Hierarchy in the Avidity and Cross-reactivity of Anti-citrullinated Protein Antibodies in the Serum of Patients With Rheumatoid Arthritis

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Abstract

Background

Fine specificity of anti-citrullinated protein antibodies (ACPAs), in which cross-reactivity exists, varies among patients with rheumatoid arthritis (RA), but it is unclear whether the mechanism of ACPA production is same or different among individuals. Since avidity of serum antibody reflects the direction of immune response, we compared the levels of avidity and cross-reactivity between various ACPAs in a cohort of RA patient.

Methods

Sera from 180 RA patients positive for anti-cyclic citrullinated peptide (CCP) 2 antibody were screened for positivity of antibodies against CCP1, and citrullnated fibrinogen (cFib), enolase (cEno), and vimentin (cVim) peptides. Avidity of the four ACPAs, and some autoantibodies and antibodies against foreign antigens was determined by an elution assay using sodium thiocyanate solution. Cross-reactivity between different ACPAs was estimated by measuring the inhibition of binding by competitor peptides.

Results

The prevalence of anti-CCP1, anti-cFib, anti-cEno, and anti-cVim antibodies in the anti-CCP2-positive RA cohort were 37.7%, 38.3%, 15.6%, and 23.9%, respectively. The avidity of ACPAs, except for anti-cVim antibody, was significantly lower than that of antibodies against foreign antigens, while there was a large variety in the avidity of other autoantibodies. At individual levels, the avidity of anti-cVim was significantly higher than that of other ACPAs, and there was a significant correlation in the avidity of anti-CCP and anti-cFib antibodies. Substantial extent of cross-reactivity was seen between different ACPAs, which also showed a fixed hierarchy.

Conclusion

The fixed hierarchy in the avidity and cross-reactivity between different ACPAs suggests that the mechanism underlying ACPA production is common to all RA patients. Presence of a dominant antigen that induces whole ACPA response is speculated.

Background

Because anti-citrullinated protein antibody (ACPA) is specifically detected in patients with rheumatoid arthritis (RA) preceding disease onset (1-3), understanding the mechanism of ACPA production is an important step for elucidating the pathogenesis of RA. ACPA includes antibodies against numerous citrullinated proteins, such as citrullinated fibrinogen, a-enolase, vimentin, as well as filaggrin, the template for the first generation cyclic citrullinated peptide (CCP1) (4). Interestingly, the number of citrullinated antigens recognized by the serum increases with time until the onset of RA, which is denoted as epitope spreading and is implicated in the disease development (5, 6). However, fine antigen specificity

varies among patients. This might reflect inter-patient variation in the repertoire of citrullinated antigens generated during pre-RA stage. Alternatively, a common antigen induces cross-reactive ACPA response with different efficiency for each citrullinated antigen, which might be influenced by genetic, environmental as well as stochastic factors, causing individual variations. In fact, at poly- and monoclonal levels, varying extent of cross-reactivity among different ACPAs has been demonstrated (7-13). Thus, it is unclear whether the mechanism of ACPA production is same or different among RA patients.

In the germinal center (GC) of lymphoid follicles, only B cells expressing BCR with binding affinity above the selection threshold can survive (14). As a result, along with the progression of immune response, avidity of the antibody to the target antigen increases, the affinity maturation. Thus, the avidity of antibody indicates the direction of immune response, and therefore measuring avidity of ACPA could be a clue to identify the initiating antigen. In fact, analysis of ACPA-producing B cell clones revealed highly mutated heavy- and light-Ig chain genes (8-12). Reverting the mutation to the germ-line sequence lost the binding to the citrullinated peptides, further indicating the importance of affinity maturation (9, 10, 13, 15). On the other hand, it has been shown that the avidity of ACPA is lower than antibodies against recall antigens (16). This might be because the immune response producing ACPA is ongoing, and newly recruited B cells are continuously involved in the antibody production. Alternatively, self-tolerance mechanisms prevent the production of high-avidity ACPA, although it is unknown whether low-avidity binding is unique to ACPA or common to autoantibodies in general.

