Affinity Purification and Comparative Biosensor Analysis of Citrulline-Peptide Specific Antibodies in Rheumatoid Arthritis

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Abstract:

Background: In rheumatoid arthritis (RA), anti-citrullinated protein/peptide antibodies (ACPAs) are responsible for disease onset and progression, however, our knowledge is limited on ligand binding affinities of autoantibodies with different citrulline-peptide specificity.

Methods: Citrulline-peptide specific ACPA IgGs were affinity purified and tested by ELISA. Binding affinities of ACPA IgGs and serum antibodies were compared by surface plasmon resonance (SPR) analysis. Bifunctional nanoparticles harboring a multi-epitope citrulline-peptide and a complement activating peptide were used to induce selective depletion of ACPA producing B cells.

Results: Ko values of affinity purified ACPA IgGs varies between 10⁻⁶⁻¹⁰⁻⁸ M and inversely correlate with disease activity. Based on their cross-reaction with citrulline-peptides we designed a novel multi-epitope peptide, containing Cit-Gly and Ala-Cit motifs in two-two copies, separated with a short, neutral spacer. This peptide detects antibodies in RA sera with 66 % sensitivity and 98 % specificity in ELISA and is recognized by 90% of RA sera, while none of the healthy samples in SPR. When coupled to nanoparticles, the multi-epitope peptide specifically targets and depletes ACPA producing B cells ex vivo.

Conclusions: The unique multi-epitope peptide designed on the basis of ACPA cross-reactivity might be suitable to develop better diagnostics and novel therapies for RA.

Keywords: rheumatoid arthritis; Citrulline-peptides; autoantibody; affinity; cross-reaction; targeting
1. Introduction

Anti-citrullinated protein/peptide antibodies (ACPA) are highly specific and sensitive clinical marker of rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease [1]. Approximately 70-80 % of RA patients are ACPA positive, and high level of ACPA has been associated with bad prognosis of the disease [2]. ACPA is produced against citrullinated self-proteins, emerging as a result of the activation of peptidyl arginine deiminase (PAD) enzymes [3, 4]. PADs are activated at inflammatory sites and in the presence of high level of intracellular free calcium in activated cells. PADs deiminate arginine to citrulline and this post-transcriptional modulation drastically modify the structure of the protein, initiating unfolding [5]. The newly appearing citrullinated epitopes will then be recognized by the immune system, resulting in the production of ACPA [6].

Most of the citrullinated target molecules of ACPA are localized in the joints, such as collagen II [7], fibrinogen/fibrin [8, 9], and the intracellular protein, vimentin [10, 11], which is released from macrophages in the inflamed synovial [12]. Binding of ACPA to synovial target proteins in vitro and in vivo has been demonstrated [13]. Deposition of ACPA and ACPA containing immune complexes induce the release of inflammatory cytokines, such as TNFα, eventually leading to the escalation of inflammation [8, 14, 15]. ACPA appears many years before the disease onset in the sera of patients, and its detection is extremely important for the early diagnosis. ACPA is not only a specific and sensitive disease marker, but also play key role in the pathogenicity of RA [16].

A wide variety of citrulline containing peptides (Cit-peptides) are recognized by ACPA, these include peptides representing citrullinated sequences in the major proteins of the joints, such as fibrinogen [8] and type II collagen [7], the intracellular structural protein, vimentin, which is also detected in the synovium [11, 17, 18], as well as peptides derived from bacterial (α-enolase) [19] and viral (Epstein-Barr virus nuclear antigen (EBNA)) origin [20]. An ACPA cross-reacting, citrullinated peptide, derived from filaggrin produced by epithelial cells has also been described [9], [21] and used in the first diagnostic tests of RA.

Citrullination in general is a physiological process, it may occur for example upon gene regulation [22], apoptosis [23], inflammation [24] and NET formation [25] without inducing an autoimmune response. In RA, however, probably due to the abnormal humoral immune response, citrullinated proteins induce ACPA production in the vast majority of patients. Since ACPA is a crucial player and has a pathological role in RA, detailed characterization of this autoantibody would be of great importance both for diagnostic accuracy and prognostic evaluation.

