Serum IgG ACPA-IgM RF immune complexes were detected in rheumatoid arthritis patients positive for IgM ACPA

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Abstract

Objective

Since the presence of IgM antibodies is a hallmark of ongoing immune response, we aimed to identify immunologically active rheumatoid arthritis (RA) patients by detecting IgM anti-citrullinated protein antibody (ACPA) levels.

Methods

IgM ACPA levels were determined in the serum of 176 RA patients by enzyme-linked immunosorbent assay, in which parameters of reactivity against citrullinated and non-citrullinated peptides were compared to ensure the specificity. Influence of IgM rheumatoid factor (RF) on IgM ACPA detection was examined by removing IgG, using protein G-conjugated beads, or by purifying ACPA, using citrullinated peptide-conjugated beads.

Results

Although IgM specific for citrullinated proteins was detected in some patients (11%), IgM molecules reactive to both citrullinated and non-citrullinated peptides were detected in a substantial number of patient samples (12%). IgM ACPA-positive reactions were associated with the presence of IgG ACPA and IgM RF. Surprisingly, protein G-mediated removal of IgG from the serum eliminated positivity for IgM ACPA, suggesting that IgG ACPA-IgM RF complex was being detected. This assumption was confirmed by the detection of IgM RF in the eluate of protein G beads and citrullinated peptide-conjugated beads.

Conclusion

In an attempt to detect IgM ACPA, we mostly revealed false positive reactions due to the presence of IgM molecules, which were not specific for citrullinated proteins, and IgG ACPA-IgM RF immune complex. The latter complex had been proposed to play a role in the pathogenesis of RA, and here, for the first time, we have demonstrated its presence in the sera of RA patients.

Key words

rheumatoid arthritis, anti-citrullinated protein antibody, rheumatoid factor, immune complex, enzyme-linked immunosorbent assay, immunoprecipitation

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Introduction

IgM antibodies are transiently produced during the induction phase of immune responses by short-lived plasma cells (PC) that arise from newly primed naive B cells. Meanwhile, some of the primed B cells migrate into the germinal centre (GC), where they undergo isotype class-switching and somatic hypermutation, leading to the generation of long-lived PCs that secrete class-switched, high-affinity antibodies. Therefore, the presence of IgM antibodies usually implies an ongoing immune response, *i.e.* active infection. Such IgM antibodies bind the antigen with low affinity and could be polyreactive.

Anti-citrullinated protein antibodies (ACPAs) are autoantibodies specifically detected in rheumatoid arthritis (RA) years before the disease onset, suggesting an involvement of ACPAs in the pathogenesis of RA (1). Although ACPAs are primarily of IgG isotype, IgM ACPAs have been also detected in some RA patients (2). It is postulated that, in IgM ACPA-positive RA patients, newly developed naive B cells are exposed to the ACPA-inducing antigen(s). Therefore, identifying RA patients producing IgM ACPAs might provide clues to understand the mechanism that initiates autoimmune reactions in RA.

However, some data conflict with the notion that IgM ACPA production reflects immunological activity of RA. Like IgG ACPAs, IgM ACPAs are detected before the onset of RA, but the appearance of IgG ACPAs precedes that of IgM ACPAs (3, 4). Analysis of ACPA-producing B cell clones has demonstrated that their specificity for citrulline depends on somatic hypermutation of the immunoglobulin gene (5, 6). However, if IgM ACPAs are also produced by B cells that underwent GC reaction, but not by newly recruited naive B cells, then the presence of IgM ACPA does not imply ongoing immune response. In addition, detecting antigen-specific IgM is sometimes problematic owing to the promiscuity in antigen specificity. In fact, IgM showed higher levels of reactivity to non-citrullinated peptides compared IgG (7, 8). The presence of IgM rheumatoid factor (RF) that forms immune complexes with IgG is another confounding element in measuring antigen-specific IgM concentrations. IgM RF is detected in the majority of ACPA-positive RA patients, and the putative roles of IgG ACPA-IgM RF immune complex in the pathogenesis of RA have been discussed previously (9-11), although the actual presence of such complex has not been formally demonstrated.