Cross-reactivity of ACPA might be another explanation for the low avidity as well as the diverse specificity. In this regard, if a dominant antigen, which is common to all patients, induces the whole ACPA response, there should be a correlation or a fixed hierarchy in the avidity of different ACPAs in the serum. On the other hand, if each ACPA response, which cross-reacts with other citrullinated antigens to some extent, is induced by different antigen or if the antigen is different among patients, the hierarchy in the avidity of ACPAs may vary according to the maturation level of each immune response. Furthermore, in this case, avidity might be higher in the antibody with lower cross-reactivity, i.e. higher specificity.

In order to understand the mechanism of ACPA production, we compared the avidity of various ACPAs in the serum of a cohort of RA patients, based on the assumption that avidity of antibodies in the serum reflects the direction of immune response. We also examined the relationship between avidity and cross-reactivity of different ACPAs.

Methods

Patients

Two hundred and thirty six consecutive serum samples from RA patients, who fulfilled the 1987 American College of Rheumatology (ACR) classification criteria (17), were screened, and those positive for CCP2 were used in the subsequent analysis (n=180). Subjects with other autoimmune diseases (Sjogren's

syndrome, n=11; systemic lupus erythematosus (SLE), n=8; chronic thyroiditis, n=4, mixed connective tissue disease (MCTD), n= 7; CREST syndrome, n=12) were also included for the analysis. To define the cutoff levels of the antibodies, 40 healthy control subjects were recruited. Serum was isolated and stored at −20°C until use. This study complies with the Declaration of Helsinki, and the study protocol was approved by the Regional Committee of Ethics for Human Research at the Faculty of Medicine of the Kyushu University (24-174). All subjects provided written informed consent before participating in the study.

Enzyme-linked immunosorbent assay(ELISA)

To detect antibodies specifically bound to CCP1, citrullinated fibrinogen (cFib), citrullinated a-enolase (cEno), and citrullinated vimentin (cVim) peptides, each pairs of citrullinated and control non-citrullinated peptides were used (Supplementary Table 1). All peptides were produced through amino acid synthesis and conjugated with biotin at the C-terminal. Ninety-six well avidin-coated plates (Avidin Plate, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were incubated with the peptides (2 mg/mL) overnight at 4°C, and after blocking non-specific binding by an incubation with Blocking One solution (Nacalai Tesque, Kyoto, Japan), the plates were incubated with the serum diluted at 1:30 by LowCross-Buffer (CANDOR Bioscience GmbH, Wangen, Germany), for 2 h at 37°C. Peroxidase-conjugated $F(ab)_2$ goat anti-human IgG (Rockland Immunochemicals, Limerick, PA, USA) was used for the detection. Antibody binding was visualized using tetramethylbenzidine substrate solution (Interchim, Montluçon, France), and the absorbance was measured at 450 nm. The antibody level was expressed as DOD between citrullinated and native peptides. Sera with DOD higher than the mean plus 2SD of the healthy control sera were considered positive. To measure the levels of IgG against CCP2, we used a commercially available ELISA kit (Euro-Diagnostica, Malmö, Sweden). For the detection of anti-influenza, and anti-diphtheria toxin antibodies, 96-well plates were coated with 1 µg/ml of A/H1N1 subunit (Bio-Rad, Hercules, CA, USA) and diphtheria toxin (List Biological Laboratories, Campbell, CA, USA), respectively. We utilized precoated commercial kits to measure the avidity of anti-SS-A/Ro, anti-dsDNA, anti-centromere, and anti-U1-RNP antibodies (all from IBL INTERNATIONAL, Hamburg, Germany).