Many groups described the multiple specificity and cross-reactivity of ACPA [26-30], and several citrullinated peptide epitopes have been identified, however, only limited data are available so far on the binding affinity of individual purified ACPA IgGs. Therefore our aim was to analyze in parallel by Surface Plasmon Resonance (SPR) and ELISA both the affinity and the specificity of affinity purified autoantibodies from RA patients.

In accordance with previous data, we detected a high degree of cross-reactivity between affinity purified ACPA IgGs, with apparent dissociation constants (K_D) between 10^-6 -10^-8 M. Based on the pattern of cross-reaction we designed a novel multi-epitope peptide that was specifically recognized by RA sera and was able to deplete specific B cells from in vitro culture. Thus we suppose that this multi-epitope peptide might be further developed for RA diagnosis and therapy.

2. Results

2.1. Affinity purification of ACPA

We have synthesized a panel Cit-peptides corresponding to known ACPA epitopes, listed in Table 1., and prepared insoluble matrixes for the immune-purification of ACPA from the sera of selected RA patients. Patients’ selection was based on the positivity of the sera in an in house Cit-peptide-based ELISA [31, 32].
Table 1. List of Citrulline containing peptides used in the assays

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequences</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filaggrin (306-326)</td>
<td>Ac-SHQESTX'CXSXGRSGSK-NH₂</td>
<td>[33]</td>
</tr>
<tr>
<td>Collagen II (359-369)</td>
<td>Ac-AXGLTGXPGDA-NH₂</td>
<td>[7]</td>
</tr>
<tr>
<td>Fibrin β (60-74)</td>
<td>H-XPAPPPISGGGYXAX-NH₂</td>
<td>[8, 34]</td>
</tr>
<tr>
<td>Vimentin (65-77)</td>
<td>Ac-SAVRAXSVPVRK-NH₂</td>
<td>[10]</td>
</tr>
<tr>
<td>α-enolase (5-21)</td>
<td>Ac-KIHAEIFDSXGNPTVE-NH₂</td>
<td>[35]</td>
</tr>
<tr>
<td>EBNA-2 (341-361)</td>
<td>Ac-GQSXGQSXGXCXGXGKG-NH₂</td>
<td>[36]</td>
</tr>
<tr>
<td>multi-epitope</td>
<td>H-Ttds²-AXAXGSGXG-XG-NH₂</td>
<td></td>
</tr>
</tbody>
</table>

¹X stands for citrulline. ²Ttds - linker 1,13-diamino-4,7,10-trioxatridecan-succinamic acid. For screening the serum samples by ELISA both citrulline and arginine containing variants of the same peptides were used.

The citrulline containing filaggrin19, collagen II, fibrin β60-74 and vimentin peptides were already thoroughly tested with our cohort of serum samples [31, 32, 37]. Therefore we only show here the screening of sera of RA patients (n=177) and healthy volunteers (n=104) with arginine and citrulline containing EBNA-2 and α-enolase peptides, respectively (Fig.1).

Figure 1. Recognition of citrulline-containing EBNA-2 and α-enolase peptide epitopes by sera of patients (RA, n=177) and healthy volunteers (H, n=104). (a), (c), OD index: OD with Cit-peptide/OD with Arg peptide. (b), (d), Receiver operating (ROC) curves. *** p ≤ 0.001.

EBNA-2 and α-enolase peptides detected autoantibodies in RA serum samples by 48 % and 27 % sensitivity, respectively. For comparison, the sensitivity values for the previously tested Cit-filaggrin, fibrin, collagen II and vimentin peptides were 62 %, 56 %, 44 % and 41 %, respectively [31, 32]. Based on these data we have selected the sera, which had a high OD index at least with one of
the peptides and purified the anti-peptide antibodies by affinity chromatography. ACPAs were purified in two steps [38]. First the IgG fractions were obtained on a protein G column and this was followed by the affinity purification of anti-peptide antibodies on the corresponding citrulline containing filagrin19-, collagen II-, fibrin-, vimentin- and α-enolase-peptide-coted matrixes, respectively.

2.2 Cross-reaction of affinity purified ACPA IgG fractions

To see if the affinity purified anti-peptide antibodies are able to recognize a different peptide, ELISA plates were coated with the individual peptides directly (fibrin, enolase, EBNA-2) or indirectly, using neutravidin coated plates and biotinylated peptides (filaggrin, collagen II, or vimentin) and the binding of the affinity purified IgG fractions was monitored (Fig. 2).

