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Rheumatoid factors do not preferentially bind to ACPA-IgG or IgG with altered galactosylation

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Abstract

Objectives. Recent reports describe interactions between the two most prominent RA-related autoantibodies, RFs and ACPAs. The main aim of the present study was to investigate whether RFs preferentially interact with ACPA-IgG over non-ACPA IgG. Additionally, interactions of RFs with IgG with altered galactose content in the Fc domain were examined, since ACPA-IgGs have been shown to have decreased Fc galactose content in RF^+ patients.

Methods. (Auto)antibody interactions were studied in a surface plasmon resonance imaging assay and with ELISA. Target antibodies were isolated from RA patient plasma (polyclonal ACPA- and non-ACPA-IgG) or recombinantly produced to obtain monoclonal IgG with well-defined Fc galactose content. Interacting autoantibodies were studied using autoantibody positive patient sera and two recombinantly produced IgM-RFs.

Results. The sera from 41 RF^+ RA patients showed similar RF binding to ACPA- and non-ACPA-IgG and no differences in binding to IgG with normal, high or low levels of Fc galactosylation. Two monoclonal IgM-RFs, one interacting with the CH2–CH3 interface and one binding close to the C-terminal end of the CH3 domain showed no influence of the Fc glycan on IgG binding by IgM-RF.

Conclusion. Although interactions between RF and ACPA may play a role in inflammatory processes in RA, RFs do not preferentially interact with ACPA-IgG over non-ACPA-IgG nor with agalatosylated IgG over IgG with normal or high galactosylation.

Key words: rheumatoid factor, anti-citrullinated protein antibodies, autoantibodies, Fc glycans, galactosylation

BASIC SCIENCE

Rheumatology key messages

- RF binding to IgG is independent of the level of Fc domain galactosylation.
- ACPA-IgGs are not inherently better targets for RF than non-ACPA-IgGs.

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Introduction

RFs are autoantibodies, primarily of the IgM isotype, that bind to the constant (Fc) domain of IgG. RFs were the first autoantibodies discovered to be associated with RA [1], are part of the RA classification criteria [2] and have prognostic value [3]. ACPAs recognize arginine amino acid residues converted enzymatically into citrulline and were linked to RA more recently [4]. Both RFs and ACPAs have been implicated in the pathogenesis of RA. Recently there has been increased interest in the effects of RF-ACPA

interactions [5, 6], since RF can bind IgG-ACPAs and RF and ACPA are often found together in RA [7]. Likewise, several studies suggest an interaction between ACPA and RF, as their combined presence is found in patients, but their presence is discordant in their (seropositive) healthy relatives [8]. The combined presence is associated with enhanced bone marrow oedema as well as with higher levels of pro-inflammatory cytokines and increased acute phase proteins and disease activity [7, 9]. *In vitro* it was shown that RF can enhance monocyte activation by ACPA-containing immune complexes [7] and unpublished data suggest that crosslinking of ACPA-Fcs by RF may enhance binding of ACPAs to their citrullinated targets by creating immune complexes with higher avidity.

However, it is unknown whether RFs preferentially bind ACPA-IgG over non-ACPA-IgG. We hypothesized that RFs might preferentially bind ACPAs over non-ACPA-IgGs based on results showing that ACPA-IgGs in RF^+ patients have a lower galactose content of the glycans in the IgG Fc domain compared with ACPAs in RF^- patients [10]. This is relevant in light of other studies showing better binding of RF to IgG with lower Fc galactosylation [11, 12]. Here we used ELISAs and a surface plasmon resonance imaging (SPRi) array to compare binding of RF to ACPA-IgG and non-ACPA-IgG isolated from plasma of RA patients. Furthermore, we investigated whether the binding of RFs is influenced by the degree of galactosylation of the IgG-Fc domain by evaluating the binding of serum RF and recombinant monoclonal RFs to recombinant monoclonal IgG with different galactosylation levels.

