IgG anti-hinge antibody against IgG4 F(ab')2 generated by not matrix metalloproteinase-3 but pepsin is a useful diagnostic marker for rheumatoid arthritis, even seronegative rheumatoid arthritis.

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Abstract

Background The pepsin agglutinator, discovered over 50 years ago, has recently referred to be an anti-hinge antibody (AHA) because of main reacting with the IgG hinge epitope. AHA shows different reactivity for each hinge epitope generated by each protease that cleaves the hinge region at different sites. Moreover, AHA shows different reactivity against different hinge epitopes derived from each IgG subclass even when the same protease is used. Since production of matrix metalloproteinase-3 (MMP-3) is enhanced in rheumatoid arthritis (RA), the specific AHA might be increasingly produced. The purpose of this study is to determine whether AHA against IgG hinge epitopes produced by MMP-3 is specifically elevated in RA.

Methods Serum IgG or IgA class of AHA against the IgG1- or IgG4 F(ab') 2 generated by either matrix metalloproteinase 3 (MMP-3) or pepsin was measured by ELISA in 81 healthy controls (HC) and 111 patients with RA. Receiver operating characteristic (ROC) analysis was used for obtaining optimal cutoff values and cutoff values indicating high specificity (>95%) of the AHA. Targeted epitope of the AHA was investigated by inhibition ELISA.

Results Seven AHAs were statistically higher in RA than HC, except IgG AHA against IgG1 F(ab') 2 generated by proteolytic cleavage of MMP-3. The areas under the curve of ROC curve were 0.66-0.80, although the sensitivities at high specificity were low (5.4-24.3%). The cumulative number of positive AHA in each individual was statistically higher in RA than HC, suggesting the extreme extent of AHA repertoires in RA. Inhibition studies revealed that IgG AHA against IgG4 F(ab') 2 generated by pepsin cross-reacted with IgG1 F(ab') 2 generated by pepsin. Although development of IgA AHAs in RA seems to be remarkable and specific, multivariate logistic regression analysis identified IgG AHA against IgG4 F(ab') 2 generated by pepsin as an independent variable for RA diagnosis, even in RA patients who were negative for both RF and ACPA [odds ratio 1.18 (95% confidence interval 1.06-1.32); P =0.003].

Conclusion In RA, we observed diversification and amplification of AHA repertoires and diagnostic utility of the specific AHA against IgG4 F(ab') 2 generated by pepsin, but not MMP-3.

Background

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by persistent synovitis, destruction of bone and cartilage in multiple joints, and disability [1]. RA is also a systemic autoimmune disease representing autoantibodies such as rheumatoid factor (RF) being autoantibodies to IgG Fc, anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein antibodies [2]. Although RF is the leading anti-IgG antibodies in RA, antibodies to IgG hinge epitopes have been also interested [2].

Multiple proteases cleave IgG at the lower hinge region and produce $F(ab')_2$ fragments [3, 4]. Antibodies to the IgG $F(ab')_2$, once designated as pepsin agglutinator, have been detected not only in patients with autoimmune diseases, but also healthy subjects [5, 6]. Lately they have been called anti-hinge antibodies (AHA), since the majority of the antibodies bind strongly to IgG $F(ab')_2$ and not at all to the whole intact

IgG [7]. Furthermore, AHA usually targets a C terminus of amino-acid sequence in lower hinge region [8, 9], implying it reacts specifically to hinge neoepitope but not other epitopes existing in the IgG $F(ab')_2$.

AHA reacting with human IgG1 or IgG4 hinge epitopes that appeared after cleavage with pepsin showed higher positivity rate and titer in RA patients than in healthy controls (HC) [10]. However, AHA against IgG1 or IgG4 $F(ab')_2$ generated by other protease such as IgG-degrading enzyme of Streptococcus pyogens (IdeS) did not show the difference, suggesting a certain AHA may be associated with a specific disorder.

Matrix metalloproteinase-3 (MMP-3) is produced in synovial cells and has been pointed out the relation to RA disease activity [11, 12]. It seems likely in RA to result in excessive production of IgG $F(ab')_2$ fragment by MMP-3 followed by overproduction of the specific AHA, since activated MMP-3 generates human IgG1 $F(ab')_2$ fragment in vitro and in vivo [3, 13].

The purpose of the present study is to measure IgG or IgA AHA against IgG1, IgG2, and IgG4 monoclonal therapeutic biologics cleaved by MMP-3 or pepsin, and to evaluate their characteristics in RA. Clinical study of serum AHA against IgG1- and IgG4 $F(ab')_2$ fragment generated by MMP-3 seems to be the first time to our knowledge.

Methods

RA patients and healthy controls (HC)

In this cross-sectional and case-control study, serum samples were collected from 111 patients with RA who met the ACR classification criteria [14] and from 81 HC. All of the patient sera were leftovers and 5 sera were shortage for testing IgA class of anti-hinge antibodies. All the samples were stored at -80°C until use.

Characterizations of the RA patients and the HC were shown in Table 1. The RA was significantly older than the HC group, although no gender difference between the two groups. Many RA patients had long disease duration and treated with biologics. Positive for rheumatoid factor and anti-CCP2 antibodies due to routine laboratory examination were 67/111 (60.4%) and 77/111 (69.4%), respectively.

Proteolytic cleavage for generating $F(ab')_2$ fragments

The following biologics were used: tocilizumab (TCZ;IgG1), infliximab (IFX;IgG1), panitumumab (PAN;IgG2) and natalizumab (NTZ;IgG4). All the biologics except IFX were humanized monoclonal IgG. Although IFX is chimeric, the hinge region and CH2 domain were derived from human IgG1 according to the database from the national center for biotechnology information (NCBI, USA). We used pepsin

(Sigma-Aldrich, USA) and human MMP-3 as proteases to cleave the biologics. The pro-MMP-3 was a kind gift from Daiichi-Fine Chemical, Toyama, Japan.

The biologics were dialyzed in the 0.1 M sodium citrate buffer (SCB, pH3.5), and then 100 μg of pepsin (Sigma-Aldrich, USA) in SCB was incubated with 10 mg of TCZ or NTZ overnight and of PAN for 2 hours. For stopping the digestion, 1M Tris was added to the IgG solution until the pH increased to 7.4. Proteolysis by MMP-3 was performed after activation of pro-MMP-3 by incubation at 55°C for 25 min [15]. The activated MMP-3, 50μg, in 50mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 10 mM $CaCl_2$ was mixed with 5mg of each biologic at 37°C. After 2 hours incubation, each small amount of the reaction mixture (20 μL) was removed and the reaction was stopped by rapid freezing. The remaining reaction mixture was continued for 24 hours and stopped by adjustment to 20 mM ethylenediaminetetraacetic acid (EDTA).