Measuring avidity of antibodies

Avidity of antibodies was determined by an elution assay using sodium thiocyanate (NaSCN) according to a previous report by Suwannalai et al. (16) with minor modifications. Serum was serially diluted (1:25, 1:50, 1:100, 1:200, 1:400) with Low cross buffer (CANDOR Bioscience GmbH) to define the dilution at which the antibody response was in the linear part of the curve. Sera with OD value of native peptide higher than 0.5 were excluded from the analysis in order to ensure data quality. Antigen-coated plates

were incubated with the diluted serum for 2 h at 37°C. After washing, the plate wells were incubated with NaSCN at the concentration of 0, 0.125, 0.25, 0.5, 1.0, 2.0 or 4.0 M for 15 min at 37°C. The plates were washed, and remaining bound antibodies were detected using peroxidase-conjugated goat anti-human IgG. We calculated the avidity index (AI) at each concentration of NaSCN as follows:

(OD value of serum treated with given M of NaSCN − OD value of serum treated with 4M of NaSCN) / (OD value of serum treated with 0 M of NaSCN − OD value of serum treated with 4 M of NaSCN) × 100.

The strength of avidity is depicted as $AI_{AUC,}$ the area under the curve of AI at seven reference points, in order to minimize the bias in the avidity of antibodies with different concentrations (dilutions). AI_{AUC} was calculated according to Perciani et al. with minor modifications (Supplementary Figure 1) (18).

Inhibition assay

The extent of cross-reactivity between different ACPAs was examined by a peptide inhibition assay as reported previously with minor modification (7). In brief, serum in appropriate dilution at which the antibody response was in the linear part of the curve as defined above, were incubated with 100 mg/ml of competitor peptide (non-biotinylated peptide) overnight at 4°C with continuous mixing. Then the serum samples were incubated in the plates precoated with the biotinylated test peptides, and subsequent ELISA steps were performed as described above. Only sera with OD value lower than 0.5 for the native peptides and those with DOD value larger than 0.2 were analyzed. The inhibition rate of antibody binding was calculated as follows:

(OD value of untreated serum − OD value of serum pretreated with competitor peptide / OD value of untreated serum) × 100.

Statistics

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (19), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). Difference between groups was analyzed by Steel-Dwass test, and correlation was determined by Spearman's correlation coefficient. P values less than 0.05 were considered significant.

Results

Prevalence of various ACPAs in a cohort of Japanese RA patients

We first examined the prevalence of various ACPAs in a cohort of Japanese RA patients who were positive for anti-CCP2 antibody (n=180). Baseline characteristics of these patients are shown in Table 1. To exclude false positive samples from the analysis, we expressed the antibody levels after subtracting the values of non-citrullinated peptides. The prevalence of anti-CCP1, cFib, cEno, and cVim antibodies were 37.7%, 38.3%, 15.6%, and 23.9%, respectively (Figure 1A). Sixty one percent (n=110) of the serum samples reacted at least one peptide, in which 44.5% (n=49) reacted to single peptide only, while 7.3% (n=8) reacted to all four peptides (Table 2). There is a positive trend between the number and the level of each ACPA, as reported previously (Figure 1B)(20). However, there was no correlation in the levels between different ACPAs in any combination (Figure 1C).

Characteristic	n=180
Age,years±SD	64±13.7
Female, n(%)	166(92.2)
Disease duration(years)	18.4±12.3
RA stage I/II/III/IV (%)	13/29/6/52
Functional class I/II/III/IV (%)	12/82/6/0
Bio DMARDs use, n(%)	55(30.6)
Glucocorticoids use, n(%)	81(45)
MTX use, n(%)	140(77.8)
CRP mg/dl±SD	0.29±0.8
RF-IgM, n(%)	157(87.2)

Table. 1 Baseline characteristics

number of recognized peptide	CCP1	cFIb	cEno	cVim	n	%
1					**49**	**44.5**
	CCP1				20	18.1
		cFib			14	12.7
			cEno		7	6.4
				cVim	8	7.3
2					**34**	**30.9**
	CCP1	cFib			17	15.5
	CCP1		cEno		2	1.8
	CCP1			cVim	3	2.7
		cFib	cEno		4	3.6
		cFIb		cVim	6	5.5
			cEno	cVim	2	1.8
3					**19**	**17.3**
	CCP1	cFIb	cEno		4	3.6
	CCP1	cFIb		cVim	14	12.7
	CCP1		cEno	cVim	0	0
		cFIb	cEno	cVim	1	0.9
4					**8**	**7.3**
	CCP1	cFIb	cEno	cVim	8	7.3
				Total	110	

