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Short title: IgG Rheumatoid Factors in Early Rheumatoid Arthritis

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ABSTRACT

Rheumatoid factor (RF), *i.e*. a family of autoantibodies against the Fc part of IgG, is an important seromarker of rheumatoid arthritis (RA). Traditional particle agglutination without disclosing the antibody isotype remains the predominating diagnostic method in clinical routine. Although IgG-RF attracts pathogenic interest, its detection remains technically challenging. The present study aimed at developing a set of tests identifying IgG-RFs directed against the four IgG subclasses. IgG-RF against either subclass of human IgG-Fc were analyzed with four novel enzyme-linked immunosorbent assays (ELISAs) utilizing four recombinant human Fc-gamma fragments (hIgG1-4) as sources of antigen. Sera from 40 patients with recent-onset RA (20 seropositive and 20 seronegative by IgM-RF and IgA-RF-isotype specific ELISA) were analyzed. Sera from 20 healthy blood donors served as reference. Among the IgM-/IgA-RF positive RA-sera, IgG-RF was found directed against hIgG1 and hIgG2, but not against hIgG3 or hIgG4. Significant correlations were seen between IgG-RF against hIgG2-Fc and IgM-RF ($r = 0.666$) levels. Further prospective studies are warranted to elucidate any correlation to disease course and outcome.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease. Insufficiently treated, it causes permanent tissue damage, joint deformities, functional impairments, reduced quality of life and premature death, mainly due to cardiovascular disease [1-4]. Although a positive test for rheumatoid factor (RF), *i.e*. autoantibodies against the Fc portion of immunoglobulin G (IgG-Fc), is a diagnostic and prognostic seromarker of RA [5-7], the serum levels of RF do not reflect disease activity. Most studies comparing RF with IgG-class antibodies to citrullinated proteins/peptides (ACPA), show that the latter are not only superior as diagnostic markers of RA, but also better predictors of disease course and outcome, and identify a uniform group of RA patients [8-12]. Both of these tests, without specifying antibody isotype or target antigen, serve as markers of RA according to the 2010 Euro-American Classification criteria [13], but "seropositive RA" still refers to RF-positive cases according to the World Health Organization International Classification of Diagnoses (ICD-10).

To most part, the clinical experience of RF refers to antibodies agglutinating IgG-sensitized particles. Although agglutination tests do not specify the RF-isotype, a positive test mainly implies IgM-class antibodies. Isotype-specific RF tests are also available, and it has been claimed that IgA-RF (especially in combination with IgM-RF) is a better prognostic seromarker than IgM-RF alone [14]. To qualify as an autoantibody, human RF with affinity for *human* IgG-Fc (hIgG-Fc) should be demonstrated. Although this was done already in the pioneering experiments by Kunkel *et al.*, and although IgG-RFs were suggested by this group already in the early 1960s [15], IgG-RF tests detecting antibodies against hIgG-Fc are not readily accessible in clinical routine diagnostics, mainly due to

methodological limitations. Specifically, assays for IgG-RF using human IgG as the antigen cannot utilize anti-human IgG-Fc detection antibodies, since they *per se* act as RFs. Nevertheless, it has been argued that IgG-RF is of special pathogenic interest [16, 17].

The present study was undertaken to develop novel IgG-RF analyses without specifying the RF subclass, but to analyze RF-specificity against recombinant human IgG-Fc of the four different subclasses in early RA.

MATERIALS AND METHODS

Subjects

The serum bank of the prospective Swedish early arthritis study "TIRA-1" [9] was utilized, *i.e.* sera from clinically well characterized patients with a diagnosis of early (onset of first joint swelling $\leq$ 12 months) RA. The sera were drawn at the time for inclusion in the TIRA-1 cohort during 1997-1998, and stored at -70°C. Before the planning of the present study, inclusion sera had been analyzed for the presence of RF measured by latex-particle agglutination, by ELISA regarding IgA- and IgM-class RF, and for IgG-class antibodies against the second generation cyclic citrullinated peptides (anti-CCP2, EuroDiagnostica, Arnhem, Holland) [9]. For the present study, inclusion sera from 20 patients seropositive regarding IgM- and IgA-RF together with a positive test for IgG anti-CCP (henceforth designated 'double-positive') were selected, as well as 20 sera from patients testing negative with the same autoantibody analyses ('double-negative'). Characteristics of the 40 TIRA-1 patients are summarized in Table 1. Sera from 20 healthy blood donors were used as reference. All studies involving the TIRA-based

material and control subjects have been approved by the regional ethics committee in Linköping, Sweden (Dnr 96-035; 01-169; M32-05; M144-05; M168-05). The TIRA biobank is approved by the Swedish National Board of Health and Welfare (SoS-1).

