

Concise Report

IgG1 and IgG4 are the predominant subclasses among auto-antibodies against two citrullinated antigens in RA

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Objective. Antibody subclasses reflect specific immunological processes and may be indicative of the underlying pathological pattern in an autoimmune disease like RA. We therefore quantified anti-cyclic citrullinated peptides (CCP) and anti-citrullinated vimentin (MCV) IgG subclass titres in RA patients and compared them with the respective titres of antibodies directed against the varicella zoster virus (VZV) and to total serum titres.

Methods. Sera of 77 patients fulfilling the ACR criteria for RA were collected. An IgG subclass-specific ELISA system was then established and combined with commercially available MCV, CCP and VZV pre-coated microtitre plates.

Results. Even though IgG1 is the predominant subclass among antibodies against CCP and MCV in RA patients, IgG4 is second with respect to titres and frequencies. This increase in IgG4 among RA-specific antibodies is independent of disease duration and does not reflect a general skewing of the immune response in these patients as overall serum titres and antibodies directed against VZV show a normal distribution of IgG1, IgG2, IgG3 and IgG4.

Conclusion. Elevated IgG4 titres are specific for auto-antibodies against citrullinated antigens in RA and are indicative of a Th2-biased environment during the generation of auto-reactive plasma cells. We discuss here an indirect role for IgG4 auto-antibodies in hindering the elimination of auto-reactive B and plasma cells and thus driving the autoimmune process.

KEY WORDS: Cyclic citrullinated peptides, Mutated citrullinated vimentin, Rheumatoid arthritis, Auto-antibodies, IgG subclasses.

Introduction

RA is a chronic autoimmune disease primarily affecting the joints with both, the innate and the adaptive immune system contributing to its pathogenesis [1]. Auto-antibodies are of particular interest as they help to diagnose the disease, yet are also indicative of tolerance gone astray. Interestingly, mutated citrullinated vimentin (MCV) and cyclic citrullinated peptides (CCP) have recently been identified as target antigens in RA and anti-MCV and anti-CCP antibodies turned out to be highly specific for RA and of good prognostic value [2, 3]. Analysing these auto-antibodies in detail may help to elucidate their specific involvement in RA and will help to unravel previous and ongoing pathogenic processes.

Auto-antibodies can directly trigger pathogenic processes as in types II and III hypersensitivity. However, the various IgG subclasses mediate different immunological effector functions. Since investigations on the IgG subclasses of anti-CCP antibodies are rare [4, 5] and missing in case of the anti-MCV antibodies, the objective of the present study was to quantify anti-CCP and anti-MCV IgG subclass titres in RA patients and compare them to the respective titres of antibodies directed against the varicella zoster virus (VZV) and to the total serum level of each IgG subclass.

Methods

Patients

Our cohort comprises 77 RA patients from Rostock and Berlin. All patients fulfill the ACR revised criteria for the classification

of RA. The patients were selected for being positive for CCP and none of them had undergone a therapy based on B-cell depletion. They were between 22 and 86 yrs of age and had a disease duration ranging from recent onset to 38 yrs. This study was approved by the local ethics committee and informed consent was given by all patients prior to serum sampling.

IgG subclass-specific ELISA

An IgG subclass-specific ELISA was established using commercially available ELISA plates coated with CCP (EUROIMMUN, Luebeck, Germany), MCV (Orgentec, Mainz, Germany) and VZV antigen (Virion/Serion GmbH, Wuerzburg, Germany) and peroxidase-conjugated human IgG subclass-specific antibodies for detection. To quantify CCP- and MCV-specific antibodies, the sera were diluted 1:400 to analyse IgG1 and 1:20 for IgG2, IgG3 and IgG4. To quantify VZV-specific antibodies, the sera were diluted 1:100 for IgG1 and 1:5 for IgG2, IgG3 and IgG4. Dilutions of the human IgG subclass-specific antibodies were 1:15 000, 1:10 000, 1:20 000 and 1:25 000 for sheep-anti-human IgG1 (Binding Site Inc., Schwetzingen, Germany), mouse-anti-human IgG2, -IgG3 and -IgG4 (AbD Serotec, Oxford, UK), respectively. All sera and detection antibodies were diluted in PBS with 1% BSA (Carl Roth GmbH, Karlsruhe, Germany) and incubated for 1.5 h at room temperature. Specific binding of detection antibodies was visualized by tetramethyl benzidine (Dako, Glostrup, Denmark). The control sera delivered with the kit systems for CCP, MCV and VZV were used for normalization of the data.