Figure 2. Reactivity of affinity purified IgG fractions with the relevant and irrelevant citrulline containing peptides. OD ratios that are OD with Cit /OD with arg containing peptides are shown. Cut-off values for each Cit and Arg containing peptide pair were calculated from OD indexes of 120 healthy samples (the means of OD indexes + 2*SD). These were below 1.5 for all peptides. Results of a typical experiment.

Interestingly, the affinity purified IgG prepared on Cit-filaggrin19 peptide recognized citrulline containing collagen II, fibrin β, EBNA-2 and α-enolase as well, beside the filaggrin19; IgG prepared on Cit-collagen II peptide bound to citrulline containing filaggrin, fibrin β, and EBNA-2 peptides, while IgG purified on Cit-vimentin peptide recognized all Cit-peptides tested, although at different level. ACPA purified on the Cit-fibrin β peptide has shown the lowest degree of cross-reactivity in ELISA.

We compared the Cit-peptide sequences and observed that certain short motifs containing Ala-Cit and/or Cit-Gly residues are present in all peptides. We supposed that both of these short motifs might be important for the recognition. Thus we designed and synthesized a novel “multi-epitope” peptide consisting of two-two copies of Ala-Cit and Cit-Gly motifs separated with a neutral spacer, SGSG. As expected, IgG purified on citrulline-containing multi-epitope peptide coated matrix recognized all other peptides, but with different intensity (Fig. 2., dark blue columns). Vice versa, the multi-epitope peptide bound to almost all other ACPA IgG, fibrin β was the exception. These data verify earlier data suggesting that ACPAs are highly cross-reactive and indicate that Ala-Cit as well as Cit-Gly motifs have importance in recognition.
2.3. Analysis of the multi-epitope peptide by ELISA screening of RA sera

Next, we screened 210 RA sera and 90 healthy control samples using the biotinylated, citrulline or arginine containing multi-epitope peptide as a coat in ELISA.

Figure 3. The Cit-multi-epitope peptide identifies RA sera with the highest specificity and 66 % sensitivity. (a), ELISA, OD indexes (OD with Cit-peptide/OD with Arg-peptid) of RA (n=210) and healthy (n=90) samples, (b) ROC curve of ELISA. Area under the curve (AUC): 0.7843.

The designed multi-epitope peptide identified ACPA in RA serum samples with 66 % sensitivity, while none of the healthy control sera showed binding (Fig. 3). This sensitivity value is somewhat higher than that of fibrin $\beta$ peptide, and the shape of the ROC curve (AUC 0.7843) suggest that a diagnostic test based on the multi-epitope peptide would be more accurate.

2.4. SPR analysis of affinity purified antibodies and sera of RA patients

For affinity measurements of IgGs purified from RA sera on insolubilized citrulline containing filaggrin19, collagen II, fibrin $\beta$, vimentin and multi-epitope peptides first we had to test the ability of the individual peptides to immobilize on GLH sensor chip. Amine coupling immobilization strategy was used. Immobilization buffers were selected separately for each peptide, according to their isoelectric point. From these five peptides only filaggrin19, vimentin and the multi-epitope peptide could couple covalently to the surface of the GLH chip. Therefore fibrin $\beta$, and collagen II peptides were biotinylated and immobilized using the neutravidin coated NLC chip.

First, apparent Kd of affinity purified IgG fractions of anti-peptide antibodies were tested on various immobilized Cit-peptides. The dissociation constants are in the micromolar - 10 nanomolar range, and as well as in the ELISAs, a high level of cross-reactivity can be observed (Table 2.).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Filaggrin</th>
<th>Collagen II</th>
<th>Fibrin $\beta$</th>
<th>Vimentin</th>
<th>Multi-epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>filaggrin19 ACPA</td>
<td>4.05E-07</td>
<td>2.10E-06</td>
<td>1.65E-06</td>
<td>nb *</td>
<td>1.33E-06</td>
</tr>
<tr>
<td>collagen II ACPA</td>
<td>5.32E-08</td>
<td>6.10E-08</td>
<td>8.44E-08</td>
<td>1.31E-07</td>
<td>7.17E-08</td>
</tr>
<tr>
<td>fibrin $\beta$ ACPA</td>
<td>3.56E-07</td>
<td>1.21E-06</td>
<td>8.34E-07</td>
<td>3.86E-07</td>
<td>2.75E-07</td>
</tr>
<tr>
<td>vimentin ACPA</td>
<td>5.58E-07</td>
<td>nb</td>
<td>1.65E-06</td>
<td>nb</td>
<td>nb</td>
</tr>
<tr>
<td>$\alpha$-enolase ACPA</td>
<td>nb</td>
<td>8.96E-08</td>
<td>2.47E-08</td>
<td>nb</td>
<td>2.94E-08</td>
</tr>
</tbody>
</table>