Methods

Detailed methods are described in the supplementary methods, available at *Rheumatology* Online. All patients signed informed consent forms for use of their samples; study approval was obtained from the local ethics committee Slotervaartziekenhuis and Reade.

Isolation of ACPA-IgG and non-ACPA-IgG from plasma

ACPA-IgG and non-ACPA-IgG were isolated from plasma of three RA patients by affinity chromatography, as previously reported [13]. The ACPA-IgG fractions showed high anti-citrulline reactivity in ELISA, whereas the non-ACPA-IgG fractions showed anti-citrulline reactivity at background (anti-arginine) levels (supplementary Table S1, available at *Rheumatology* Online).

Production of mAbs with different galactosylation levels

Anti-human rhesus D heavy and light chain were sequenced from a single human B cell clone from a hyperimmunized donor [14]. A single-gene vector containing anti-D or anti-2,4,6-trinitrophenol (TNP) IgG1 heavy and kappa light chain encoding sequences was cloned as described previously [15]. IgGs were produced in human embryonic kidney freestyle cells. Glycoengineering of IgGs and analysis of Fc glycans (supplementary Table S2, available at *Rheumatology* Online) by mass spectrometry was performed as previously described [16].

Production of monoclonal RFs

Two monoclonal IgM-RFs (mRFs) were produced. Variable heavy and variable light chain amino acid sequences for mRFs RF 61 [17] and RF-AN [18] were retrieved from the Protein Data Bank [19] (accession codes 2J6E and 1ADQ).

SPRi

For the SPRi experiments the IBIS MX96 imager was used. ACPA-IgG and non-ACPA-IgG isolated from three patients and the three differently galactosylated monoclonal anti-D antibodies were spotted at 30, 10 and 3 nM spotting concentrations onto pre-activated Easy2Spot G-type sensors. Samples were flowed over the sensor for 5 or 10 min in the association phase, followed by a 5 min dissociation phase and regeneration of the sensor with a 12 s pulse of 10 mM glycine-HCl.

Serum samples

Serum from 46 early RA patients from the Amsterdam region was used in the SPRi experiments, 41 RF^+ and 5 RF^-, determined by commercial assays. All patients signed informed consent forms for use of their samples. The RF^+ standard sample RELARES used in the experiments is a national reference serum with a defined IgM-RF level of 200 IU/ml [20].

ELISAs

For the ELISAs, target anti-TNP antibodies were diluted to 1 μg/ml and incubated for 2 h on 96-well flat-bottom plates that had been coated overnight at 4 °C with 10 μg/ml TNP-ylated human serum albumin in PBS.