The specificities of the detection antibodies were verified on ELISA plates coated with each of the IgG subclasses (Sigma-Aldrich, Munich, Germany) using dilutions from 10 µg/ml to 5 ng/ml. Optical densities (ODs) were calculated as OD₄₅₀ (specific wave length) minus OD₆₅₀ (reference wave length) as determined by an automated plate reader (Millenia Kinetic Analyser, DPC, CA, USA). For the CCP-, MCV- and VZV-specific ELISA, an OD > 0.211 > 0.101 and > 0.085, respectively, was considered positive (4-fold the minimum OD). All assays were run in duplicates.

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Determination of total IgG subclass levels

Standard nephelometry was used to determine the absolute amounts of IgG subclasses in patients' sera.

Statistics

The Mann–Whitney U-Test was used to calculate the significance levels of the OD differences between the IgG subclasses. Fisher's exact test was used to calculate odds ratios (OR), 95% CIs and levels of significance for comparison of subclass-positive and -negative sera. Pearson's correlation was used to analyse an association between disease duration and IgG4 levels.

Results

Even though IgG1 is the predominant subclass among antibodies against CCP and MCV in RA, IgG4 is conspicuously elevated

We investigated here the IgG subclasses of antibodies directed against CCP and MCV. To that end we analysed the sera of 77 RA patients who were positive for both autoantibodies. In a first step, we used an IgG subclass-specific ELISA system combined with standard ELISA plates to quantify titres, using the ODs as an

indirect indicator. Figure 1A and B summarize the ODs of the anti-CCP and anti-MCV antibodies. Medians were 0.849, 0.095, 0.085, 0.158 and 0.095, 0.061, 0.064, 0.065 for anti-CCP- and anti-MCV-specific IgG1, IgG2, IgG3 and IgG4, respectively. As expected, IgG1 is the most abundant subclass for both antibodies. Interestingly, the anti-CCP-specific IgG4 titre is significantly higher than the ones for IgG2 and IgG3 with *P*-values of 0.008 describing the differences (Fig. 1A). As for the anti-MCV-specific IgG subclass titres, IgG4 is slightly elevated compared with IgG2 and IgG3; however, the differences do not reach statistical significance (Fig. 1B).

In a second step, we analysed the percentages of RA patients positive for the four IgG subclasses of anti-CCP and anti-MCV. Again, IgG1 is the most prominent subclass (data not shown). Table 1 summarizes the results on IgG2-, IgG3- and IgG4-positive patients. While 33 of the 77 patients carrying anti-CCP antibodies are positive for the IgG4 subclass, it is only 14 and 20 who are positive for IgG2 and IgG3, respectively. The differences between IgG4 and IgG3 and between IgG4 and IgG2 are statistically significant with ORs of 2.12 and 3.35 describing the difference. The corresponding 95% CIs are 1.03, 4.49 and 1.53, 7.61, respectively (Table 1). The same 33 patients positive for IgG4 antibodies against CCP are also positive for the IgG1. Among the anti-MCV antibody-positive patients, 33 are positive for IgG4,