*nb: no binding
2.4.1. KD determination of serum IgG on GLH chip

Fig. 4. shows representative sensograms of IgG from an RA patient purified by affinity chromatography on immobilized Cit-filaggrin19 peptide. As a negative control, intravenous immunoglobulin (IVIG) was used, pooled from the plasma of more than a thousand healthy blood donors, which clearly did not show any specific binding.

To calculate the KD values for serum samples, the concentration of an individual peptide specific IgG was estimated in all sera by ELISA, using a standard concentration series from the given affinity purified IgG fraction. Typical binding curves of two serum samples are shown on Fig. 4. c) and d).

Figure 4. Representative sensograms of (a) Cit-filaggrin19-affinity purified IgG; (b) negative control IVIG and two typical sera with (c) higher and (d) lower affinity on Cit-filaggrin19 coupled GLH chip.

The distribution of the apparent KD values of serum antibodies measured on Cit-vimentin, Cit-filaggrin19 and multi-epitope peptides immobilized by covalent coupling is shown on Fig. 5. The majority of KD values were in the range between $10^4$ - $10^7$ M/L. All sera selected for this assay (n=68) were highly positive for the given Cit-peptide in ELISA. SPR analysis has shown that 92 % of sera bound to Cit-vimentin and all samples bound to the multi-epitope peptide on the chip. The distribution of KD values were similar, however, only 41 % of the Cit-filaggrin19 peptide positive sera bound to this peptide under the flow condition on the chip. The $K_a / K_d$ plots shown at the lower panels of the figure represent the data of individual sera. In general, more sera have shown a lower dissociation rate from the multi-epitope peptide as compared to the other two.
2.4.2. Binding of serum IgG to the biotinylated peptides immobilized on neutravidin coated chip (NLC)

Fibrin and collagen II peptides that did not immobilize on GLH chip, were biotinylated and immobilized on a neutravidin coated surface. Additionally, multi-epitope peptide was also biotinylated and re-tested on NLC chip (Fig. 6.)

When the biotinylated peptides were immobilized on the NLC chip, 31 % of selected RA sera bound to Cit-collagen peptide with apparent $10^{-6} - 10^{-8}$ $K_d$, while 60 % of sera bound to the Cit-fibrin peptide. 

**Figure 5.** Distribution of $K_d$ values of serum antibodies as measured from RA samples on GLH chip, upper panels; isoaffinity diagrams, lower panels

**Figure 6.** Distribution of $K_d$ values measured from RA sera on biotinylated Cit-collagen II, Cit-fibrin $\beta$ and multi-epitope peptides immobilized on NLC chip.
β peptide with $10^{-5} - 10^{-8}$ KD. Interestingly, although a lower proportion of sera (53%) showed binding to the multi-epitope peptide immobilized on the NLC chip and among those who bound, a larger proportion showed higher KD ($10^{-8}$ M/L).

Low avidity ACPA was previously shown to associate with a higher rate of joint destruction [39]. Therefore we tested if the disease activity score, DAS28, correlates with the apparent KD (Fig. 7). We found an inverse correlation between DAS and KD of IgG purified on Cit-filagrin19 peptide, and on Cit-fibrin β peptide, indicating that low KD auto-antibodies are more pathogenic.

![Fig. 7.](image)

**Fig. 7.** Correlation between the disease severity (DAS28) and the apparent binding affinity (Kd) of serum antibodies from RA patients.