Detection of human IgG fragments

Cleaved human IgG fragments were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer using 10% gels under non-reducing conditions. Samples were heated at 100°C for 2 min in 50 mM Tris-HCl buffer (pH 6.8) with final concentration of 20% glycerin and 1% (w/v) SDS. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (0.25% (w/v); Nacalai Tesque, Kyoto, Japan) in 50% (v/v) methanol and 10% (v/v) acetic acid.

Purification of IgG $F(ab')_2$

At the outset, human IgG digested by pepsin or MMP-3 was separated by gel filtration on Sephadex G-150 (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Estimated IgG $F(ab')_2$ fractions were concentrated by Vivaspin 20 (Sartorius Stedium, Goettingen, Germany). Finally to remove IgG possessing Fc, the concentrated crude IgG $F(ab')_2$ were applied to a Protein G Mag Sepharose (GE Healthcare, Uppsala, Sweden) in 50 mM Tris, 150 mM NaCl pH 7.5. Purification of the IgG $F(ab')_2$ was confirmed by the SDS-PAGE described above. Each purified $F(ab')_2$ fragment was denoted by addition of an italic subscript meaning the protease responsible for the cleavage (e.g. IgG1 $F(ab')_{2MMP\text{-}3}$).

Measurement of AHA by enzyme linked immunosorbent assay (ELISA)

ELISA plates (Sumilon, Sumitomo-Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 μL/well of IgG $F(ab')_2$ of 0.5 μg/ml in 0.1 M carbonate/bicarbonate buffer, pH 9.6. After washing with 10mM Tris buffer containing 0.9% NaCl, pH 7.4,with 0.05% Tween-20 (TBST), serum samples diluted at 1:200 in the TBST were added to the plate (100 μL/well). Plates were incubated for 2 hours at room

temperature (RT). After washing, 100 µL/well of alkaline phosphatase (ALP)-conjugated anti-human IgG Fc (Sigma-Aldrich) diluted 1:10,000 with the TBST, or ALP-conjugated anti-human IgA (Sigma-Aldrich) diluted 1:10,000 with the TBST was added and incubated for 1 hour at RT. After washing, the AHA was visualized with 100 µL/well at 1mg/mL of nitrophenyl phosphate tablets (Sigma-Aldrich) in diethanolamine buffer, pH 9.8, for 30 minutes except 2 hours for IgA AHA measurement. Absorbance was measured at 405 nm using a microplate reader.

Levels of IgG AHA were calculated by a calibration curve using pooled human IgG purified by 40% ammonium sulfate and DEAE sephadex. We arbitrarily defined 1 mg/mL of the pooled IgG as containing 800 arbitrary units (AU)/mL of IgG AHA to IgG $F(ab')_{2pepsin}$. We also used this calibration curve for measuring IgG or IgA AHA to other IgG $F(ab')_2$ fragments.

Inhibition study for specificities of IgG AHA against IgG1- or IgG4 $F(ab')_{2pepsin}$

Same volume of various concentrations of inhibitors (5,000, 1,000, 200, 40, 8, 1.6, 0.32, 0 µg/mL) and 1:200 diluted IgG AHA positive serum from RA patient was mixed well in the tube, followed by incubation for 2h at RT. The mixtures were added to ELISA plate (100 µL/well) coated with IgG1- or IgG4 $F(ab')_{2pepsin}$, and then allowed to react for 2h at RT. The subsequent procedure was the same as the measurement of AHA described above. The extents of inhibition by inhibitors were expressed as percent inhibition of the AHA responses, calculated as follows:

Percent inhibition

=1-(absorbance in the presence of inhibitors/absorbance in the absence of inhibitors) x 100

Statistical analysis

We used the Mann-Whitney U test and the Kruskal-Wallis test to compare the difference between 2 groups and among multiple groups, respectively. We also used Fisher's exact χ^2 test for nominal characteristic. To elucidate the independent variables associated with RA diagnosis, univariate logistic regression followed by multivariate logistic regression analysis was performed. Age and gender were forcedly entered into the model. Two-tailed *P* value <0.05 was considered significant. All of the data were analyzed on a personal computer using SPSS version 19 (IBM Japan, Tokyo, Japan) and StatFlex version 6 (Osaka, Japan).

Results

Cleavage of the biologics by MMP-3

Monoclonal IgG1 (TCZ, IFX), IgG2 (PAN) and IgG4 (NTZ) were cleaved by MMP-3 and the products were analyzed using non-reducing SDS-PAGE (Additional file 1: Figure S1). To easily understand the generated human IgG1 fragments, their schematic diagrams were shown in Additional file 1: Figure S2. We finally could obtained the purified IgG1 $F(ab')_{2MMP\text{-}3}$ and IgG4 $F(ab')_{2MMP\text{-}3}$.

Different levels of serum AHA against various IgG $F(ab')_2$

Levels of serum AHA against the 4 different IgG $F(ab')_2$ excluding AHA2 (IgG anti-TCZ IgG1$F(ab')_{2MMP\text{-}3}$) were significantly higher in RA (n=111 or 106) than HC (n=81) (Figure 1). Median values of IgG anti-IgG1 $F(ab')_2$ (AHA1, AHA2) were about 8-10 times higher than those of IgG anti-IgG4 $F(ab')_2$ (AHA3, AHA4). Furthermore, when used same $F(ab')_2$ as antigen, median value of IgG AHA were 2-6 times higher than those of IgA AHA (e.g. AHA1 vs AHA5). Among IgG AHAs, AHA3 possessed highest discriminative power (P=3.52x10^{-11}). In general, all of IgA AHAs (AHA5-AHA8) seemed to be superior to IgG AHAs as to discriminating between RA and HC. Next, AHA levels in RA patients were compared after stratification according to positive/negative for RF and anti-CCP2, namely double positive RA (DPRA), double negative RA (DNRA), RA with single positive RF (SPRA (RF)) and RA with single positive anti-CCP2 (SPRA (CCP)). As shown in Figure 2, the Kruskal-Wallis test revealed significant difference of AHA levels among the four groups in AHA1, AHA2 and AHA6. Moreover, the Mann-Whitney U test was used to compare the difference between the groups and the AHA levels of the DNRA in AHA, AHA2 and AHA6 were significantly lower than those of the DPRA (AHA1; P=0.004, AHA2; P=0.003, AHA6; P=0.03).