In the present study, we aimed to identify and characterie RA patients producing IgM ACPAs. However, the results of our analysis rather indicate that putative IgM ACPA reactions were false positive due to the presence of IgM molecules, which were not specific for citrullinated proteins, and IgG ACPA-IgM RF immune complex.

Materials and methods

Patients

One hundred and seventy-six patients with RA, who fulfilled the 1987 American College of Rheumatology (ACR) classification criteria (12), were included in the study. To define antibody cut-off levels, 23 healthy control subjects were also recruited. 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

ACPAs were measured by enzyme-linked immunosorbent assay (ELISA). Pairs of citrullinated and control non-citrullinated peptides used for the assay are produced through amino acid synthesis (Table I) (7, 13-16). All peptides are conjugated with biotin at C-terminal. Avidin-coated plates (Avidin Plate, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were incubated with the peptides (2 μg/mL), and after blocking non-specific binding by an incubation with Blocking One solution (Nacalai Tesque, Kyoto, Japan), the plates were incubated with sera diluted at 1:50, for 1 h at room temperature. IgG and IgM antibodies were detected using peroxidase-conjugated $F(ab)_2$ goat anti-human IgG (Rockland Immunochemi-

Table I. List of biotin-conjugated synthetic peptides.

Peptide name	Sequence	Corresponding base peptide	reference
cfc0-cyc	HQCHQESTRGRSRGRCGRSGS-biotin	Filaggrin (306-324)	[13,14]
cfc1-cyc	HQCHQEST**X**GRSRGRCGRSGS-biotin		
nFib	NEEGFFSARGHRPLDKK-biotin	Fibrinogen-β (36-52)	[7,15]
cFib	NEEGFFSA**X**GHRPLDKK-biotin		
nEno	KIHAREIFDSRGNPTVE-biotin	α-enolase (5-21)	[16]
cEno	KIHA**X**EIFDS**X**GNPTVE-biotin		

X: citrulline.

cals, PA, USA), and goat anti-human IgM (Sigma-Aldrich, MO, USA). Antibody binding was visualised using tetramethylbenzidine substrate solution (Interchim, Montluçon, France), and the absorbance was measured at 450 nm. The cut-off absorbance level in each assay was defined as mean value of measurements in control subjects +2SD. To detect IgG and IgM against cyclic citrullinated peptide 2 (CCP2), we used a commercially available ELISA kit (Euro-Diagnostica, Malmö, SWE), with a minor modification for anti-CCP2 IgM detection. We also used commercial kits to measure the levels of IgM RF (ORGENTEC Diagnostika GmbH, Mainz, Germany) and total IgM (Human IgG ELISA Quantitation Set, Bethyl Laboratories, TX, USA).

Serum treatment with protein G-conjugated beads

To deplete IgG, serum samples were incubated with protein-G-conjugated magnetic beads (ProteinG Mag Sepharose Xtra; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Briefly, 5 μg of the beads were incubated overnight with 1 μl of serum samples diluted with 19 μl of tris buffered saline (TBS) on a shaker at 4°C. After magnetic separation of the beads, the concentration of IgM reactive to cfc1-cyc in the liquid fraction was measured by ELISA.

To analyse the antibodies bound on protein G-conjugated beads, 5 μg of the beads were incubated overnight with 4 μl of serum samples diluted with 16 μl of TBS on a shaker at 4°C. After washing five times with TBS, the beads were incubated twice with 40 μl elution buffer (1 M glycine-HCl, pH 2.7). The eluate was collected and immediately neutralised with 1 M Tris-HCl (pH 9) before measuring the concentration of IgM RF.

Serum treatment with streptavidin-conjugated beads

To purify antibodies that bind to cfc1-cyc, 40 μl of streptavidin-conjugated magnetic beads (GE Healthcare, Buckinghamshire, UK) were first incubated with 4 μl of biotinylated cfc1-cyc (1 μg/μl). After washing out the excess of peptides, the beads were incubated with 8 _l of serum samples. Then, bound antibodies were eluted by the same procedure as above, and the levels of IgM RF in the eluate were measured.