IgG-RF enzyme-linked immunosorbent assay (ELISA)

All serum samples were analyzed in triplicate, and the individual results expressed as mean values. The RF target antigens, hIgG-Fc subclasses 1-4, respectively, were expressed in a eukaryotic host (*Pichia pastoris*) and purified as previously described [18]. Microtitre plate wells (Corning Costar 96-well half area, Corning Inc. Corning, NY, USA) were incubated over night with the different subclasses of hIgG-Fc (5 µg/ml) prepared in 50 mM carbonate-bicarbonate buffer, pH 9.6. The wells were washed four times using phosphate-buffered saline supplemented with Tween-20 (PBS-T: 10 mM sodium phosphate, 140 mM NaCl, 2.7 mM KCl, 0.05% Tween-20, pH 7.4) between each step of the protocol and all incubations were performed at room temperature. PBS-T was added and the plate was incubated for 30 min. Serum samples were added in triplicates, diluted 1:100 in HEPES-buffered saline supplemented with EDTA and Tween-20 (HBS-ET: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20, pH 7.5). The plate was incubated for 1 h. As primary detection antibody, a polyclonal chicken IgY anti-human IgG-Fab (Gallus Immunotech Inc., Fergus, ON, Canada) was added, diluted 1:10000 in PBS-T and the plate was incubated for 1 h. The risk of cross-reactivity with light chains was avoided by solid-phase pre-adsorption of the primary antibody to a human IgM support. As secondary antibody, a biotinylated donkey IgG anti-chicken IgY (Gallus Immunotech Inc.,) was added, diluted 1:8000 in PBS-T and the plate was incubated for 1

h. For revelation, alkaline phosphatase-conjugated streptavidin was added, using the standard ABC amplification method (Vector Laboratories Inc., Burlingame, CA, USA). Then substrate (SIGMAFAST™ p-Nitrophenyl phosphate Tablets, Sigma, St. Louis, MO, USA) was added for approximately 30 minutes - until a control serum reached optical density (OD) of approximately 1.0. OD was monitored at 405 nm in a Multiscan Ascent plate reader (Thermo Labsystems Oy, Helsinki, Finland). Net OD (i.e. where antigen-free OD values have been subtracted) results regarding IgG-RF against the four distinct hIgG-Fc subclasses are presented as mean values of triplicate measurements. Suitable control experiments have included analysis of primary and secondary antibody specificity as well as samples without serum (with serum-free OD < 0.2 for all four antigens).

Statistics

Statistical analysis was performed by the Kruskal-Wallis rank sum test (KaleidaGraph version 4.1.1; Synergy Software, Reading, PA, USA) by comparing the three groups (double-positive, double-negative and control). P-values < 0.05 were considered significant. Correlation analyses were performed with Spearman's rho test (PASW Statistics version 18.0.0; SPSS Inc, Somers, NY, USA).

RESULTS

IgG-RF subclass specificity

When comparing the two patient groups (RF(+)/anti-CCP(+) and RF(-)/anti-CCP(-)) and the healthy blood donors regarding IgG-RF binding to the different subclasses of hIgG-Fc (1-4), ELISA data demonstrated significantly higher levels of IgG-RF directed

against hIgG subclasses 1 and 2 (Figure 1). In the double-positive patient group, the average level of additional anti-hIgG1-Fc and anti-IgG2-Fc reactivity was of equal magnitude (0.33 and 0.28, respectively). There was no apparent increase in binding of IgG-RF to hIgG3-Fc or hIgG4-Fc. Analyses regarding IgG-RF binding to the different hIgG-Fc subclasses, in the double-positive patient group, versus IgM-RF levels revealed significant correlations between IgG-RF against hIgG2-Fc and levels of IgM-RF ($r = 0.666$; $p = 0.001$). No significant correlations were seen regarding IgG-RF against the other hIgG-Fc subclasses.