Specificity of IgG AHA responses against IgG1- or IgG4 $F(ab')_{2pepsin}$

To elucidate whether IgG AHA responses against IgG1- or IgG4 $F(ab')_{2pepsin}$ possess specificity for epitopes on each IgG subclass $F(ab')_{2pepsin}$, inhibition studies were implemented. Response of serum AHA from a patient with RA (S-47) against IgG1 TCZ $F(ab')_{2pepsin}$ was inhibited by neither IgG2- nor IgG4 $F(ab')_{2pepsin}$, but inhibited dose-dependently by both of IgG1 $F(ab')_{2pepsin}$ (Figure 3A). Meanwhile in another RA patient (S-212), AHA response against IgG4 $F(ab')_{2pepsin}$ was inhibited by not only IgG4 $F(ab')_{2pepsin}$, but IgG1 $F(ab')_{2pepsin}$ to a certain extent (Figure 3B). These results indicate possibilities that IgG AHAs against IgG1 $F(ab')_{2pepsin}$ specifically react with IgG1 $F(ab')_{2pepsin}$, but those against IgG4 $F(ab')_{2pepsin}$ cross-react with IgG1 $F(ab')_{2pepsin}$. To further ascertain the possible properties, percent inhibitions at definite concentration of inhibitors (1,000 μg/mL) were calculated in sera from 9 RA patients. IgG AHAs against IgG1 $F(ab')_{2pepsin}$ exhibited a predisposition to have specific reaction to pepsin-digested IgG1 hinge neoepitopes (Figure 3C). IgG1 $F(ab')_{2pepsin}$, however, inhibited the all reactions of five IgG AHAs against IgG4 $F(ab')_{2pepsin}$ to the same extent as IgG4 $F(ab')_{2pepsin}$ (Figure 3D). These

observations indicate that AHAs against pepsin-digested IgG4 show a tendency to cross-react with IgG1 $F(ab')_{2pepsin}$, meanwhile specificities of AHAs against IgG1 $F(ab')_{2\ pepsin}$ are mostly restricted to IgG1 $F(ab')_{2pepsin}$.

Clinical utility of AHA as laboratory test for RA

We sought clinical utility of AHA in RA by ROC curve analysis. The mean values (SE) of area under the curve were from 0.57 (0.04) of AHA2 to 0.80 (0.03) of AHA6 and AHA7 (Table 2). After calculated optimal cutoff values of each AHA by the Youden's index, we obtained sensitivity/specificity, positive predictive value (PPV+)/negative predictive value (PPV−), positive likelihood ratio (LR+) and diagnostic odds ratio (DOR) as shown in condition 1 of Table 2. The LR+ values were 1.79 (95% CI 1.23-2.62) of AHA1 to 3.44 (95% CI 2.69-4.11) of AHA3, leading to only a small shift of pretest to posttest probability. The DOR of AHA6 and AHA7 were relatively high, 10.24 (95% CI 5.40-19.42) and 11.35 (95% CI 5.65-22.79), respectively. Next, same statistical indices were calculated at high specificity conditions (96-98%) for RA as shown in condition 2 of Table 2. Increment of the LR+ values except AHA2 and AHA4 resulted in generating moderate shifts in pretest to posttest probability, although the values of sensitivity were as low as 2.7% of AHA4 to 24.3% of AHA3. Changes of the DOR values were also observed such as 7.22 to 12.70 of AHA3 and 11.35 to 4.57 of AHA7.

An expansion and diversification of the AHA response in RA

We looked into how many positive AHAs were obtained within each HC or RA patient (Figure 4). The AHA exceeding each optimal cutoff value was accepted as positive, and the cumulative number of positive AHA in each HC and RA patient was counted. The IgG and IgA AHAs were classified into 5 grades (0-4) according to the cumulative number. The grade of IgG AHAs in RA showed a tendency to be higher than that in HC (Figure 4A). Furthermore, the highest grade (cumulative number of recognized epitopes: 4) of IgA AHAs was over 50% in RA, indicating extremely extensive recognition profile (Figure 4B). These results suggest AHA repertoire in RA is expanded and diversified.

Potential of AHA as an independent diagnostic marker for RA

To further assess the potential ability of AHA in diagnostic performance of RA, we accomplished logistic regression analysis. Univariate analysis using 106 patients with RA and 81 HC showed that AHAs except AHA2 were proper to be selected for multivariate analysis as shown in analysis 1 of Table 3. Multivariate logistic regression analysis revealed that AHA3, IgG AHA against NTZ IgG4 $F(ab')_{2pepsin}$, and weakly AHA5, IgA AHA against TCZ IgG1 $F(ab')_{2pepsin}$, were selected as an independent variable contributing to RA diagnosis. Additionally, logistic regression analysis using AHAs of patients with DNRA

(n=18) revealed that only AHA3 was once more selected as an independent variable [odds ratio 1.18 (95% CI 1.06-1.32); *P*=0.003] as shown in analysis 2 of Table 3.

Discussion

There has been conflicting for a long time as concerning the specificity of anti-F(ab')$_2$ antibodies in human sera [5, 16, 17]. Nowadays, studies using human monoclonal IgG1 F(ab')$_2$ and synthetic peptide analogues of IgG1 hinge region revealed that most of anti-IgG F(ab')$_2$ antibodies targeted the lower hinge epitopes, but neither idiotopes nor other epitopes in the IgG F(ab')$_2$ fragment [9, 10, 18].

Serum AHA exists in healthy persons and in patients with a variety of diseases [19-23]. Notably, higher positive incidence and level of serum AHA in RA compared to HC have been reported [5, 6, 24], and were in line with our results. Contrary to our expectations, IgG AHA against IgG1 F(ab')$_{2MMP\text{-}3}$ was not significantly higher in RA than HC, although IgA AHA against IgG1 F(ab')$_{2MMP\text{-}3}$ and moreover both IgG and IgA AHA against IgG4 F(ab')$_{2MMP\text{-}3}$ were significantly elevated in RA. Given those significantly elevated IgA AHA against IgG1 F(ab')$_{2MMP\text{-}3}$ and IgG/IgA AHA against IgG4 F(ab')$_{2MMP\text{-}3}$ in RA, it seems likely that up-regulated immune responses by IgG1/IgG4 F(ab')$_{2MMP\text{-}3}$ fragments are resulted in RA. However, why did not the level of IgG AHA against IgG1 F(ab')$_{2MMP\text{-}3}$ show significant difference? Is there any up-regulated mechanism associated with increment of the IgG AHA in HC? MMP-12 that could cleave IgG1 at the same location in the lower hinge as do MMP-3 [7] could participate the increased AHA production in HC? Are these proteases involved in generation of IgG1 F(ab')$_{2MMP\text{-}3}$ that induce up-regulated production of the IgG AHA throughout their life? In any case, we cannot clearly explain this issue in the present situation. Importantly, it was remarkable that all IgA AHAs were significantly higher in RA than HC, which might reflect abnormal conditions of mucosal immunity resulting from dysbiosis of respiratory, gut and oral mucosa in RA [25, 26].