SDS-PAGE and western blot analysis

The eluate from the protein G beads, purified human IgM (ORIENTAL YEAST, Tokyo, Japan), and IgG (Jackson immunoResearch, PA, USA) were diluted in lithium dodecyl sulfate sample buffer (Novex, CA, USA) and subjected to denaturation and reduction at 85°C for 5 minutes. The samples were separated on a 4-12% gradient SDS–polyacrylamidegel by electrophoresis (Novex, CA, USA) and were transferred to nitrocellulose membranes (GE Healthcare Life Science, Buckinghamshire, UK). The blots were incubated overnight with blocking buffer (TaKaRa, shiga, Japan) and incubated with mouse anti-human IgM (1:1,000) (Santa Cruz Biotechnology, TX, USA) or mouse anti-human IgG (1:500) (SouthernBiotech, AL, USA) antibody for 1 hour at room temperature. After rinsing, the membrane was incubated with HRP-labeled goat anti-mouse secondary antibody (1:1,000) (Santa Cruz Biotechnology, TX, USA) on a shaker at room temperature for 1 h. Following additional rinsing, immunoreactivity of the blots was detected using an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using JMP v. 12 (SAS Institute, Cary, N.C., USA). Statistical correlation was tested by calculating the Spearman's rank correlation coefficient. Differences in the levels of IgM ACPAs between baseline and after protein G treatment were determined by paired Student's *t*-test. Differences in the levels of IgM RF in the eluate of protein G-conjugated beads or citrullinated peptide-conjugated beads were determined by the Mann-Whitney U-test.

Results

Characteristics of the study population

The study population mainly consisted of women (86.1%). Mean age (SD) and mean disease duration (SD) were 60.3 (12.9) years, and 15.5 (11.0) years. The majority of the patients (73%) were treated with disease-modifying antirheumatic drugs, among whom methotrexate and biologics was received by 64% and 37%, respectively. The mean Disease Activity Score in 28 joints, based on C-reactive protein level, was 2.0±0.8.

Lack of specificity for citrullin of IgM ACPAs from some RA patients

Since specificity of IgM ACPA toward citrullinated proteins has not been characterised in detail, we first compared the reactivity of serum antibodies to citrullinated and non-citrullinated versions of CCP1, cfc1-cyc and cfc0-cyc, respectively (12). About 40% of samples contained IgG (66/176) that selectively reacted with cfc1, but a few samples (5/176, 3%) contained IgG