Table. 2 Prevalence of various ACPAs

Avidity of different ACPAs and other autoantibodies

We next measured the avidity of serum antibodies to the four citrullinated peptides by an elution assay using gradients of NaSCN. In order to minimize the bias in comparing the avidity of antibodies with different concentrations (dilutions), the values are expressed as AI_{AUC} (Supplementary Figure 1). In addition to ACPA, we also measured avidity of some autoantibodies, including anti-dsDNA, anti-SS-A,

anti-U1-RNP, and anti-TPO, and antibodies against foreign antigens, including, influenza and diphtheria toxin (Figure 2). We found that the avidity of ACPA, except for anti-cVim antibody, was significantly lower than that of antibodies against foreign antigens. There was no difference in the avidity level between anti-CCP, anti-cFib, and anti-cEno antibodies. Among the autoantibodies examined, anti-dsDNA antibody showed the lowest avidity, even lower than any ACPA. Anti-TPO and anti-centromere antibodies, on the contrary, tended to show higher avidity than antibody against foreign antigens. Thus, low avidity is not a general feature of autoantibodies but might be unique to ACPA, in which anti-cVim antibody showed higher avidity than others.

The relationship in the avidity of different ACPAs

We analyzed the relationship in the avidity of different ACPAs at an individual level using serum samples reactive to more than two citrullinated antigens. Although, no correlation was detected in the levels (Figure 1C), there was a significant correlation in the avidity between anti-CCP1 and anti-cFib antibody (Figure 3). A trend of correlation was observed in the avidity between anti-CCP1 and anti-cEno or anti-cVim antibodies, but it was not statistically significant. Furthermore, we confirmed that the avidity of anti-cVim antibody was higher than anti-CCP1, cFib, and cEno antibodies at individual levels. Thus, there is a correlation or fixed hierarchy in the avidity of different ACPAs. We observed no correlation or fixed hierarchy in the avidity of different antibodies against recall antigens (Supplementary Figure 2).

The extent of cross-reactivity between different ACPAs

We lastly examined the relationship between the levels of avidity and cross-reactivity. The extent of cross-reactivity was examined by an inhibition assay, in which reduction rate of binding to an antigen by preincubation with another antigen was measured. Although there was a substantial variation in the extent of cross-reactivity, it exceeded 50% in most cases (Figure 4). Interestingly, there was a clear hierarchy in the cross-reactivity in some combinations of peptides. Thus, preincubation with CCP1, cFib or cEno inhibited binding to cVim more efficiently than the opposite, indicating anti-cVim antibody is more cross-reactive than the others. Similarly, serum binding to CCP1 was inhibited by preincubation with cEno more efficiently than did the preincubation with CCP1 to cEno.

Discussion

In the present study, we measured the avidity of various ACPAs in the serum of RA patients in order to understand the mechanism of ACPA production, because avidity of antibody is supposed to reflect the

direction of antigen specificity. Importantly, avidity can be compared among patients with varying antibody levels and is not likely reduced by therapeutic intervention once antibody is produced.

In line with the results of previous reports (16), we observed that the avidity of ACPAs, except for anti-cVim antibody, in a Japanese cohort of RA patients was lower than that of antibodies against recall antigens. This is not likely because those ACPAs are autoantibodies, as we found some autoantibodies even higher avidity than the antibodies against recall antigens. Another explanation is that ACPA immune response is ongoing and that a large portion of serum ACPA is produced by B cells that have not undergone extensive affinity maturation, which may not circulate in the peripheral blood. This can explain the discrepancy with the previous report showing high levels of SHM in peripheral blood B cells (8-12). However, reverting mutation of ACPA genes of those B cells to the germline sequence lost the binding to the citrullinated peptides (9, 10, 13, 15), arguing against this possibility. Recently, an alternative hypothesis has been raised. Most ACPA has N-glycosylation sites in the Fab portion, which are generated via somatic hypermutation and are assumed to provide survival advantage in the GC (21). Hence the threshold of binding affinity to the target antigen could be lowered. Although the mechanism why Fab glycosylation sites are preferentially induced in ACPA is unclear, it indicates that recognition of citrulline residue, albeit at low avidity, is independently involved in the selection of GC B cells.