About the epitopes targeted by AHA in HC, Falkenburg *et al* reported that IgG AHA against IgG1 F(ab')$_{2pepsin}$ was inhibited by IgG1 F(ab')$_{2pepsin}$ but not IgG4 F(ab')$_{2pepsin}$, although IgG AHA against IgG4 F(ab')$_{2pepsin}$ was inhibited by both IgG4 F(ab')$_{2pepsin}$ and IgG1 F(ab')$_{2pepsin}$ [10]. These findings almost agree with the results of our inhibition studies using RA patients as shown in Figure 3. However, they also reported that the specificity of AHA against IgG4 F(ab')$_{2pepsin}$ was clearly different between HC and RA, namely, the AHA in HC cross-reacted with IgG1 F(ab')$_{2pepsin}$, whereas the AHA in RA were inhibited only by IgG4 F(ab')$_{2pepsin}$. Unlike their findings, AHA responses against IgG4 F(ab')$_{2pepsin}$ showing cross-reactive with IgG1 F(ab')$_{2pepsin}$ were recognized in RA. We suspect this discrepancy owing to different methods detecting AHAs, namely, our direct coating of IgG F(ab')$_2$ to ELISA plate vs. their indirect coating that results in stable and conformational hinge epitope (anti-biotin IgG F(ab')$_2$ bound to biotinylated human serum albumin).

We found a much more extended hinge epitope recognition profile in RA compared to HC. This finding might indicate the possibility of the epitope-spreading phenomenon in which the immune response is

extended to involve new intramolecular or intermolecular epitopes [27-29], although our study was cross-sectional. The phenomenon has been revealed by observation of ACPA in RA, which occurs before clinical disease onset [30, 31]. More study using preclinical and longitudinal RA patients is needed to confirm whether the same phenomenon as ACPA is observed.

Compared to ACPA, AHA did not seem to be useful for RA diagnosis because of LR+ values indicating less than 5 at optimal cutoff values, since LR+ of anti-CCP2 in RA, when used HC and non-RA patients as control, was calculated as 71.6 and 12.1, respectively [32]. Meanwhile, LR+ of RF was not high and reported to be 4.86 [33] and seemed to approximate to the LR+ of AHAs. At cutoff value for over 95% specificity, however, two AHAs (AHA3 and AHA6) revealed LR+ close to 10, which can lead to an increase in the probability of RA diagnosis. In any case, as limitation of this study, it is impossible to obtain convincing LR+ because we used HC as control, and study using non-RA patients with joint symptom is needed.

Three AHAs (AHA3, AHA5, AHA8) were selected by univariate analysis and then AHA3, IgG anti-IgG4 $F(ab')_{2pepsin}$, and AHA 5, IgA anti-IgG1 $F(ab')_{2pepsin}$, were selected as independent variable for RA diagnosis by multivariate logistic regression analysis as shown in Table 3. Additionally, only AHA3 was also selected as independent variable when the multivariate analysis was conducted on DNRA (RF and anti-CCP2 double negative RA patients). Increment of the specific AHA reactivity against IgG4 $F(ab')_{2pepsin}$ in RA reminds us that 1) IgG4 is produced in the context of prolonged antigenic stimulation and 2) the AHA must be generated *in vivo* by other physiological proteases except pepsin, since pepsin needs activation in acidic stomach conditions and do not reach circulation. Activated matrix metalloproteinase-7 (MMP-7) seems to be a candidate of proteases for cleavage of human IgG so that it cleaves IgG4 at the same lower hinge site between F234 and L235 (EU numbering) as pepsin does [10, 13]. Interestingly, the increased MMP-7 seems to mainly originate from not articular but extra-articular lesions such as nodules and lung of RA [12, 34, 35].

What role do the AHAs play in RA? Although we can not clearly explain, it has been proposed that several biological functions of AHA such as B cell suppression due to cross-linking the B cell receptor and the FcγRIIb, complement amplification via capture of dimeric C3b due to immune-complex formation of antigen-binding IgG $F(ab')_2$ and AHA, and restoring function of cleaved IgGs without Fc [7]. One possible proposal in RA is IgG4 ACPA that is the leading IgG subclass following IgG1 [36]. The IgG4 ACPA themselves would enervate IgG1-mediated ACPA-associated pathogenic progression through complement activation and triggering Fcγ receptors. In this situation, it has been proposed that specific AHAs bind to IgG4 $F(ab')_2$ with ACPA reactivity, form immune complexes and potentially progress inflammatory processes [10]. Further studies will be needed to evaluate the pathogenic or protective participation of AHA in addition to RF and ACPA in joints and/or lung of RA.

Conclusions

We have found an extended epitope recognition profile of AHA in RA, suggesting maturation of AHA-producing immune cells. IgG AHA against IgG4 $F(ab')_2$ generated by pepsin as an alternate protease of MMP-7, but not MMP-3, seemed to be a potential diagnostic marker for RA, even for seronegative RA.