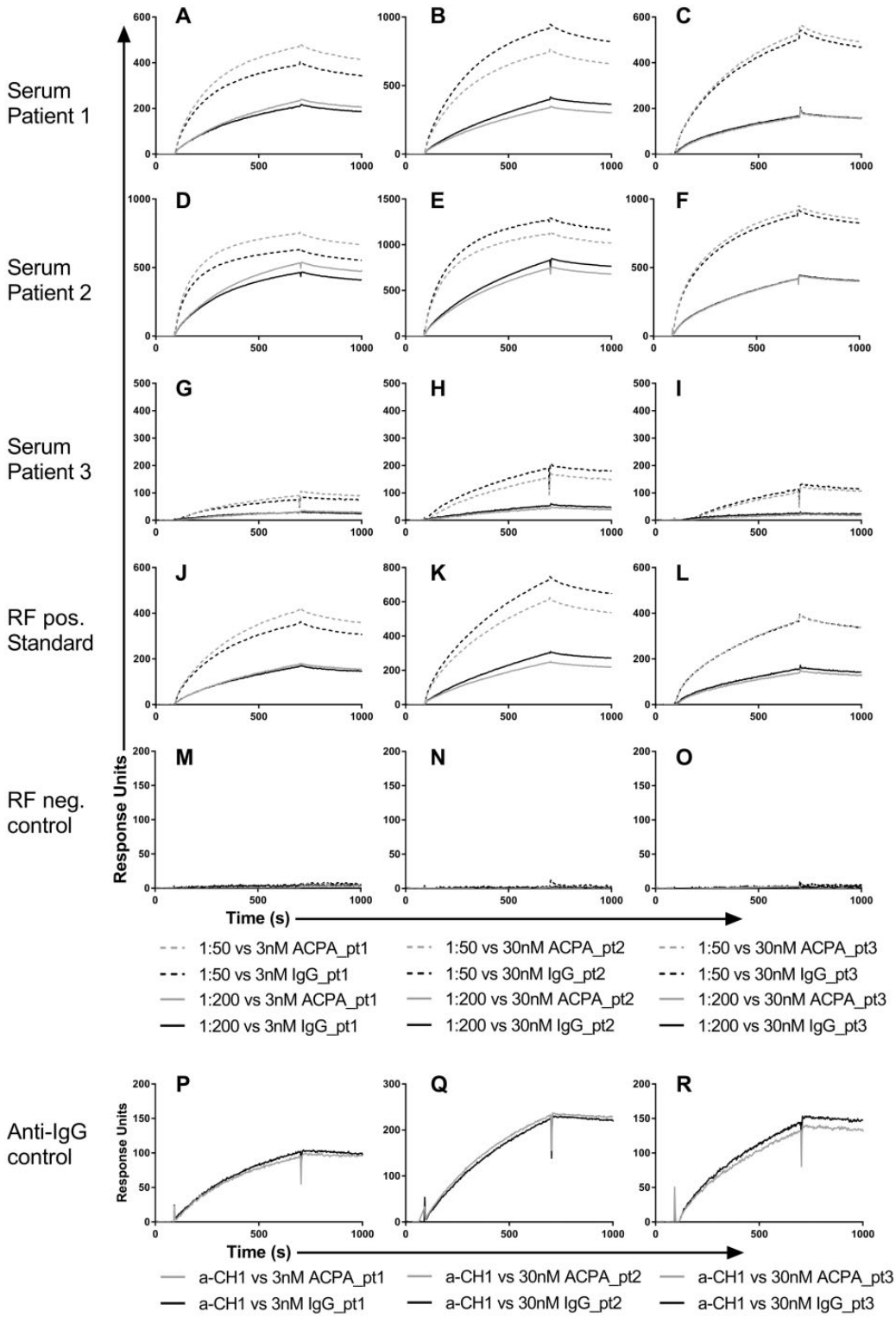
Results

RFs do not preferentially bind ACPA-IgG over non-ACPA-IgG

To compare the interactions of serum RFs with ACPA-IgG and non-ACPA-IgG, serum from three RF^+ $ACPA^+$ RA patients was flowed over a sensor to which ACPA-IgG and non-ACPA-IgG isolated from these same three patients had been coupled. The sensorgrams in Fig. 1 show that the RF response in the patient sera, as well as in the RF^+ reference serum, bound the ACPA-IgG and non-ACPA-IgG targets similarly. No binding was observed when RF^- serum was used. Comparing the two targets, the association and dissociation phases were similar. The shapes of the binding curves were not different for the RFs in the sera binding to autologous *vs* allogenic (non-)ACPA-IgG.

To determine whether differential binding of RFs to ACPA-IgG or non-ACPA-IgG could be a feature of a subpopulation of RF^+ RA patients, we next flowed diluted serum from 41 RF^+ and 5 RF^- RA patients over the sensor. The change in refractive index, expressed as

Fig. 1 Interactions of RF with ACPA-IgG and non-ACPA IgG



Sensorgrams show the interaction of RFs in sera from three different RF^+ RA patients (**A**–**I**), RF^+, (**J**–**L**) or RF^- (**M**–**O**) reference sera with ACPA-IgG and non-ACPA-IgG isolated from the same three RA patients. The association phase of the curves represents RF in sera (diluted either 1:50 as dotted lines or 1:200 as solid lines) binding under flow to IgG (ACPA, grey lines; non-ACPA, black lines) spotted on the SPR sensor. The dissociation phase starts when the flow of diluted serum is exchanged for buffer and RFs start to dissociate from their targets. Similar coupling of ACPA-IgG and non-ACPA-IgG was confirmed using an anti-CH1 llama antibody fragment flowed over the sensor (P-R). pt: patient.