Although numerous citrullinated proteins/peptides have been demonstrated to be the target of ACPA, it is unlikely that all these antigens induce the corresponding specific antibodies. Instead, cross-reactivity generates the diversity of ACPA to a substantial extent, which might also be involved in the low avidity of ACPA. A question is whether the ACPA-inducing antigens are same or different among patients. The variety in ACPA fine specificity fits better with the latter hypothesis. Sequential appearance of different antigens might be the mechanism of the epitope spreading. In this case, hierarchy in the avidity of different ACPAs might vary among patients. However, our data actually showed a fixed hierarchy or positive correlation in the avidity of different ACPAs. It is thus hypothesized that a dominant and common antigen induces the whole ACPA response, in which cross-reactivity between different antibody clones may also exist. In support of this, an amino acid motif critical for ACPA binding has been identified (22). Crystal structure of monoclonal ACPAs demonstrated the conformation that binds different citrullinated peptides (23). Taken together these speculate that the avidity of ACPA is primarily determined by its binding strength to the core-epitope.

As reported previously (6, 7), we observed cross-reactivity between different ACPAs. We further found that anti-cVim antibody is more cross-reactive than the other three ACPAs, indicating that the high avidity of anti-cVim antibody is not owing to low levels of cross-reactivity, i.e. high specificity. Interestingly, it was reported that affinity maturation of ACPA increased cross-reactivity, rather than shaping up the specificity (13). However, it is premature to conclude that cVim is the dominant target antigen inducing whole ACPA response, because anti-cVim reactivity was not always detected in the serum, and, the cVim peptide used in the experiments is not a natural product. Further studies comparing the avidity of antibodies against a variety of natural antigens might identify such dominant antigen.

Our results might not be as clear-cut as those from studies analyzing monoclonal ACPAs, because whole serum contains polyclonal antibodies with different avidity in different amount. However an advantage of using serum samples is that it reflects the whole picture of in vivo immune response and avoids the risk of missing any antibody clone. Thus, it is important to integrate the notion obtained from studies using monoclonal antibodies and polyclonal serum. Another limitation of our study is the small number of citrullinated peptides used for the analysis. In addition, increasing the number of patient samples could more clearly demonstrate the statistical relationship.

In conclusion, we found in this study that there is a fixed hierarchy in the avidity and cross-reactivity of different ACPAs despite large variations in the specificity and levels. This emphasizes the importance of low-affinity cross-reactive binding in the generation of diverse antigen specificity of ACPA and suggests the presence of dominant antigen that induces ACPA production. Interestingly, cross-reactivity of ACPA to carbamylated or acetylated peptides has also been revealed (12, 24, 25), raising a possibility that, not only ACPA, but all these RA-related antibodies are generated by the common mechanism.

Conclusions

We detected fixed hierarchy in the avidity and cross-reactivity between different ACPAs in the serum of a cohort of RA patients, suggesting that the mechanism underlying ACPA production is common to RA patients. Presence of a dominant antigen that induces whole ACPA response is speculated.

Abbreviations

1. ACPA: Anti-citrullinated protein antibody

2. RA: rheumatoid arthritis; CCP1

3. cyclic citrullinated peptide 1

4. GC: germinal center; ACR

5. American College of Rheumatology

6. CCP2: cyclic citrullinated peptide 2

7. SLE: systemic lupus erythematosus

8. MCTD: mixed connective tissue disease

9. ELISA: enzyme-linked immunosorbent assay

10. cFib: citrullinated fibrinogen-β

11. cEno: citrullinated α-enolase

12. cVim: citrullinated vimentin

13. OD: Optical Density

Declarations

Ethics approval and consent to participate:

This study complies with the Declaration of Helsinki, and the study protocol was approved by the Regional Committee of Ethics for Human Research at the Faculty of Medicine of the Kyushu University (24-174). All subjects provided written informed consent before participating in the study.