Abbreviations

ACPA: anti-citrullinated protein antibodies, AHA: anti-hinge antibody, ALP: alkaline phosphatase, AU: arbitrary units, DNRA: RF and anti-CCP2 double negative RA, DOR: diagnostic odds ratio, DPRA: RF and anti-CCP2 double positive RA, EDTA: ethylenediaminetetraacetic acid, ELISA: enzyme linked immunosorbent assay, HC: healthy controls, IdeS: IgG-degrading enzyme of *Streptococcus pyogens*, IFX: infliximab, LR+: positive likelihood ratio, MMP-3: matrix metalloproteinase-3, MMP-7: matrix metalloproteinase-7, NTZ: natalizumab, PAN: panitumumab, PPV−: negative predictive value, PPV+: positive predictive value, RA: rheumatoid arthritis, RF: rheumatoid factor, ROC: receiver operating characteristic, RT: room temperature, SCB: sodium citrate buffer, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis, SPRA (CCP): RA with single positive anti-CCP2, SPRA (RF): RA with single positive RF, TBST: 10mM Tris buffer containing 0.9% NaCl, pH 7.4,with 0.05% Tween-20, TCZ: tocilizumab

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Tables

Table 1. Characteristics of patients with RA and healthy controls (HC)

	RA (n=111)	HC (n=81)	*P*
Age, years *	63.0 (53.3-66.0)	57.0 (49.8-64.0)	0.004
Women, n (%)	91 (82.0)	60 (74.1)	0.21
Duration of disease, years*	5.0 (3.0-12.0)		
bDMARDs, n (%)	77 (69.4)		
CDAI *	3.4 (1.4-8.3)		
HAQ score*	0.13 (0-0.75)		
Steinbrocker's stage, n (%)			
I	18 (16.2)		
II	35 (31.5)		
III	15 (13.5)		
IV	43 (38.7)		
Smoking, n (%)	34 (30.6)		
Autoantibodies, n (%)			
RF (+)/RF (-)	67 (60.4)/44 (39.6)		
Anti-CCP2 (+)/Anti-CCP2 (-)/NA	77 (69.4) /28 (25.2)/6 (5.4)		

*Values are the median (interquartile range) unless otherwise indicated. NA= not available

Table 2. Evaluation of AHA at optimal cutoff value (condition 1) and at cutoff value for high specificity (condition 2)*

	Cutoff	AUC (SE)	Sen/Spe	PPV+/PPV−	LR+ (95% CI)	DOR (95% CI)
Condition 1						
AHA1	22.5	0.69 (0.04)	82.0/54.3	71.1/68.8	1.79 (1.23-2.62)	5.41 (2.89-10.1)
AHA2	204	0.57 (0.04)	29.7/86.4	75.0/47.3	2.19 (1.74-2.75)	2.69 (1.29-5.63)
AHA3	4.70	0.78 (0.03)	59.5/82.7	82.5/59.8	3.44 (2.69-4.41)	7.02 (3.66-14.5)
AHA4	4.10	0.66 (0.04)	70.3/65.4	73.6/61.6	2.03 (1.52-2.72)	4.47 (2.46-8.13)
AHA5	10.3	0.74 (0.04)	78.3/66.7	75.5/70.1	2.35 (1.64-3.36)	7.22 (3.86-13.5)
AHA6	5.40	0.80 (0.03)	79.3/72.8	79.3/72.8	2.92 (2.02-4.22)	10.2 (5.40-19.4)
AHA7	1.20	0.80 (0.03)	89.6/56.8	73.1/80.7	2.07 (1.21-3.56)	11.4 (5.65-22.8)
AHA8	1.10	0.74 (0.04)	73.6/67.9	75.0/66.3	2.29 (1.66-3.16)	5.89 (3.19-10.9)
Condition 2						
AHA1	277	0.69 (0.04)	16.2/97.5	90.0/45.9	6.57 (5.37-8.03)	7.65 (2.10-27.9)
AHA2	766	0.57 (0.04)	5.40/96.3	66.7/42.6	1.46 (0.90-2.36)	1.49 (0.36-6.08)
AHA3	14.8	0.78 (0.03)	24.3/97.5	93.1/48.5	9.85 (8.24-11.8)	12.7 (3.85-41.8)
AHA4	39.7	0.66 (0.04)	2.70/97.5	60.0/42.3	1.10 (0.53-2.26)	1.10 (0.18-6.72)
AHA5	89.8	0.74 (0.04)	16.0/97.5	89.5/47.0	6.50 (5.27-8.01)	7.55 (2.05-27.7)
AHA6	32.5	0.80 (0.03)	23.6/97.5	92.6/49.4	9.55 (7.93-11.5)	12.2 (3.66-40.6)
AHA7	12.6	0.80 (0.03)	10.4/97.5	84.6/45.4	4.20 (3.21-5.50)	4.57 (1.11-18.8)
AHA8	5.37	0.74 (0.04)	16.0/97.5	89.5/47.0	6.50 (5.27-8.01)	7.55 (2.05-27.7)

* The cutoff values represent as AU/mL. AUC= area under the curve; SE= standard error; Sen/Spe= sensitivity/specificity; PPV+/PPV−= positive predictive value/negative predictive value; LR+= positive likelihood ratio; 95% CI= 95% confidence interval; DOR= diagnostic odds ratio; AHA1= IgG anti-TCZ IgG1 F(ab')$_{2pepsin}$; AHA2= IgG anti-TCZ IgG1 F(ab')$_{2MMP\text{-}3}$; AHA3= IgG anti-NTZ IgG4 F(ab')$_{2pepsin}$; AHA4= IgG anti-NTZ IgG4 F(ab')$_{2MMP\text{-}3}$; AHA5= IgA anti-TCZ IgG1 F(ab')$_{2pepsin}$; AHA6= IgA anti-TCZ IgG1 F(ab')$_{2MMP\text{-}3}$; AHA7= IgA anti-NTZ IgG4 F(ab')$_{2pepsin}$; AHA8= IgA anti-NTZ IgG4 F(ab')$_{2MMP\text{-}3}$.

Table 3 Univariate and multivariate logistic regression analyses of the association between AHA and RA*