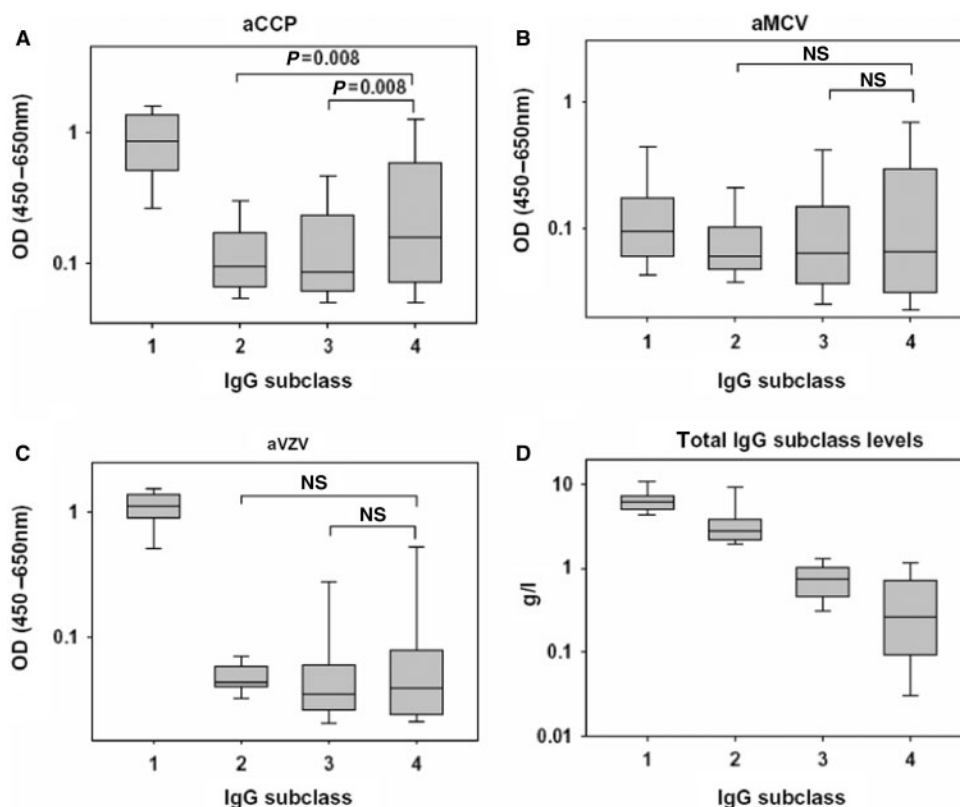


FIG. 1. IgG1 followed by IgG4 are the predominant subclasses among autoantibodies in RA. Box plots show the ODs corresponding to the various IgG subclass titres against CCP (A), MCV (B) and VZV (C). (A and B) 77 RA sera were tested and dilutions were 1 : 400 (IgG1) and 1 : 20 (IgG2, IgG3, IgG4). (C) 19 RA sera were tested and dilutions were 1 : 100 (IgG1) and 1 : 5 (IgG2, IgG3 and IgG4). Box plot (D) demonstrates the overall serum levels of 10 patients tested for the IgG subclasses. *P*-values were calculated by Mann–Whitney U-test, NS: not significant.

TABLE 1. RA patients positive for IgG4 auto-antibodies outnumber the ones positive for IgG2 and IgG3

Antigen	IgG2 <i>n</i> (%)	IgG3 <i>n</i> (%)	IgG4 <i>n</i> (%)	IgG4 vs IgG3 OR (95% CI)	IgG4 vs IgG2 OR (95% CI)
CCP	14 (18)	20 (26)	33 (43)	2.12 (1.03, 4.49)*	3.35 (1.53, 7.61)**
MCV	20 (26)	26 (34)	33 (43)	1.47 (0.73, 2.98) ^{NS}	2.12 (1.03, 4.49)*

Significance level indicated by NS (not significant), * (0.05 > *P*-value > 0.01) and ** (0.01 > *P*-value > 0.001).

22 are positive for both IgG1 as well as for IgG4 and 20 and 26 are positive for IgG2 and IgG3, respectively. Here, the difference between IgG4 and IgG2 reaches statistical significance with an OR of 2.12 and a 95% CI of 1.03, 4.49 indicating that for both target antigens IgG1 followed by IgG4 are the predominant antibody subclasses.

Importantly, no correlation was found between the duration of the disease and the level of IgG4 titres for both, CCP ($P=0.2$; $r=-0.13$) and MCV ($P=0.6$; $r=-0.06$).

Antibody responses against VZV and total sera of RA patients are not skewed towards IgG4

To find out whether the predominance of IgG4 over IgG2 and IgG3 is specific for auto-antibodies or is a general characteristic of the immune response in RA patients, we analysed the IgG subclass distribution against the common VZV as well as total serum IgG1, IgG2, IgG3 and IgG4 levels. We therefore selected the IgG1-, IgG2-, IgG3- and IgG4-high producers for anti-CCP and anti-MCV and tested 19 of them for anti-VZV antibodies and 10 for total serum Ig subclasses. For VZV antibodies, the median OD was 1.123 for IgG1 and 0.044, 0.035 and 0.039 for IgG2, IgG3 and IgG4, respectively with no significant differences among the latter three. In total serum, the mean concentrations for IgG1, IgG2, IgG3 and IgG4 were 6.24, 2.75, 0.74 and 0.266 g/l, corresponding to norm values (Fig. 1D). Elevated IgG4 titres thus seem to be specific for anti-citrullinated peptide auto-antibodies in RA.