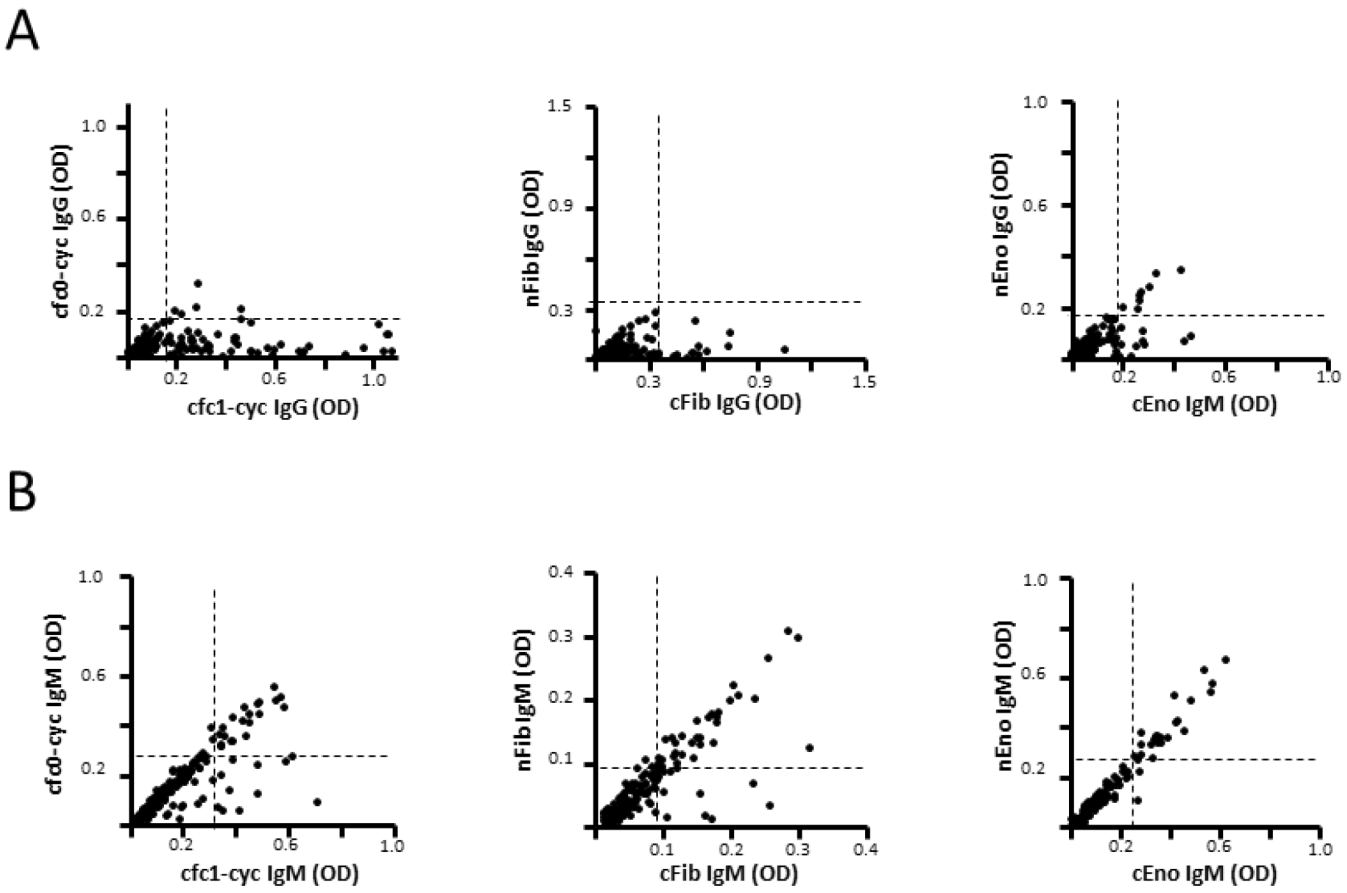

Fig. 1. Binding of IgG and IgM antibodies in the serum of RA patients to citrullinated and non-citrullinated peptides.
Reactivity of IgG (**A**) or IgM (**B**) in the serum samples to the pairs of citrullinated and non-citrullinated peptides was examined by ELISA. Absorbance values (optical density, OD) for cfc1 and cfc0, cFib and nFib, or cEno and nEno peptides are plotted on the graphs. Broken lines indicate cut-off values (mean absorbance values + 2 standard deviations determined in serum samples from healthy controls.)

that weakly reacted to both cfc1-cyc and cfc0-cyc (Fig. 1A, left). In contrast, a substantial proportion of samples (21/176, 12%) contained IgM that reacted equally to cfc1-cyc and cfc0-cyc, although some samples (20/176, 11%) contained IgM that bound specifically to cfc1-cyc (Fig. 1B, left). Similar pattern of antigen reactivity was observed when we analysed the binding to a pair of fibrinogen-derived peptides (cFib and nFib) (Fig. 1A and B, middle). We also examined α-enolase-derived peptides (cEno and nEno), but there were only a few samples containing IgG specific for cEno, and IgM anti-cEno antibody was hardly detected. (Fig. 1A and B, right).

IgM ACPA-positive reaction is associated with the presence of IgG ACPA and IgM RF

We next examined the relationship between the levels of IgG and IgM ACPAs. We expressed antibody levels after subtracting absorbance value of non-citrullinated peptides from that of citrullinated peptides. In cases of cfc1-cyc and cFib, IgM ACPA levels correlated well with IgG ACPA levels (Fig. 2A, left, middle). Virtually all IgM ACPA-positive samples were also positive for IgG ACPA. We also measured levels of IgG and IgM antibodies against CCP2, which were expressed as simple absorbance values because the non-citrullinated version of the peptide was not available. The levels of an anti-CCP2 IgM antibody were also correlated with those of IgG (Fig. 2A, right).