	Univariate analysis		Multivariate analysis	
Variable	OR (95% CI)	*P*	OR (95% CI)	*P*
Analysis 1: Total RA vs. HC				
Age	1.03 (0.99-1.06)	0.075	1.04 (1.00-1.09)	0.037
Gender	1.59 (0.79-3.17)	0.189	2.19 (0.86-5.62)	0.100
AHA1	1.01 (1.00-1.01)	0.001	1.00 (0.99-1.00)	0.992
AHA2	1.00 (0.99-1.00)	0.295	–	–
AHA3	1.18 (1.09-1.27)	1×10^{-5}	1.16 (1.07-1.26)	3×10^{-4}
AHA4	1.03 (1.00-1.06)	0.025	1.00 (0.96-1.04)	0.825
AHA5	1.03 (1.02-1.05)	8×10^{-5}	1.02 (1.00-1.03)	0.034
AHA6	1.06 (1.03-1.09)	4×10^{-4}	1.01 (0.98-1.04)	0.367
AHA7	1.16 (1.05-1.28)	0.004	1.04 (0.96-1.13)	0.339
AHA8	1.65 (1.30-2.09)	3×10^{-4}	1.20 (0.89-1.62)	0.226
Analysis 2: DNRA vs. HC				
Age	1.04 (0.98-1.11)	0.226	1.07 (0.98-1.15)	0.115
Gender	2.62 (0.59-13.21)	0.193	9.29 (1.01-85.3)	0.049
AHA1	0.99 (0.99-1.01)	0.898	–	–
AHA2	1.00 (0.99-1.00)	0.243	–	–
AHA3	1.16 (1.06-1.27)	0.002	1.18 (1.06-1.32)	0.003
AHA4	1.03 (0.99-1.07)	0.112	1.03 (0.98-1.08)	0.311
AHA5	1.01 (0.99-1.03)	0.062	1.01 (0.99-1.03)	0.186
AHA6	1.00 (0.98-1.03)	0.664	–	–
AHA7	1.06 (0.98-1.15)	0.143	–	–
AHA8	1.21 (0.83-1.70)	0.347	–	–

*The number of RA patients in analysis1 and in analysis 2 was 106 and 18, respectively. The number of HC was same as 81 in both analyses.

Declarations

Ethics approval

The Ethics Committee at the Iizuka Hospital, Iizuka, Japan, approved this study (reference number: 26143), and participants gave informed consent.

Consent for publication

N/A

Availability of data and materials

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

Competing interests

All authors declare that they have no competing interests.

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Authors' contributions

TO conceived the study, performed all of the experiments, data analysis and wrote the manuscript. SO conceived the study and done critical revision of the manuscript. AU and SN performed acquisition of data. All authors read, commented on and approved the final manuscript.

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Figures

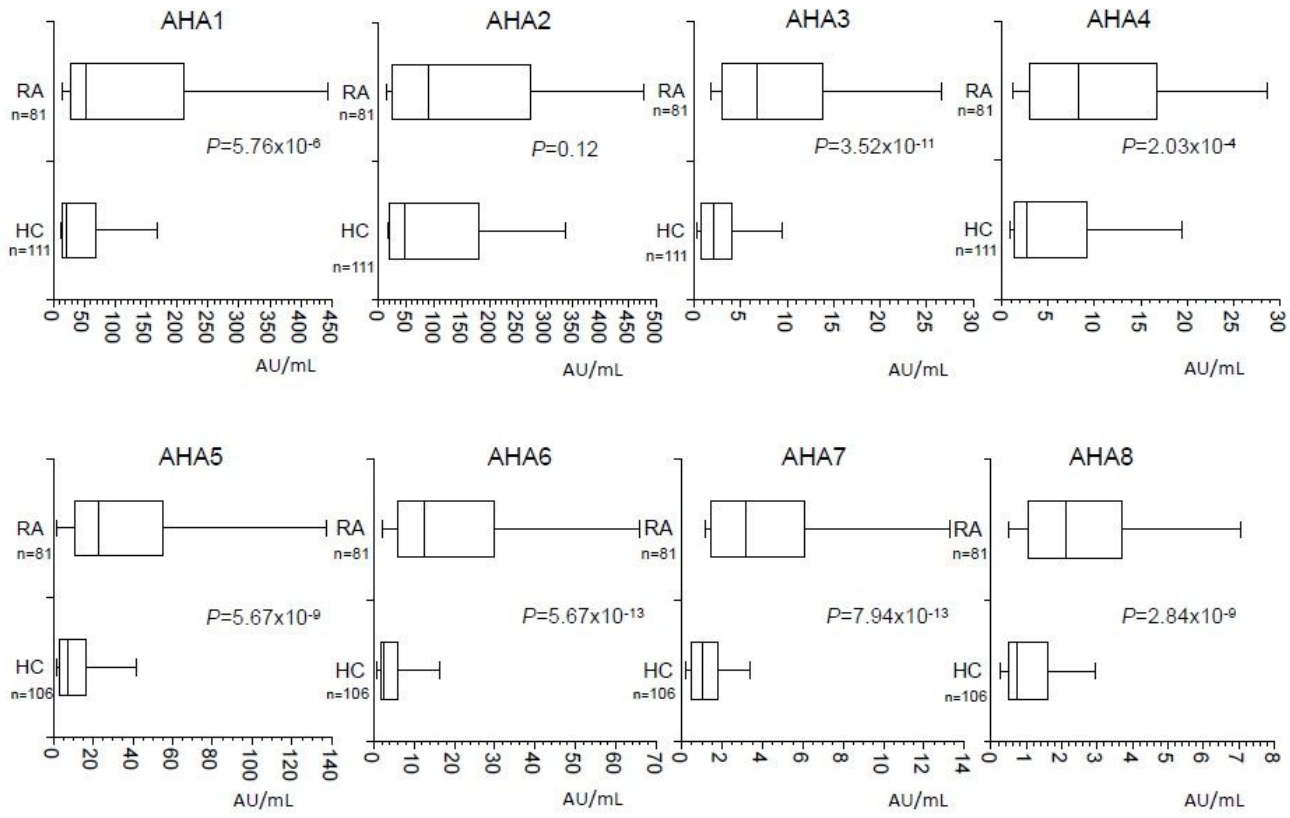

Figure 1

Comparison of AHA levels between HC and RA patients. AHA1, AHA2, AHA3 and AHA4 represent IgG AHA against TCZ IgG1 F(ab')2pepsin, TCZ IgG1 F(ab')2MMP-3, NTZ IgG4 F(ab')2pepsin and NTZ IgG4

F(ab')2MMP-3, respectively. AHA5, AHA6, AHA7 and AHA8 represent IgA AHAs against TCZ IgG1 F(ab')2pepsin, TCZ IgG1 F(ab')2MMP-3, NTZ IgG4 F(ab')2pepsin and NTZ IgG4 F(ab')2MMP-3, respectively. Differences in levels between HC and RA were analyzed with Mann-Whitney U test.