Consent for publication:

Not applicable.

Availability of data and materials:

All data generated or anayzed during this study are included in this published article and its supplementary information files.

Competing interests:

The authors declare that they have no competing interests.

Funding:

Authors' contributions:

AH and HY participated in the study design. TT and MK participated in the collection of serum samples. AH, TS and HY performed laboratory assays and led the interpretation of the study results with assistance from the remaining authors. HY and YN critically revised the manuscript and provided final approval. All authors read and approved the final manuscript.

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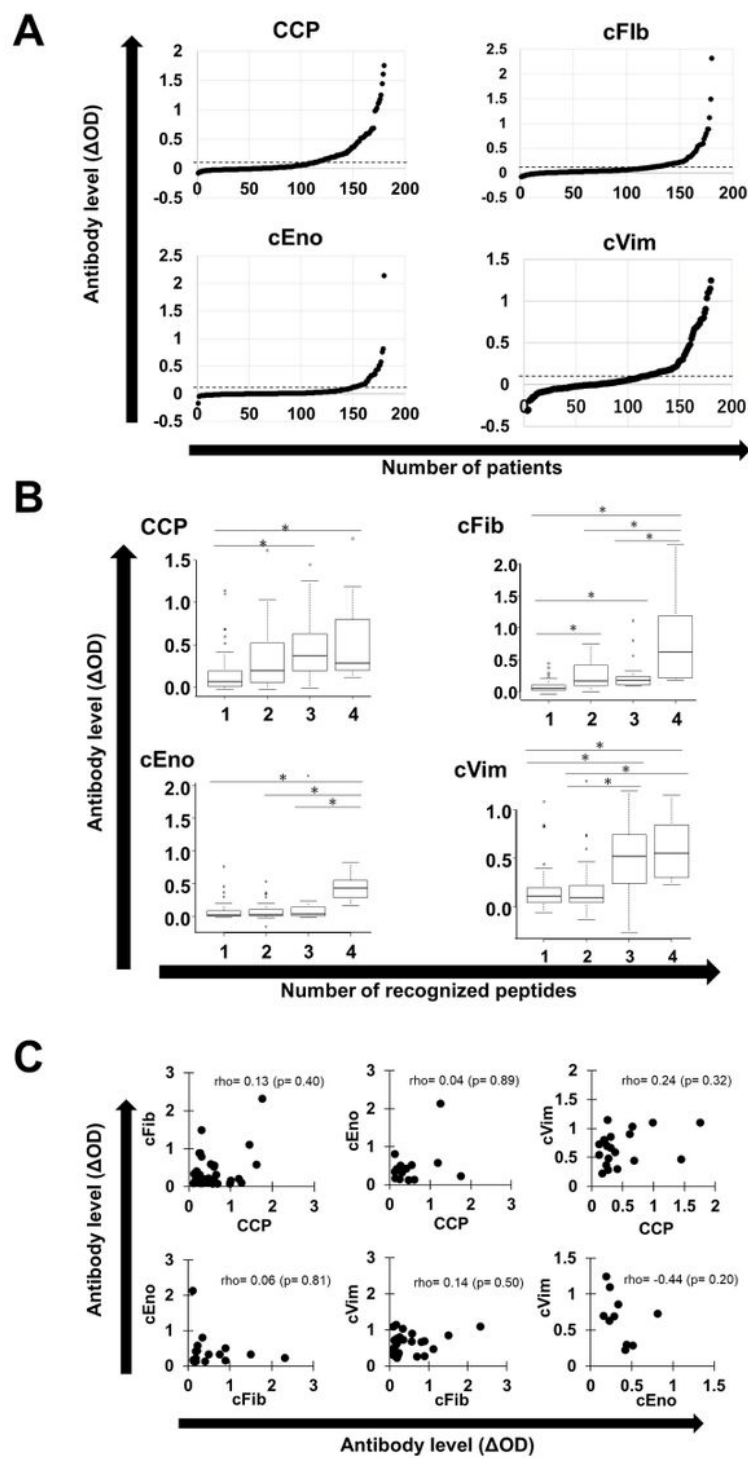
Figures

Fig. 1

Figure 1

Presence of different ACPAs in a cohort of Japanese RA patients. A, The levels of anti-CCP, cFib, cEno, and cVim antibodies, which are expressed as ⬛OD values, are shown in probability plots. Dotted lines show the cut-off level. B, The relationship between the number of ACPA-positivity and the mean level of each ACPA is shown. C, The relationship between the levels of different ACPAs is shown. *p<0.05.