response units in the sensorgrams, caused by binding of RF to the IgG ligands was compared for ACPA- and non-ACPA-IgG at one time point (350 s) at the end of the association phase (Fig. 2A). Fig. 2B shows that for the 38 RF^+ sera that gave a sufficient SPR shift, the relative binding to ACPA-IgG *vs* non-ACPA-IgG is virtually constant, despite an almost 20-fold variation in RF level, suggesting that it is unlikely that RFs preferentially binding to one over the other target make up a significant part of the RF response in sera of RA patients.

RFs do not preferentially bind to IgG with altered galactosylation

To specifically investigate the influence of the degree of galactosylation of the IgG Fc glycan in the interaction with serum RFs, we analysed the binding characteristics of the RF^+ reference serum and the RA patient sera to recombinant monoclonal IgG1 antibodies, glyco-engineered to have different levels of Fc galactosylation, previously determined by mass spectrometry [16] (supplementary Table S2, available at *Rheumatology* Online). Similar to the findings for the ACPA *vs* non-ACPA comparison, there were no major differences in RF association with or RF dissociation from IgG with normal, low or high galactose content of the Fc glycan (supplementary Fig. S1A and B, available at *Rheumatology* Online). None of the RF responses appeared to have a significant proportion of RFs exclusively binding to one of the three different glycoforms (Fig. 2C).

Interaction of two monoclonal IgM-RFs with ACPAs and differentially galactosylated IgGs

Two monoclonal IgM-RFs were used to investigate whether the specific localization of the RF-IgG interaction site on IgG-Fc influences RF binding of ACPA-IgG and non-ACPA-IgG and differently galactosylated IgGs. Monoclonal RF 61 binds IgG close to the C-terminal end of the CH3 domain [17], at a distance from the Fc glycosylation site, whereas RF-AN binds at the CH2–CH3 interface [18], where the glycan structure might influence the conformation of the RF epitopes and thereby RF binding (Fig. 2D). Both monoclonal RFs bound ACPA- and non-ACPA-IgG similarly (Fig. 2E). The observations in Fig. 2E that more RF 61 binds in the association phase compared with RF-AN and RF 61 dissociates faster are most likely caused by the higher affinity of RF 61 ($>5 \times 10^{-7}$ *vs* $>5 \times 10^{-4}$ kDa for RF-AN (supplementary Fig. S2, available at *Rheumatology* Online). Due to its higher affinity, RF 61 can probably bind to one or two IgG-Fcs using just one or two Fab domains, whereas RF-AN would need to make a more polyvalent connection for efficient binding, interacting with more IgG-Fcs, resulting in binding with a higher total avidity and slower dissociation.

Binding of RF 61 and RF-AN to three IgG targets with different galactosylation content was evaluated by ELISA. Since coating IgG targets directly to the plate might induce conformational changes and non-specific binding, we used anti-TNP antibodies specifically binding to TNP-coated plates as targets. Both RF-AN and RF 61 showed equal binding between the three differently galactosylated antibodies at different dilutions of mRF (Fig. 2F).