2.5. Selective depletion of autoantibody producing B cells ex vivo by the multi-epitope peptide covalently coupled to poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NP)

We have reported earlier that bifunctional nanoparticles covalently coupled to Cit-fibrinβ60-74 peptide and a peptide inducing cell lysis deplete the peptide specific B cells in ex vivo cultures of PBMC from RA patients. Here we tested if the newly designed multi-epitope peptide would have a similar effect. Therefore, the multi-epitope peptide and a complement activating peptide, a modified form of the peptide derived from HIV1 GP120 [32, 40] (Ac-$^{230}$C(Acm)NNQTFNGTGPC(Acm)TNV$^{247}$-K-NH$_2$, (Acm: acetamido-methyl)) were simultaneously coupled to PLGA nanoparticles, and added to pre-activated PBMC from RA patients in the presence or absence of complement source (1% human sera, NHS). The Cit-peptide specific antibody production was monitored by ELISpot assay. The bifunctional PLGA nanoparticles targeted to B cells recognizing the multi-epitope peptide significantly suppressed the peptide specific IgG secretion in the presence of complement (Fig. 8.), while total IgG production was not affected (data not shown).

These data indicate that the newly designed multi-epitope peptide might be suitable for the development of autoantigen-specific immunotherapy.
Figure 8. *Ex vivo* inhibition of the Cit-peptide specific antibody production by bifunctional PLGA nanoparticles covalently coupled to multi-epitope peptide and a complement activating peptide. Antibody synthesis was tested by ELISpot assay according to a modified procedure of Manufacturer (Mabtech, Stockholm, Sweden). As complement source 1% normal human sera (NHS) was used, for the negative control the sera were heat-inactivated (iNHS). a) Average number of peptide specific antibody secreting cells (ELISpots) from 10^6 PBMC of eight RA patients, b) ELISpot data of individual RA patients.

3. Discussion

In the present study we have analyzed the specificity and the apparent affinity of anti-peptide antibodies isolated from the sera of RA patients by affinity chromatography. The citrulline containing peptides we used for the isolation and analysis represent the immunodominant epitopes of fibrin β chain, filaggrin, collagen II, vimentin, α-enolase and EBNA-2. Most of the affinity purified IgG fractions recognized other cit-peptides, beside the ones used for the isolation, and their apparent Kᵦ were comparable. Additionally, in some cases (e.g. anti-cit-vimentin) anti-peptide antibodies bound to a different peptide with even higher affinity, indicating a high degree of cross-reactivity. Comparing the individual peptide sequences we have observed that all peptides contain Cit-Gly and/or Ala-Cit motifs, which are seemingly essential for the recognition. Thus we designed an artificial peptide, containing two copies of each small motifs separated with a neutral spacer. This “multi-epitope” peptide was recognized by sera from RA patients with 66% sensitivity and 98% specificity, and the binding affinities were comparable with that of the natural citrulline containing peptides. Antibodies purified on the multi-epitope peptide cross-reacted with most other peptides tested, although at different degree. The functional activity of the multi-epitope peptide was equivalent to that of Cit-fibrin β peptide [32], it was able to specifically deplete Cit-peptide specific B cells, thus significantly inhibit ACPA production.

Cross-reactivity of ACPA was reported by several groups, and the concept that Cit-Gly motif has importance in the recognition was also studied earlier [26, 29, 30, 36, 41]. Several combinations of Cit-peptides and ACPAs have been tested for cross-reactivity; antibodies to Cit-EBNA(35-58) strongly cross-reacted with the immunodominant epitope of citrullinated fibrin [42] and vice versa, two proteins encoded by Epstein-Barr virus (EBV), EBNA-1 and EBNA-2 were specifically recognized by antibodies of the ACPA family [43]. Additionally, monoclonal antibodies obtained from an RA patient and were specifically directed against a citrullinated fibrin peptide also recognized Cit-peptides derived from enolase and vimentin [28]. Recently Trier et al. concluded that ACPAs are a collection of largely cross-reacting but occasionally non-cross-reactive antibodies. Cross-reactivity is largely depends on Cit-Gly motif in combination with the peptide backbone, while non-cross-reactive ACPAs depend on a specific citrullinated epitopes, rather than a citrulline containing motif [41, 44].

The data presented here expand these earlier findings, first, since here a panel of affinity purified anti-peptide antibodies were screened with a panel of Cit-peptides representing immunodominant
epitopes of *in vivo* ACPA target proteins, and second, in addition to binding studies by ELISA, affinities of the interaction were also analyzed by SPR. Both the specificity and the affinity play a key role in the pathological effect of an antibody, therefore we have monitored these two parameters by analyzing their interactions with seven Cit-peptides derived from major target proteins of ACPA.