We examined other factors that could be associated with positivity for IgM ACPA. We did not detect significant correlations with sex, age, disease duration, or disease activity (data not shown). Although there was no correlation with the levels of total IgM (Fig. 2B, left), the levels of IgM ACPA were significantly associated with those of IgM RF (Fig. 2B, right).

Presence of IgG ACPA-IgM RF immune complex in IgM ACPA-positive serum

The data above indicated a possibility that IgM RF influenced IgM ACPA detection (Fig. 3A). To deplete IgG-IgM RF immune complex, serum samples were incubated with protein G-conjugated beads that specifically bind to IgG. Surprisingly, anti-cfc1 IgM was strikingly reduced after the treatment with protein G-conjugated beads in all samples that were positive for anti-cfc1 IgM (Fig. 3B). Even in the samples showing the ΔOD values above the cut-off point after the treatment, IgM was estimated to be removed more than 95%. These suggest the presence of an immune complex consisting of IgG anti-cfc1 and IgM RF. In addition, all IgM- anti-cfc1-negative samples re-

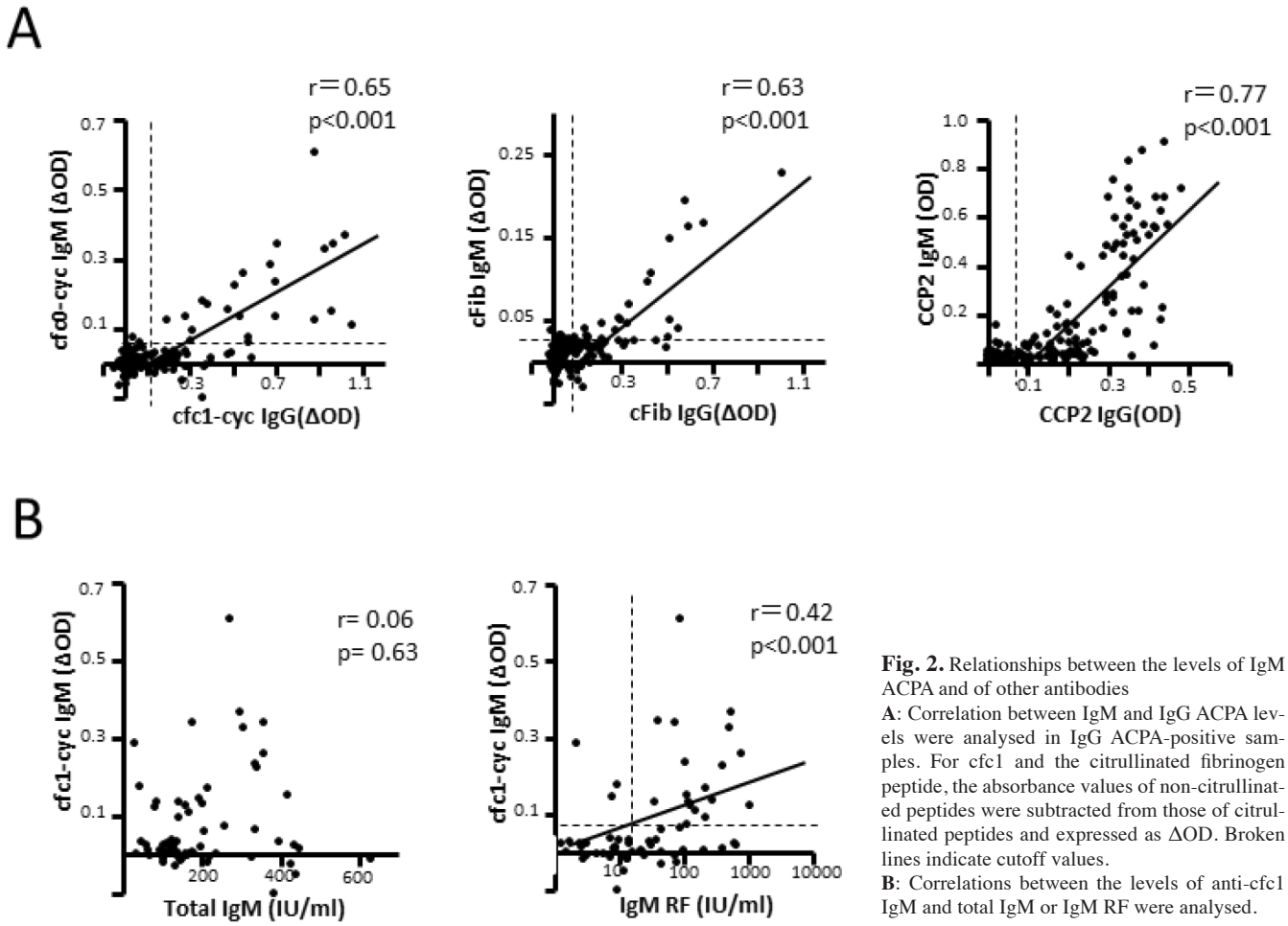

Fig. 2. Relationships between the levels of IgM ACPA and of other antibodies
A: Correlation between IgM and IgG ACPA levels were analysed in IgG ACPA-positive samples. For cfc1 and the citrullinated fibrinogen peptide, the absorbance values of non-citrullinated peptides were subtracted from those of citrullinated peptides and expressed as ΔOD. Broken lines indicate cutoff values.