Fig.2
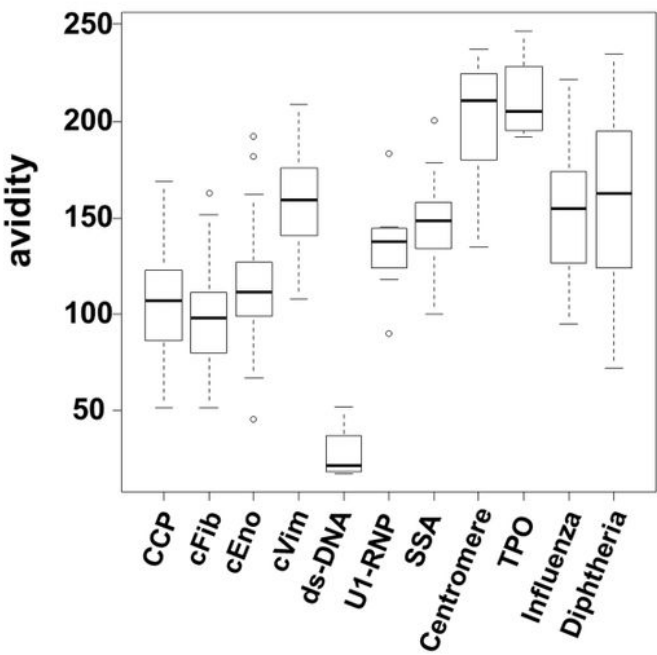
	CCP	cFib	cEno	MCV	ds-DNA	U1-RNP	SSA	Centromere	TPO	Influenza	Diphtheria
CCP				***	***		**	***	**	***	***
cFib			*	***	***		**	***	*	***	***
cEno				***	***			*	*	**	*
MCV					***				*		
ds-DNA						*	*	*		***	***
U1-RNP											
SSA											
Centromere										*	
TPO											
Diphtheria											
Influenza											

*=p<0.05   **=p<0.01   ***=p=0.001

Figure 2

Comparison of avidity of various antibodies in the serum. A, Avidity of various antibodies in the serum was measured by an elution assay using NaSCN solution and is expressed as AIAUC (see Materials and Methods). B, Statistical differences between the avidity of different antibodies are shown.
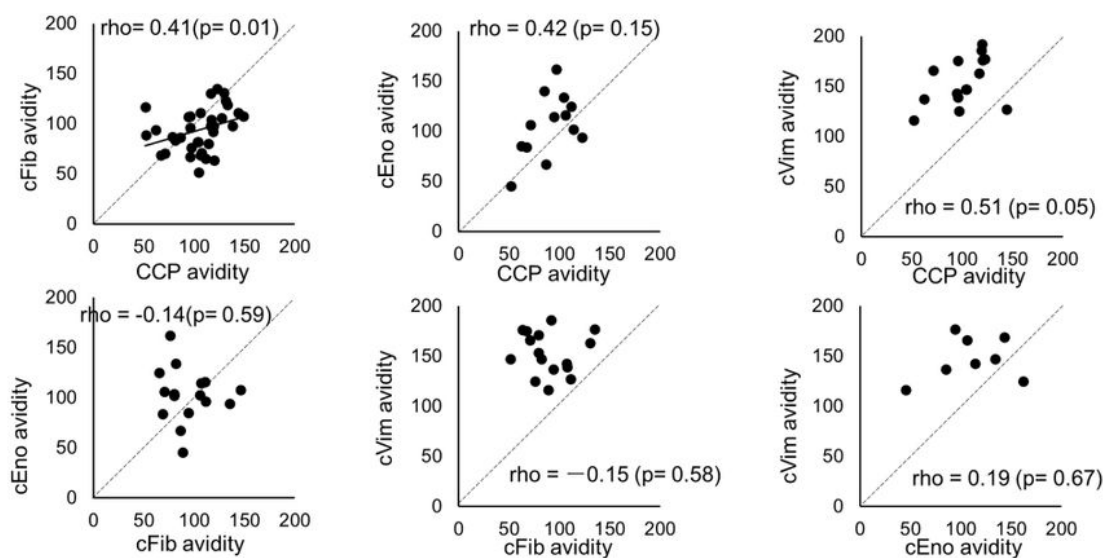
Fig. 3