The suggested central role of Cit-Gly [41, 44] in the recognition of autoantigens by ACPA does not explain how vimentin and fibrin $\beta$ peptides are recognized. Cit-vimentin and fibrin$\beta$ peptide do not contain Cit-Gly, instead has Ala-Cit motif. Similarly, collagen II and enolase peptides also have Ala-Cit motif. Although vimentin peptide had restricted reactivity with affinity purified IgG fractions, IgG purified on Cit-vimentin recognized all other peptides, even without Ala-Cit, such as EBNA-2 and filaggrin, indicating that antibodies specific for the Ala-Cit motif cross-react with Cit-Gly and thus Ala-Cit motif is also important in the recognition. Based on these, we designed an artificial peptide containing two Cit-Gly and two Ala-Cit motifs. This artificial peptide has a higher sensitivity to detect serum ACPA than fibrin $\beta$ and filaggrin peptides, has a similar binding affinity, and is able to target *in vitro* the peptide specific B cells of RA patients.

We determined the apparent binding affinities of Cit-peptides to affinity purified antibodies by SPR biosensor analysis. The IgG fractions purified by affinity chromatography show a comparable $K_0$ and a similar distribution of dissociation constants as RA serum antibodies. Most of the $K_0$ values were between 1 micromolar - 10 nanomolar range and showed high heterogeneity. While almost all serum samples bound to Cit-vimentin and to the multi-epitope peptide, only 30-60 % of RA sera reacted with citrulline containing collagen II, fibrin $\beta$ and filaggrin19 peptides on the chip, although all samples were positive in ELISA. This may be explained by the difference between the static nature of the binding to ELISA plate in contrast to the dynamic flow rate over the surface of the chip, which induce a faster dissociation of the analyte from the peptide ligand coated surfaces. Thus antibodies with low affinity might be lost.

In concert with previous findings [39] we detected an inverse correlation between $K_0$ values and the disease severity (DAS 28), in case of Cit-filaggrin19 and Cit-fibrin$\beta$ peptides, the lowest $K_0$ was associated with the most severe disease. A possible explanation could be that low binding affinity might allow higher degree of cross-reaction, leading to more extensive tissue damage.

ACPA play a pathological role in the development of RA, in the perpetuation of inflammation and the pathophysiology of the disease [45]. ACPA positive patients have a faster progress of the disease and a worst radiological outcome [27, 46] thus the detailed characterization of ACPA has high importance. These results presented here contribute to the understanding of the interactions between citrullinated epitopes and ACPA in RA patients thus may support the development of better diagnostics and precision therapy for RA.

4. Materials and Methods

4.1. Patients

Blood samples were collected from 210 RA patients (165 women/45 men; median age 63 years, interquartile range 51–70; median disease duration 6 years, interquartile range 3–13) at the Buda Hospital, Budapest, Hungary of Hospitaller Brothers of St John. The diagnosis of the disease was established on the basis of the revised classification criteria of the American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) [47].

Blood samples were taken after the patients signed a written consent, and with ethical permission provided by the National Public Health and Medical Officer Service (49468/-2013/EKU (576/2013)). Selected patients with a high level of anti-CCP antibodies were repeatedly recruited for the functional assays. A total of 120 age-matched healthy control sera were obtained from healthy volunteers.
4.2. ACPA affinity purification

We purified IgG from RA sera via Protein G agarose (Thermo Fischer Scientific, MA, USA) affinity chromatography. To isolate ACPAs, Citrulline containing peptides were covalently bound (5 mg/ml) to GE NHS HiTrap 1 ml column, according to manufacturer’s instructions. Purified IgG was diluted 2x in 0.1 M NaH2PO4, 0.15 M NaCl, pH=7 buffer and circulated through the column for 3 days. After washing, ACPA-containing fractions were eluted with 0.1M glycine buffer (pH 2.5). The pH was immediately restored by adding 10 µl 2M TRIS pH 9 to 1 ml eluted ACPA. The eluate fractions were dialyzed against PBS and after determining the IgG concentration via Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA) the samples were stored at -20°C.