B: Correlations between the levels of anti-cfc1 IgM and total IgM or IgM RF were analysed.

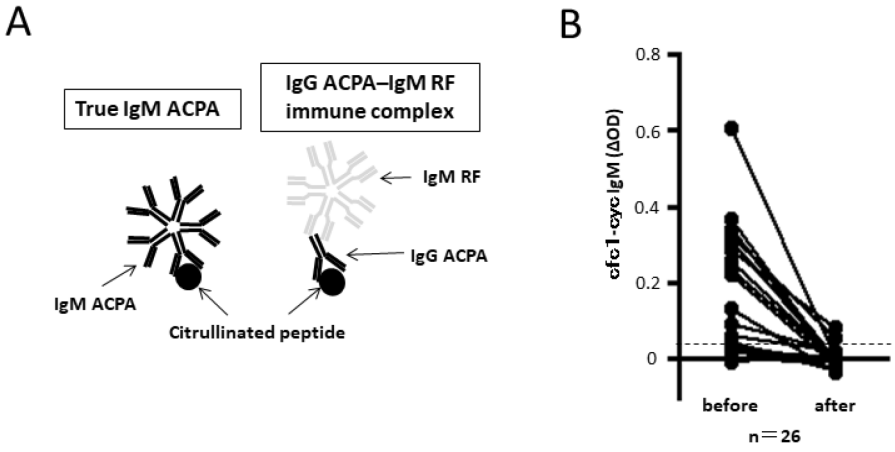

Fig. 3. Reduction of IgM ACPA activity after IgG removal by protein G-conjugated beads.
A: Illustration of IgM ACPA (left) and IgG ACPA-IgM RF immune complex (right).
B: Concentration of IgM anti-CCP1 antibody (n=26) in serum samples was measured before and after incubation with protein G-conjugated beads.

mained negative after protein G treatment, excluding possible interference of IgG in the detection of IgM ACPA.
In order to verify whether IgM RF was indeed removed from the serum by protein G beads, we measured the levels of IgM RF in the eluate from the beads. We detected IgM RF in the eluate from the beads that had been incubated with IgM CCP1-positive serum (Fig. 4A). Small amount of IgM RF was detected even in the eluate from RF-negative serum samples, if they were IgM ACPA-positive (see Fig. 2B, right panel). The presence of IgM in the eluate from protein G beads was confirmed by western blotting (Fig. 4B).
To further verify the presence of IgG ACPA-IgM RF immune complex in the serum, we incubated serum samples with cfc1-cyc-conjugated beads. We detected IgM RF in the eluate from samples that were positive for IgM anti-cfc1, but not from serum samples that did not contain IgM or IgG anti-cfc1, even if the serum was positive for RF (Fig. 4C). These results indicate the presence of IgG ACPA-IgM RF immune complex detected as IgM ACPA in the serum of RA patients.