DISCUSSION

Rheumatoid factor is a family of autoantibodies with great diversity regarding idiotypic specificity as well as antibody isotype and subclass. RFs can be induced by T-cell dependent as well as T-cell independent B-cell activation and hypermutation, probably even outside germinal centres [19]. Immunization with a variety of antigens (*e.g.* during infection or upon vaccination) can initiate RF production. This is likely to result from IgG antibody development and immune complex formation, and it has been implied that the stimulating Fc-gamma receptor (FcγR) IIa and the inhibitory FcγRIIb are crucial for RF formation and regulation [20, 21]. In this study we analyzed subclass-specific IgG-RF in sera from patients with early (<12 months from arthritis onset) RA, comparing seropositive cases (as defined by a positive IgM- and IgA-RF at inclusion) with seronegative. All of the selected IgM/IgA-RF positive cases were also positive regarding IgG anti-CCP, whereas the IgM/IgA-RF negative cases were consistently anti-CCP negative. Using human recombinant glycosylated antigens (Fcγ 1-4) and chicken anti-

human IgG-Fab, we found evidence of raised levels of IgG-RF against IgG of subclasses 1 and 2, but no evidence of IgG-RF against IgG3 or IgG4.

The first isotype-specific RF tests using ELISAs or radio-immunoassays (RIAs) were introduced already in the 1970s [22-24]. However, analysis of IgG-RF using human IgG (hIgG) as the source of antigen requires careful assay design, since both hIgG and IgG-RF may be recognized by the detection antibody. Instead, rabbit IgG is often used as source of antigen, for instance bound to microtitre plates coated with bovine serum albumin (BSA), followed by attachment of rabbit IgG anti-BSA antibodies [25]. BSA is often utilized also in other immunoassays to prevent non-specific surface attachment of immunoglobulins. In order to prevent false positive reactions due to occurrence of anti-BSA antibodies in serum, the dilution buffers also contain BSA which neutralizes the anti-BSA antibodies. Notably, however, this formation of BSA-anti-BSA complexes may in turn also neutralize RF. Since anti-BSA antibodies are commonplace [26] and considering the risks of erroneous results due to their presence [27, 28] we discourage from the use of BSA as a blocker of non-specific immunoglobulin binding in immunoassays.

An attractive IgG-RF RIA, developed by Carson *et al.* in 1977, exploited isolated hIgG-Fc as target antigen, and polyclonal $F(ab')_2$ fragments directed against IgG-Fab as detection antibody to avoid false positive results [22]. Later, McDougal *et al.* refined this concept using a monoclonal anti-Fab antibody to the Fd portion of IgG (localized partly to the variable heavy chain domain and to the first constant heavy domain, C_H1) for revelation in both RIA and ELISA formats [29]. Similarly, Elson *et al.* used a monoclonal anti-Fab detection antibody against IgG-C_H1 [30], and isolated Fcγ fractions

of IgG1 and IgG2, and intact IgG3 and IgG4, as antigen substrates for ELISA subclass-specific detection of IgG-RF.

The results presented here demonstrate the utility of recombinant hIgG-Fc fragments of human origin as the source of antigen for IgG-RF ELISA. An advantage with this approach is that no other parts of IgG than Fcγ interfere with RF-binding, thereby reducing the risk of false positive results. Also, it allows for precise control of the antigens at the molecular level, eliminating antigen heterogeneity as a source of limited assay reproducibility. In the present study we found high levels of IgG-RF directed against hIgG1-Fc, as well as raised antibody levels against hIgG2-Fc, but not against hIgG3 or hIgG4. The clinical implications of our findings of IgG1- and IgG2-specific IgG-RFs are not immediately obvious but, speculatively, IgG2 antibodies elicited by carbohydrate antigen exposure (*e.g.* of microbial origin), may be a driving force to develop IgG2-specific IgG-RF.

Similar RF affinity patterns as those presented here have previously been described for IgM- and IgG-class RFs derived from RA patients [30, 31]. Elson *et al.*, who studied the subclass specificity of human IgG-RF using intact hIgG3 and hIgG4 and heterogeneous pools of hIg1-Fc and hIgG2-Fc as antigens and BSA as a blocking agent, found positive IgG-RF with the target-antigen pattern IgG1≈IgG2>IgG3=IgG4. In their study, levels of IgG1- and IgG2-specific RF were significantly raised in relation to healthy donors, and solely in the patients with extraarticular disease (nodules, vasculitis or Felty's syndrome) [30]. Miyata *et al.*, who used intact hIgG1-4 as antigen and tested sera from 8 patients with established RA and 7 previously tested IgG-RF positive sera from healthy subjects, reported that IgG-RF was predominantly found in the IgG1 fraction [32]. Contrasting to

most other studies on IgG-RF reactivity, Tokano *et al.*, reported that a substantial proportion (8 of 49) of RA patients had IgG-RF recognizing IgG3 [33]. The reasons for the discrepancies between the different studies are not obvious, but may be sought in differences in the patient populations, medication strategies and immunoassay designs.