4.3. ELISA

ELISA was used to determine serum IgG ACPA concentrations and to test ACPA cross-reactions. The biotinylated, citrulline or arginine containing filaggrin19, vimentin, collagen II and the multi-epitope peptides were added at 1 µg/ml to the Medisorp plates (Greiner GmbH) pre-coated with 5 µg/ml Neutravidin (Pierce Biotechnology, Rockford, IL, USA) overnight, at 4 °C, and then the plates were incubated for 1h at 37 °C. EBNA2, α-enolase and fibrin β peptides were coated directly to Maxisorp (Nunc) plates at 2.5 µg/ml. After washing, the plates were blocked with 150 mM NaCl and 2 % BSA containing PBS for 1h, at 37 °C.

To determine the specific ACPA concentrations in sera a calibration curve was prepared from the affinity purified ACPA as standard, in a two-fold dilution series, starting from 0.5 mg/ml. Sera samples were diluted in 1:100. Dilution buffer was 2 M NaCl, 2 % BSA in PBS. In cross-reaction assays, ACPAs were diluted to 0.1 mg/ml. Sera/ACPA samples were incubated overnight at 4 °C, shaking. After washing, the plates were incubated with 1:15000 dilution of rabbit anti-human IgG HRP (H+L) (Southern biotech) for 1h at 37°C. Signal was developed with TMB substrate (Sigma) and reaction was stopped with 2 N H2SO4. Plates were read at 450 nm with THERMO Multiscan EX ELISA-reader.

4.4. SPR analysis

All experiments were conducted using a ProteOn™ XPR36 Protein Interaction Array System from Bio-Rad. All solutions and sensor chips were purchased from Bio-Rad. Citrulline containing peptides were immobilized on GLH sensor chip, with amine coupling strategy. Immobilization buffers were selected separately for each peptide, according to the isoelectric point of the peptide. Running buffer was 0.005% PBST pH 7.4.

For immobilization, sensor chip surface was activated with 1:1 mixture of 400 mM EDAC and 100mM Sulfo-NHS for 300 sec at a flow rate 30 µl/min. Immobilization buffers were selected separately for each peptide using the pH scouting procedure, using the following buffers: sodium acetate buffer (10 mmol/L, pH 4, 4.5, 5.0), phosphate buffer (10 mmol/L, pH 6, 6.5, 7, 7.5 and 8) and borate buffer (10 mmol/L, pH 9 and 9.5). Peptides were solubilized in each buffer at a final concentration of 10 µg/ml. The selected buffer and immobilized peptide quantities are reported in Table 2. Each peptide, solubilized in the previously selected immobilization buffer (10 µg/ml), was injected for 300 s at a flow rate of 30 µl/min. To deactivate free reactive sites, ethanolamine–HCl (1 mol/L, pH 8.5) was injected for 300 s at a flow rate of 30 µl/min. Reference channel was activated injecting NHS/EDC (1:1) and PBST and deactivated with ethanolamine–HCl.

Peptides that were not able to bind to the surface of the GLH chip were biotinylated and then coupled to neutravidin functionalized NLC sensor chips (Bio-Rad). After the immobilization of the biotinylated peptides (2.5 µg/ml, 30 µl/min, 300 s) the excess of neutravidin binding sites were neutralized by biocytin (Sigma) in concentration of10 µg/ml for 200 s at a flow rate of 30 µl/min.

Affinity purified anti-citrullinated peptide antibodies were diluted in running buffer to final concentrations of 2000, 1000, 500, 250, 125 mmol/L. Sera samples were used in two-fold dilutions, starting dilution was 10x in running buffer. Diluted samples were injected over each immobilized peptide for 120 s contact time at a flow rate of 50 µl/min. Dissociation was performed over a 600 s at a flow rate of 50 µl/min, and finally the chip surface was regenerated by injecting a glycine solution.
(10 mmol/L, pH 2) for 30 s, 100 µl/min and washed with running buffer at 90 µl/min flow rate for 150 s. Binding kinetics and data analysis were calculated with Bio-Rad ProteOn Manager software, Langmuir kinetic model.

The chip surface was regenerated by adding Glycine-HCl (10 mM, pH 2.5), and then PBST solution at a flow rate of 30 µl/min. The stability of the chip surface was controlled throughout the experiments.