Figure 3

Correlation and hierarchy in the avidity of different ACPAs. The relationship between the avidity (AIAUC) of different ACPAs in serum samples reactive to more than two citrullinated antigens are analyzed.

Dashed diagonal lines indicate the points of equal value, and solid lines indicate the correlation curve.
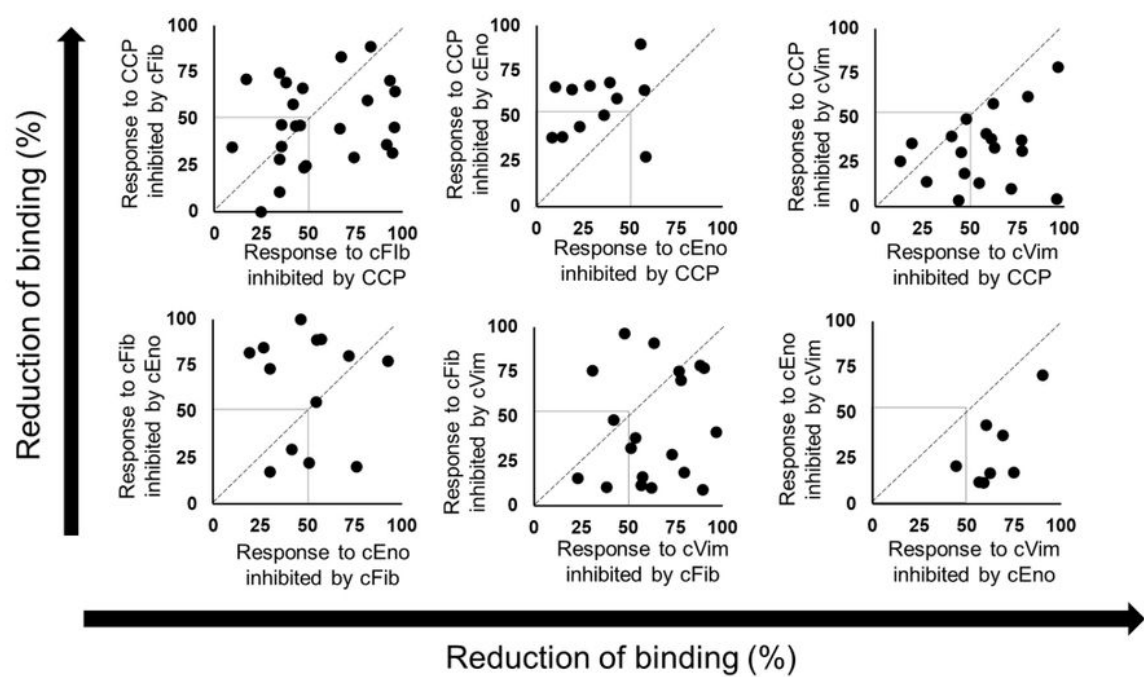
Fig.4

Figure 4

The extent of cross-reactivity between different ACPAs. Cross-reactivity between different ACPAs was estimated by measuring reduction rate of peptide binding by preincubation with inhibitor peptide. Combinations of inhibitor and test peptides are indicated in the panels. Dashed diagonal lines indicate the points of equal value.

Supplementary Files

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- renamed0ec16.TIF
- renameda7f20.TIF
- renamedb4c9a.TIF