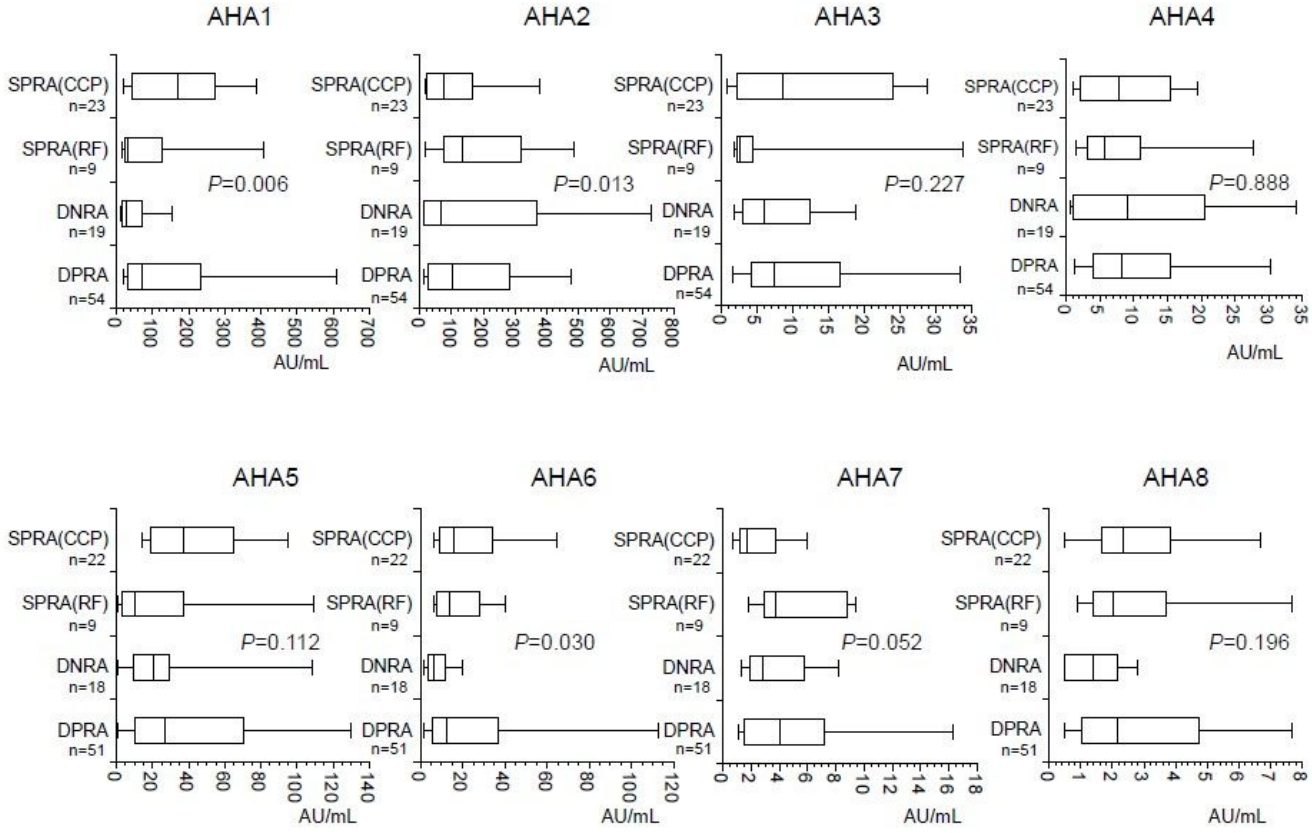

Figure 2

Comparison of AHA levels in RA stratified by presence or absence of RF and anti-CCP2 antibodies (CCP). Double positive RA (DPRA) indicates RA patients being positive for both RF and CCP. Double negative RA (DNRA) indicates RA patients being neither. Single positive RA (SPRA) indicates RA patients being either positive for RF [SPRA (RF)] or positive for CCP [SPRA (CCP)]. The number of patients in each RA group is shown in the figure. The non-parametric Kruskal-Wallis test was used to compare the 4 groups.