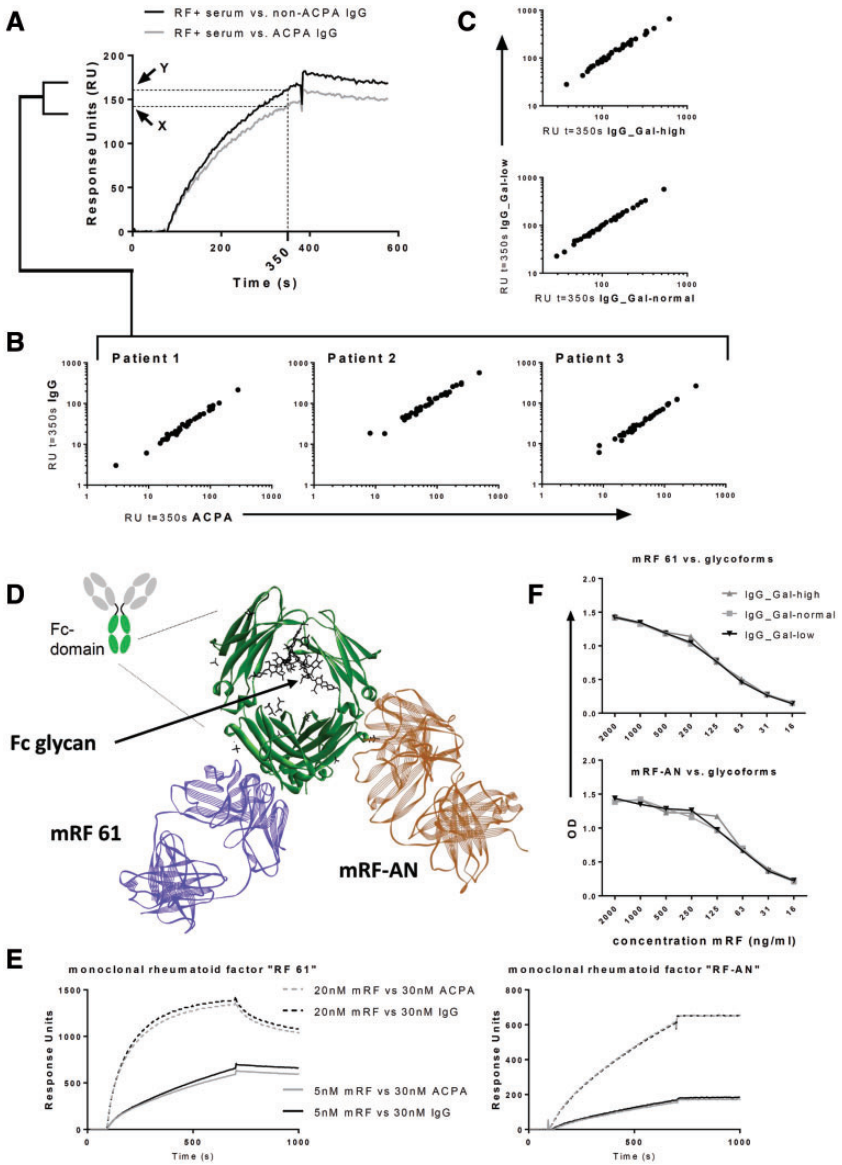
Discussion

RF and ACPA are the two major classes of autoantibodies assumed to play a role in RA. Both have predictive value for RA onset and severity. Interestingly, their combined presence is a better marker for severe disease than the presence of only RF or only ACPA [7]. While pathogenic properties of RF and ACPA in isolation have been studied in detail, only recent studies have investigated the combination [5–7]. In the present study we investigated whether RFs would preferentially interact with ACPA-IgG compared with non-ACPA-IgG. This hypothesis is supported by the finding that ACPA-IgG is highly agalactosylated, especially in RF^+ patients [10]. This may expose epitopes for RFs by inducing conformational changes in IgG-Fc [21]. Using a biosensor system, we found that serum RF showed comparable binding profiles when interacting with ACPA-IgG or non-ACPA-IgG. Among 41 RF^+ RA patients there were none with a dominant RF response preferentially binding to ACPA-IgG or non-ACPA-IgG. We conclude that ACPA-IgGs are not inherently bound better by RF than non-ACPA-IgGs. Still, RF–ACPA-IgG complexes may preferentially form over RF–non-ACPA-IgG complexes because of high local synovial production and the fact that a multiplicity of ACPAs binding to their citrullinated targets provides RFs with multiple Fc targets, facilitating a multivalent, high-avidity interaction. Furthermore, since our SPR setup measures total RF and our monoclonals are IgM-RF, it remains possible that IgA- or IgG-RF responses have a different fine specificity.

