was little cross-
reactivity between the different epitopes. The fine specificity of the ACPA response as
investigated in the current study, was also associated with the presence of HLA SE alleles
(data not shown). Furthermore, in the current study several patients were single-positive for
each specific peptide, which rules out a large extent of antibody cross-reactivity. Affinity
maturation, which leads to a higher antibody binding affinity and thus makes antibodies
easier to detect by ELISA, could in this manner, have contributed to the increase in epitope
recognition which we measured using this method. With regard to our understanding of
the immune response in vivo, however, the distinction between epitope spreading and
affinity maturation is, in this case, largely inconsequential, as the effect would be the
same: increased antibody reactivity to a certain citrullinated epitope.

The in vitro methodology employed in this study cannot mimic the way in which antigen
is presented in vivo and was therefore not intended as a technique by which to identify
a specific ACPA fine specificity as the culprit of disease onset or exacerbation. Instead,
the study was designed to assess the extent to which epitope spreading occurs in associa-
tion with disease progression by using a selected set of peptides as a model for ACPA
diversification. It was not possible to discern clusters of peptides which were preferentially
recognized together, nor was it possible to identify a specific sequence in which the ACPA
reactivities developed over time.

In summary, our findings indicate that the recognition of citrullinated epitopes expands
before the development of arthritis. Furthermore, at the time of clinical presentation, UA
patients who will later develop RA already recognize more citrullinated antigens than UA
patients who will not develop RA. During disease progression from UA to RA, as well as
during the further disease course of RA, we did not observe additional changes in anti-
citrulline reactivity. Together, these data indicate that an important part of the evolution of
the ACPA response may take place before disease onset.
REFERENCES