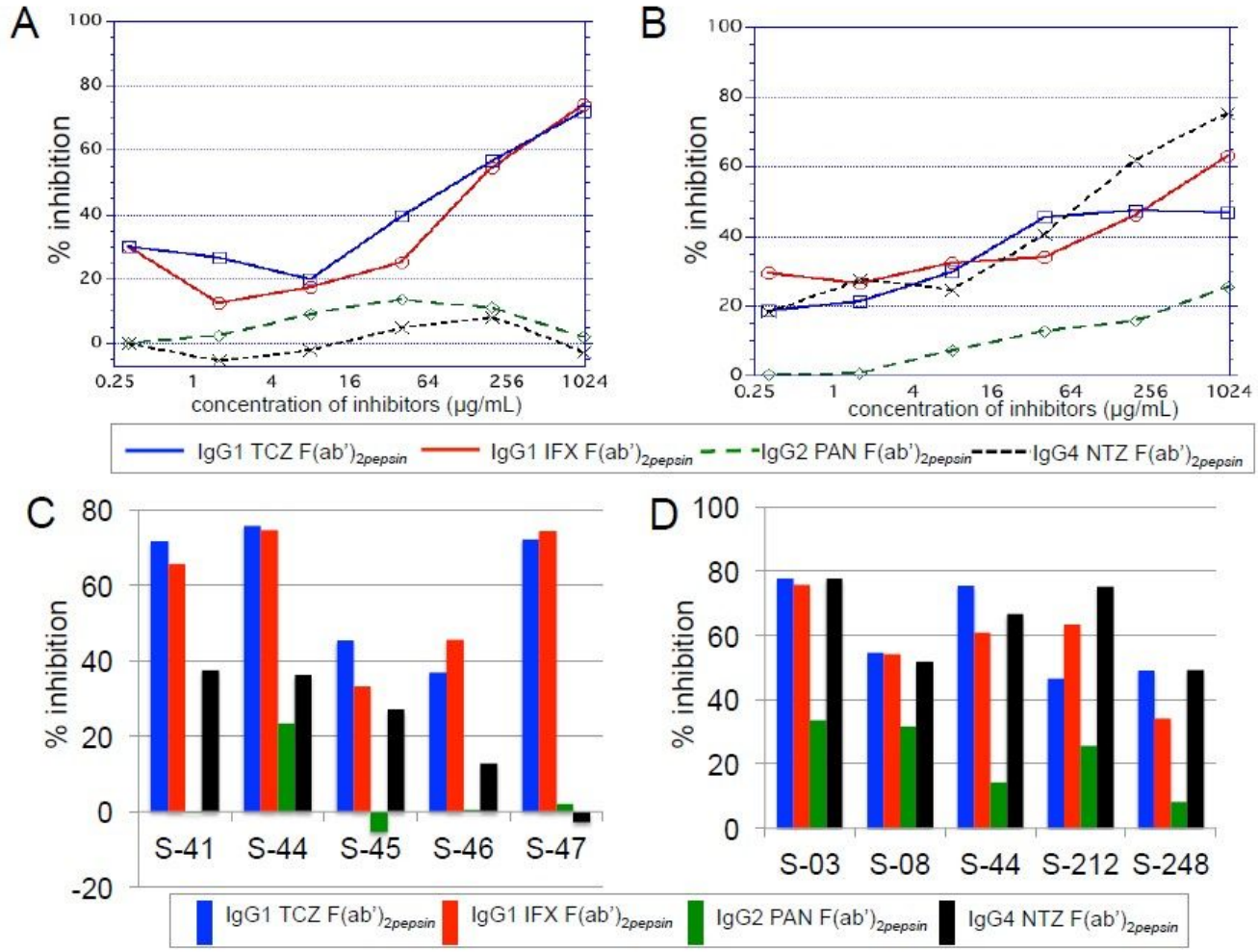

Figure 3

Inhibition studies for hinge-epitope specificity of IgG AHA. Typical cases were shown in (A) and (B). (A) IgG AHA from RA patient (S-47) against IgG1 TCZ F(ab')2pepsin was neither inhibited by IgG2 PAN- nor IgG4 NTZ F(ab')2pepsin, but dose-dependently by both TCZ-and IFX F(ab')2pepsin. (B) IgG AHA from RA patient (S-212) against IgG4 F(ab')2pepsin was dose-dependently inhibited by IgG4 F(ab')2pepsin, followed by IgG1 IFX- or TCZ F(ab')2pepsin, and slightly by IgG2 F(ab')2pepsin. (C) Percent inhibitions of IgG AHAs from five RA patients (S-41, S-44, S-45, S-46, S-47) against IgG1 TCZ F(ab')2pepsin by inhibitors (four different IgG F(ab')2pepsin) at 1,000 μg/mL were 36.8-75.6 for TCZ F(ab')2pepsin, 33.2-74.5 for IFX F(ab')2pepsin, −5.4-23.4 for PAN F(ab')2pepsin and −2.8-37.4 for NTZ F(ab')2pepsin. (D) When used IgG4 NTZ F(ab')2pepsin as coating antigen and four inhibitors at 1,000 μg/mL, percent inhibitions of IgG AHAs from RA patients (S-03, S-08, S-44, S-212, S-248) were 46.6-77.5 for TCZ, 34.2-75.8 for IFX, 8.2-33.5 for PAN and 49.2-77.6 for NTZ.

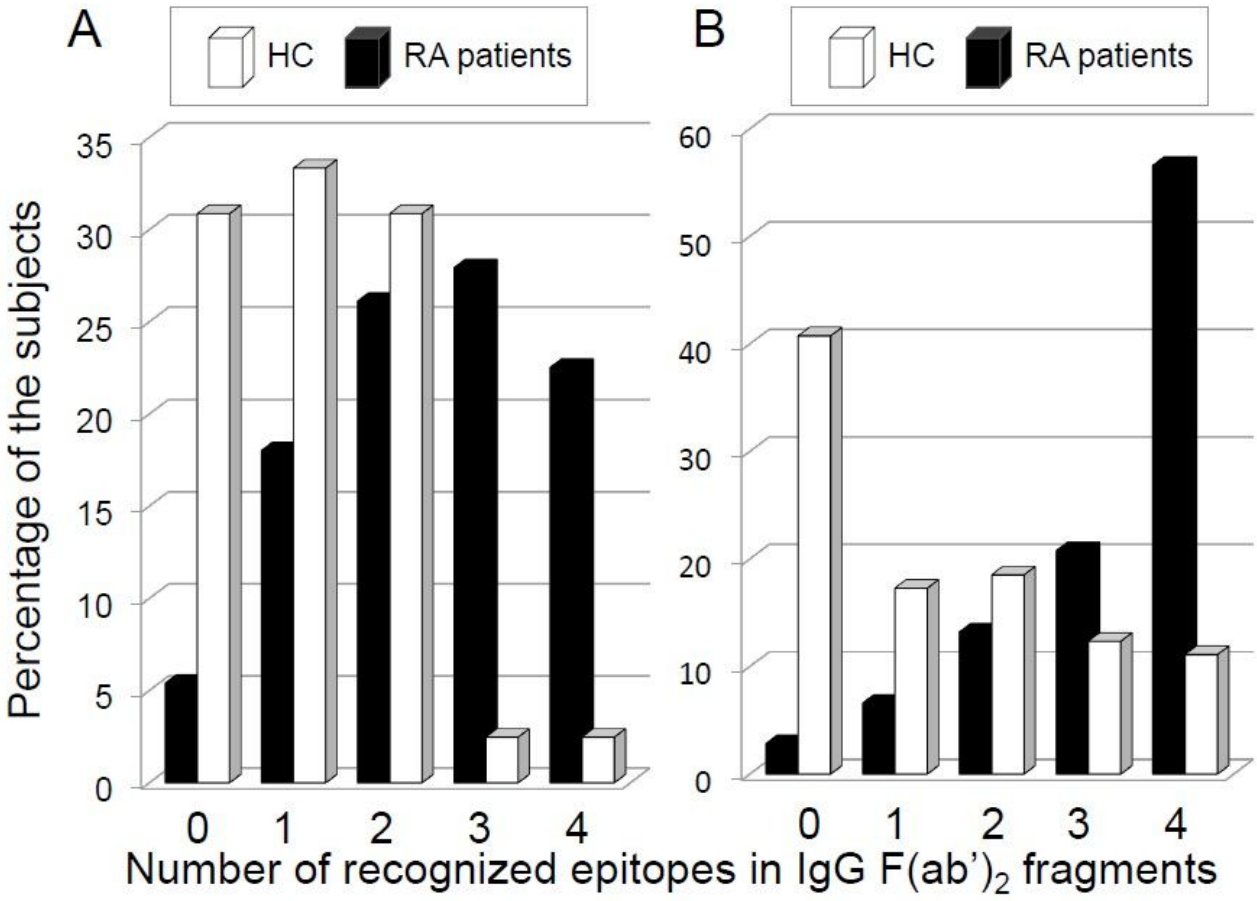

Figure 4

Frequency distribution of cumulative number of hinge epitopes recognized by IgG AHA (A) or IgA AHA (B) from each patient with RA or each HC. Depicted are the percentages of RA patients and HC.

Supplementary Files

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- FigureS2.pdf
- FigureS1.pdf