Discussion

We show here that IgG4 significantly outweighs IgG2 and IgG3 among anti-CCP antibodies in RA patients. This result holds true for both, serum titres and frequency of patients positive for the various subclasses. Likewise, IgG4 among the anti-MCV antibodies also significantly outweighs IgG2 with respect to the frequencies of positive patients while the situation is less clear-cut for serum titres of subclasses: the upper quartile of IgG4 among anti-MCV antibodies does show an obvious increase compared with IgG3 and IgG2; however, these differences do not quite reach statistical significance (Fig. 1). So far, there is only one previous publication directly comparing titres of IgG subclasses among anti-CCP antibodies and our data are in line: Chapuy-Regaud *et al.* [4] analysed anti-citrullinated fibrinogen antibodies and also showed that IgG4 outweighed IgG2 and IgG3 with respect to both serum titres and frequencies of patients positive for the various subclasses. However, our data do not seem to confirm the notion that prolonged antigenic stimulation promotes IgG4 production as subclass titres did not correlate with disease duration [6]. Any additional studies on IgG subclasses among anti-citrullinated peptide antibodies either correlate a specific subclass to the course of disease or the response to therapy but do not allow the direct comparison of subclass titres [5, 7].

While IgG1 in general is the most abundant IgG subclass, IgG4 titres outweighing IgG2 and IgG3 are extraordinary and have so far only been described for ANCA in WG and anti-dsDNA antibodies in SS [8, 9]. In contrast, in SLE anti-dsDNA antibodies of the IgG2 and IgG3 subclass outweigh IgG4 [9]. In our patients, skewing of the antibody response towards IgG1 and IgG4 seems to be specific for auto-antibodies as the overall serum titres are in the norm range with IgG4 being the least abundant subclass and IgG4 among the antibodies against the common VZV IgG4 seems to be negligible.

Clearly, elevated IgG4 titres among auto-antibodies in RA are indicative of a Th2-biased environment [10]. Whether ectopic germinal centres as described in the pannus tissue of RA patients can provide this kind of environment or whether autoantibody-producing plasma cells are generated centrally is still a matter of debate [11]. Likewise, the pathological function of IgG4

autoantibodies in driving the autoimmune process needs to be clarified. The extremes presenting in the clinic range from individuals completely lacking IgG4 to hyper IgG4 disease. While the former are either asymptomatic or may show an increased susceptibility to encapsulated bacteria [12], hyper IgG4 disease is a systemic condition characterized by an organ-restricted inflammation with infiltrates of IgG4-positive plasma cells and fibrosis [13]. So far, an active pathological role for IgG4 has only been described for blistering skin disease [14].

While IgG1 mediates all the obvious immunological effects like classical complement activation and Fc receptor (FcγR)-mediated phagocytosis, antibody dependent cellular cytotoxicity (ADCC), degranulation and release of inflammatory mediators, IgG4 seems to be the most inert among the subclasses [15]. An indirect effect though may be mediated through the dynamic Fab arm exchange that renders the antibodies monovalent. Due to the failure to cross-link, monovalent antibodies prevent the formation of immune complexes required to trigger the inhibitory FcγRIIB on auto-reactive B- and plasma cells. Thus, the predominance of IgG4 may indirectly obstruct the elimination of auto-reactive cells of the B lineage and support the accumulation of autoantibodies of the IgG1 subclass [6, 15]. In fact, an FcγRIIB variant that causes reduced immune complex uptake has recently been shown to be a strong predictor of joint damage in RA [16].

Independent of whether the prevailing subclasses are cause or consequence of pathogenic processes, they may provide a tool to classify and eventually to develop novel treatment plans for autoimmune diseases.

Rheumatology key messages

- IgG1 is the predominant subclass and IgG4 is conspicuously elevated among antibodies against CCP and MCV in RA.
- Elevated IgG4 titres among auto-antibodies in RA are indicative of a Th2-biased environment.

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