The importance of Fc galactosylation for binding of RF has been studied before with differing results. Soltys *et al.* [12] analysed the binding of synovial tissue–derived monoclonal RFs to polyclonal IgG preparations of varying galactosylation status. They found that some RFs bound better to IgG preparations with lower galactosylation content, whereas others bound independently of galactosylation content. Newkirk *et al.* [22] reported that monoclonal RFs bound equally to the Fc of polyclonal IgG from normal controls and polyclonal IgG with a lower galactosylation content from RA patients. Limited information is available on the binding properties of polyclonal IgM-RFs. Imafuku *et al.* [11] found higher IgM-RF reactivity in three of nine RA patients against agalactosyl IgG. The present study involved the serum of >40 RF^+ RA patients and used defined targets to evaluate the binding of RF to differently galactosylated IgG. The glycans on the recombinant monoclonal IgGs used as targets have been analysed by mass spectrometry to accurately define their galactosylation percentage [16].

Using an SPRi setup, RF-IgG interactions were studied in real-time. A limitation of the SPRi is that the amount of IgG target coupled to the sensor during the spotting procedure can vary. Therefore only sensor spots showing comparable signals with control anti-IgG-CH1 antibodies were used for comparing RF-IgG and RF-ACPA

Fig. 2 Serum RF and monoclonal RFs binding to ACPA-IgG, non-ACPA IgG and differently galactosylated IgG



(**A**) Representative sensorgram of RF$^+$ RA patient serum (1:200 dilution) binding to ACPA (grey line) and control IgG (black line). (**B**) The response at 350 s was recorded and plotted. Comparison of RF responses generated by incubating 38 RF$^+$ RA sera with an SPR sensor equipped with control IgG and ACPA-IgG isolated from three different patients as targets. (**C**) Comparison (as in B) of RF binding to IgG with low to IgG with high (upper panel) or normal Fc galactosylation. (**D**) Interaction sites of mRF 61 (purple) and mRF-AN (orange) Fab domains with IgG-Fc (green). Figure was created with Discovery Studio 4.5 software, using structures 2J6E and 1ADQ from the RSCB Protein Data Bank. (**E**) Interaction of monoclonal RFs with ACPA-IgG and non-ACPA-IgG. Representative examples of experiments with ACPA-IgG and non-ACPA-IgG from three patients. (**F**) Monoclonal RFs binding to monoclonal anti-TNP antibodies with low, normal or high

interactions. Since frequent regeneration of the sensor can affect the coupled IgG targets, we ran a standard sample before and after the experiments with the patient sera (supplementary Fig. S1, available at *Rheumatology* Online) to ensure signal stability. Using these tools we found that the degree of galactosylation of the Fc domain does not appear to influence binding dynamics of polyclonal RF responses in RA patients. Furthermore, no patients showed a dominant RF response recognizing only IgG with low, normal or high Fc galactosylation. Moreover, using two recombinant monoclonal IgM-RFs, one (mRF 61) that binds far away from the glycosylation site and one (mRF-AN) that binds much closer at the CH2–CH3 interface, we show that potential

conformational changes induced by regulating Fc galactosylation did not influence RF binding.

While changes in Fc glycosylation have been suggested to increase the pathological potency of ACPAs [23], a potential amplifying effect of RF through binding to the ACPAs does not seem to be dependent on these changes. This suggests that in the pathophysiology of RA, amplification of ACPA-mediated inflammation by RF can occur before ACPAs acquire a more pro-inflammatory phenotype.

In conclusion, although recent literature suggests that interactions between RF and ACPA have a catalytic effect on inflammation, the present study demonstrates that RFs do not preferentially bind to ACPAs over non-ACPA-IgG or agalatosylated IgG over IgG with normal or high galactosylation.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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