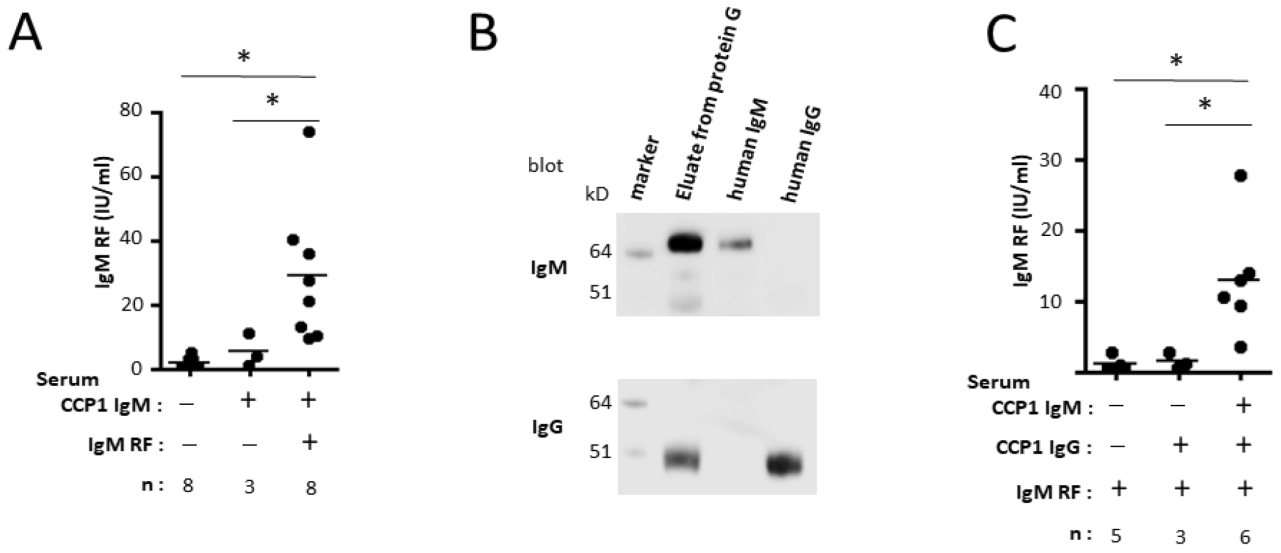

Fig. 4. Detection of IgM RF bound to IgG ACPA in IgM ACPA-positive serum samples (A) Concentrations of IgM RF in the eluate from the protein G-conjugated beads that had been pre-incubated with serum samples with various IgM RF and IgM ACPA status were measured. The number of the samples in each group was 8 in CCP1 IgM-, IgM RF-; 3 in CCP1 IgM+, IgM RF-; 8 in CCP1 IgM+, IgM RF+. Statistical significance is indicated as follows: *p<0.05. (B) Western blot analysis of the presence of IgM (upper panel) and IgG (lower panel) in the eluate from protein G beads incubated with serum from IgM ACPA positive patient. Molecular marker (lane 1), eluate from protein G beads (lane 2), purified human IgM (lane 3), purified human IgG (lane 4). (C) Concentrations of IgM RF in the eluate fractions from cfc1 peptide-conjugated beads that had been pre-incubated with serum samples with various IgM RF and IgM ACPA status were measured. The number of the samples in each group was 5 in CCP1 IgM-, CCP1 IgG-, IgM RF+; 3 in CCP1 IgM-, CCP1 IgG+, IgM RF-; 6 in CCP1 IgM+, CCP1 IgG+, IgM RF+. Statistical significance is indicated as follows: *p<0.05.

Discussion

On the basis of the assumption that IgM ACPA production represents active immune response in RA, we aimed to detect and characterise RA patients producing IgM ACPAs. However, we failed to detect serum samples containing "true" IgM ACPA, but instead revealed the presence of two types of false positive reactions for IgM ACPA mediated by: a) IgM molecules not specific for citrullinated proteins, and b) IgG ACPA-IgM RF immune complex.