In contrast to previous studies on subclass-specificity of IgG-RF, we investigated sera from patients with recent onset arthritis at the time for RA diagnosis (before treatment with disease-modifying agents), and consistently used molecularly homogenous, recombinant hIgG-Fc fragments as antigen substrates and Tween-20 to prevent non-specific immunoglobulin binding. In our setup with subclass-specific hIgG-Fc fragments, we conclude that IgG-RF specific for IgG2 is pronounced in seropositive RA (as defined by IgM- and IgA-RF ELISAs) and that it coincides strongly with IgM-class RF. We also found evidence of IgG1-specific IgG-RF occurrence without significant correlation to IgM- or IgA-RF, but no signs of IgG3- or IgG4-specific IgG-RF using sera from healthy blood donors as reference material. It cannot be excluded however, that IgM-RF and/or IgA-RF interfere with IgG-RF binding (and vice versa). As regards the level of IgG1-specific RFs, it may have been overestimated due to the tendency of IgG1 form Fc-Fc interactions in the solid-phase assay [34], a phenomenon that has recently been reported to be inhibited by the addition of Fc-derived peptides from IgG1, IgG2, and IgG4 [35]. Furthermore, IgG4, which constitutes only a small fraction of total circulating IgG, has often been recognized as an "RF", but this is probably to great extent explained by the tendency of IgG4 to form aggregates due to Fc-Fc interactions [36]. In the present study, however, IgG4-RFs were rare. Yet, it is possible that such Fc interactions could contribute to the relatively high OD values for healthy donors and double-negative

patients. Furthermore, it cannot be excluded that the high background levels observed in the reference sera regarding reactivity with IgG1, IgG3 and IgG4, may actually reflect occurrence of IgG-RFs in healthy individuals. Apart from additional studies on IgM- and IgA-depleted sera, inclusion of trace amounts of pepsin [36] may give further information on the antigen-specificity of IgG-RFs.

To conclude, we present a novel approach for subclass-specific IgG-RF analysis using a panel of recombinant hIgG(1-4)-Fc fragments. Our study showed IgG1- and IgG2-specific IgG-RF in seropositive rheumatoid arthritis. Future studies on larger patient groups, and analysis of consecutive serum samples together with data on disease course and outcome measures will be needed to evaluate the diagnostic and prognostic potentials of subclass-specific IgG-RF analysis.

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TABLES

Table 1. Baseline characteristics of the early RA patient groups

	RF(+)/anti-CCP(+)	**RF(-)/anti-CCP(-)**
Number of subjects	20	20
Females	15 (75%)	13 (65%)
Age, mean years (SD)	53.7 (13.1)	60.3 (18.8)
DAS28, mean (SD)[a]	5.31 (0.97)	5.59 (0.9)
Mean number of ACR 1987 criteria[b]	4.7	4.35
Subcutaneous rheumatoid nodules	2 (10%)	1 (5%)
Bony erosions on X-ray (hands/feet)	4 (20%)	2 (10%)
Anti-CCP[c], mean , U/mL (SD)	786 (545)	< 25
IgA-RF[d], mean, U/mL (SD)	112 (134)	4.4 (3.4)
IgM-RF[d], mean, U/mL (SD)	384 (239)	15.2 (5.9)

[a]DAS28 = 28-joint count disease activity score, [b]ACR 1987 criteria = American College of Rheumatology classification criteria, [c]CCP = cyclic citrullinated peptides, [d]RF = rheumatoid factor enzyme immunoassay.

FIGURE LEGENDS

Figure 1. IgG-RF ELISA with different human IgG-Fc subclasses used as antigen. Filled circles represent the net OD signals expressed for the patient-group denoted double-positive, *i.e.* IgM-RF(+), IgA-RF(+) as well as IgG anti-CCP(+). Open circles represent the net OD signals expressed for the patient-group denoted double-negative, *i.e.* IgM-RF(-), IgA-RF(-) as well as IgG anti-CCP(-). Open diamonds represent the net OD signals expressed for the control-group consisting of 20 healthy blood-donors. (NS = not significant).

FIGURES

Figure 1