Table 3. Characteristics of peptide immobilization on GLH chip

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Immobilization buffer</th>
<th>Immobilized peptide (RU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filaggrin (306-326)</td>
<td>sodium acetate pH 4</td>
<td>500</td>
</tr>
<tr>
<td>Vimentin (65-77)</td>
<td>borate pH 9</td>
<td>5700</td>
</tr>
<tr>
<td>Multi-epitope</td>
<td>borate pH 9.5</td>
<td>3100</td>
</tr>
</tbody>
</table>

4.5. Peptide synthesis

All peptides were synthesized by solid-phase peptide synthesis as described previously [31, 37, 42]. The N-terminus of peptides was free or acetylated. Some of the peptides (filaggrin 19, vimentin, collagen II) were C-terminally labeled and some (fibrin β and multi-epitope peptide) were N-terminally labeled by biotinyl-aminohexanoic acid [37]. The –COOH group on the C-terminus was always amidated (Table 1). Peptides were purified by semipreparative reversed-phase high-performance liquid chromatography (HPLC) and were characterized by analytical reversed-phase HPLC and electrospray ionization mass spectrometry (ESI-MS).

4.6. Preparation and characterization of bifunctional PLGA NPs

Carboxylate-functionalized PLGA NPs were prepared by the nanoprecipitation method as described [32]. The NPs were dispersed in doubly distilled water, finally containing 1.6 × 10¹¹ NPs/ml. The average diameter of NPs was 160–180 nm, each NP containing approximately 4–5000 carboxyl groups available for covalent binding of peptides. NPs were converted to PLGA-active ester derivatives with high excess N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The activated particles were covalently linked to a 1:1 (mol:mol) mixture of complement activating and targeting peptide. This strategy resulted in uniformly oriented peptides of the two types.

4.7. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from RA patients and healthy volunteers as described previously [38]. B cells were purified by negative selection using magnetic bead-activated cell sorting (MACS) according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA, USA). The purity of the resulting B-cell population was 85–98 %. B cells (10⁶/ml) were cultured with 1 µg/ml CD40L, 1 µg/ml R848 (Mabtech, Stockholm, Sweden) 10 ng/ml interleukin (IL)-2, and 50 ng/ml IL-21 (ImmunoTools GmbH) for 48 hours to increase the frequency of activated memory B cells.

4.8. Detection of IgG secreting cells by enzyme-linked immunospot assay (ELISpot)

PBMCs were cultured in RPMI-1640 containing 10 % FCS in the presence of 10 ng/ml recombinant IL-2 and 1 µg/ml R848 polyclonal activator provided with the enzyme-linked immunospot assay (ELISpot) kit (Mabtech, Stockholm, Sweden). The cells were harvested at day three, and incubated with the bifunctional PLGA NPs at 1000-fold excess for 1 hour. After removing the unbound NPs, the cells were incubated at 37 °C for 30 minutes in the presence of 1 % pooled normal human serum (NHS) as a complement source or in heat-inactivated serum. After washing, 4 ×10⁶ PBMCs were transferred into the wells of ELISpot plates (Millipore) pre-coated with the citrulline e containing multi-epitope peptide and with anti-IgG, respectively. The spots were
developed after 18 hours according to the manufacturer’s instruction (Mabtech, Stockholm, Sweden).

The frequency of Cit-containing multiepitope peptide-specific IgG and non-specific IgG producing cells was determined using a C.T.L. Immunospot analyzer (CTL-Europe GmbH, Bonn, Germany).

4.9. Statistical analysis

For the statistical analysis of data the two-tailed t test and Pearson correlation were used and the results were analyzed with GRAPHPAD PRISM 4 software (GraphPad Software, La Jolla, CA, USA)

In all tests, p < 0.05 was considered significant.

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Abbreviations

ACPA Anti-Citrullinated Protein/peptide Antibodies
Citrulline
DAS Disease Activity Score
EBNA Epstein-Barr virus Nuclear Antigen
EBV Epstein-Barr virus
IVIG Intravenous Immunoglobin
NHS Normal Human Sera
NP Nanoparticles
PBMC Peripheral Blood Mononuclear Cells
PLGA Poly (lactic-co-glycolic acid)
RA Rheumatoid arthritis
SPR Surface Plasmon Resonance

References