Previous reports describing IgM ACPAs showed relatively high levels of reactivity to control, non-citrullinated peptides (7, 8). However, the relationship between the levels of reactivity to citrullinated peptides and control, non-citrullinated peptides has not been characterised in detail. In this study, we plotted the absorbance values of reactions with citrullinated peptide against those of reactions with non-citrullinated peptide and found that a substantial portion (12% for CCP1) of RA serum samples contained IgM that bound equally well to both peptides. Thus, these were citrulline-nonspecific antibodies, which likely recognised the conformation shared by the two peptides with low affinity. Binding of IgG ACPAs to non-citrullinated proteins has been also reported in certain conditions, such as infection and hepatic disease (17, 18). Thus, care should be taken to exclude binding to non-citrullinated proteins during measurements of ACPA levels.

Although IgM antibodies in some patients (11% for CCP1) seemed to be specific for citrullinated proteins, these were also false positive results due to the formation of IgG ACPA-IgM RF immune complex. This conclusion was made on the basis of several observations, namely, the loss of IgM ACPA activity when IgG was removed from the serum by protein G-conjugated beads (Fig. 3B), detection of IgM RF in the eluate from the protein G-conjugated beads (Fig. 4A, B), and finally, the presence of IgM RF in the eluate from citrullinated peptide-coated beads (Fig. 4C). Although we could not exclude a possibility that polyreactive IgM RF binds to citrullinated peptides, it is unlikely because most IgM RF positive serum are IgM ACPA negative. On the other hand, we detected a small amount of IgM RF in the eluate from the beads incubated with the serum from IgM ACPA-positive, RF-negative patients (Fig. 2B, right panel; Fig. 4A). Since RF is identified by its binding to IgG, false negative result for RF might be possible if all RF antigen-binding sites were already occupied by IgG, including IgG ACPA. This might also explain why some IgM ACPA-positive serum samples were negative for IgM RF, as reported previously by Lakos *et al*. (19). It is well known that ACPAs, as well as RF, are produced before the onset of RA. ACPA production in the pre-RA stage is characterised by a gradual increase of the level, and the number of epitopes and isotypes of the antibodies. Notably, the appearance of IgG precedes that of IgM (3, 4). This unusual kinetics of antibody production can now be explained on the basis of our current observations: the gradual increase of IgG ACPAs and IgM RF leads to the formation of immune complexes, which can, at a certain time point, be detected as IgM ACPAs.

Although our data showed a correlation between the levels of IgG ACPAs with those of IgM "pseudo" ACPAs (Fig. 2), Suwannalai *et al*. reported that IgG and IgM ACPAs have different antigen specificity (7). This apparent discrepancy could be due to the differences between patient cohorts, but might also result from the difference in the threshold of different ACPAs. In similarity to our data, Suwannalai *et al*. found that

IgM ACPAs showed much higher absorbance values against non-citrullinated peptides and significant differences in reactions with citrullinated and non-citrullinated peptides were detected only in the samples with higher IgG levels. Thus, our data are similar to the observations of Suwannalai *et al*.

Although we did not detect "true" IgM ACPAs in our cohort, it does not exclude the occurrence of "true" IgM ACPAs, especially at pre-RA stage or even before that. Unfortunately, samples from such patients were not included in our analysis, but it will be of interest to analyse them in future. Nevertheless, it is worth noting that specificity of ACPA to citrullinated proteins depends on somatic hypermutation (5, 6), suggesting that IgM-expressing B cells do not recognise citrulline before entering GC. At present, it is unknown what kinds of antigens are recognised by such originally IgM-expressing naive B cells, but this might be the very first step of ACPA production. Although it remains possible that IgM ACPA is produced by long-lived plasma cells originated from GC B cells, in this case, the presence of IgM does not imply an ongoing immune response.

The importance of IgG ACPA-IgM RF immune complex in the pathogenesis of RA had been suggested previously. IgM RF enhanced IgG ACPA-induced TNF-α production by macrophages (9-11). The presence of IgG ACPA and IgM RF correlated with the extent of disease progression or joint destruction (11, 20). The levels of IgG ACPA and IgM RF decrease after anti-TNF-α treatment (21). In this regard, our data, which demonstrate for the first time the presence of IgG ACPA-IgM RF immune complex in serum samples of RA patients, provide support for its relevance in the pathogenesis of RA.

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