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**Author**: Scherer, Hans Ulrich  
**Title**: Basic disease mechanisms in rheumatoid arthritis  
**Issue Date**: 2013-04-25
Immunoglobulin 1 (IgG1)
Fc-glycosylation profiling of anti-citrullinated peptide antibodies from human serum.

Scherer HU, Wang J, Toes RE, van der Woude D, Koeleman CA, de Boer AR, Huizinga TW, Deelder AM, Wuhrer M.

ABSTRACT

In several autoimmune disorders, including rheumatoid arthritis (RA), autoantibodies are thought to be the driving force of pathogenicity. Glycosylation of the Fc-part of human immunoglobulins is known to modulate biological activity. Hitherto, glycosylation of human IgG-Fc has been analyzed predominantly at the level of total serum IgG, revealing reduced galactosylation in RA. Given the pathogenic relevance of autoantibodies in RA, we wished, in the present study, to address the question whether distinct Fc-glycosylation features are observable at the level of antigen-specific IgG subpopulations. For this purpose, we have developed a method for the micro-scale purification and Fc-glycosylation analysis of anti-citrullinated peptide antibodies (ACPA). ACPA represent a group of auto-antibodies that occur with unique specificity in rheumatoid arthritis patients. Their presence associates with increased inflammatory disease activity and rapid joint destruction. Results indicate that ACPA of the IgG1 subclass vary considerably from total serum IgG1 with respect to Fc-galactosylation, with galactosylation being higher on ACPA than on serum IgG1 for some patients, while other patients show higher galactosylation on serum IgG1 than on ACPA. Using this method, studies can be performed on the biological and clinical relevance of ACPA glycosylation within rheumatoid arthritis patient cohorts.
INTRODUCTION

Human immunoglobulin G (IgG) occurs in four subclasses, which share a conserved N-glycosylation site in their Fc region. Notably, Fc-N-glycosylation is very different from Fab N-glycosylation, the latter occurring in the variable regions of approximately 20% of the polyclonal IgG population from human serum. Fab N-glycans show a high degree of galactosylation and sialylation whilst Fc-N-glycans are predominantly non-sialylated and truncated structures (i.e., biantennary N-glycans lacking galactose residues). This difference has been found for IgGs from the human circulation\textsuperscript{1,2} as well as for monoclonal IgGs expressed in mammalian cell culture\textsuperscript{3-6}.

Fc-glycosylation varies much between individuals: Fc-N-glycan galactosylation shows a pronounced age dependency, with a rather low degree of galactosylation for young children, a maximum of galactosylation around age 25, and a decrease in galactosylation at higher age\textsuperscript{7-9}. During pregnancy the degree of IgG Fc-galactosylation increases and drops again after delivery\textsuperscript{10-12}, and various inflammatory diseases including rheumatoid arthritis (RA) and tuberculosis are associated with decreased galactosylation of IgG\textsuperscript{10,12,13}.

Fc-glycosylation modulates various biological activities of IgG: first, fucose-deficient IgG1 is binding with higher affinity to the Fcγ receptor IIIA than its core-fucosylated counterpart\textsuperscript{14,15}. Sialylation does likewise influence the binding of the various IgG subclasses to different activating and inhibitory Fcγ receptors\textsuperscript{16}. This modulation of the interaction with Fcγ receptors by IgG Fc-glycosylation features strongly influences the efficacy of IgGs in antibody dependent cellular cytotoxicity (ADCC) assays\textsuperscript{14,17-20}. Second, in a mouse model, sialylation of the Fc-part N-glycan has been shown to be involved in the anti-inflammatory properties of human IgG\textsuperscript{16}, which seems to be at the basis of the beneficial effects of donor IgG (intravenous immunoglobuline; IVIG) for the treatment of various inflammatory and autoimmune diseases. In this study Kaneko\textit{et al.} showed in a mouse model that a change in total IgG Fc-glycosylation characterized by an altered sialylation is underlying the pro-versus anti-inflammatory activity of antibodies\textsuperscript{16}.

Most studies on IgG glycosylation have analyzed N-glycans released from total human serum IgG, which includes Fc N-glycans and Fab N-glycans. IgG glycosylation, however, is different for the Fc-part and the Fab part\textsuperscript{1-6}, and varies between IgG subclasses\textsuperscript{12}. Only recently, Mehta\textit{et al.} have for the first time analyzed IgG glycosylation at an antigen-specific level\textsuperscript{21}, while all the other studies have not addressed the occurrence of possible specific glycosylation features of antigen-specific subpopulations of IgG.

In order to obtain a deeper insight into antibody glycosylation, we decided to analyze the Fc N-glycans of antigen-specific subpopulations of serum IgG. We chose for analyzing the Fc-glycosylation of anti-citrullinated peptide antibodies (ACPA), which are
autoantibodies of predominantly the IgG1 subclass that occur in RA-patients with high specificity. For this purpose, we have established a micro-scale capturing assay for ACPA from serum followed by monitoring of Fc-glycosylation at the glycopeptide level in an IgG-subclass specific manner. Our results clearly indicate that ACPA IgG1 differs from total serum IgG1 in the degree of galactosylation of the Fc N-glycan.

MATERIALS AND METHODS

IgG purification from total human serum

Protein A-Sepharose beads (GE Healthcare, Eindhoven, The Netherlands) were washed three times with 10 volumes of PBS. 15 μl of beads per well were applied to a 96-well filter plate (Multiscreen Solvinert, 0.45 μm pore-size low-binding hydrophilic PTFE; Millipore, Billerica, MA). The volume was brought to 150 μl with PBS, and 2μl of serum was applied per well. The plate was sealed with tape and incubated on a shaker for 1 h. The beads were washed 5x with 200 μl PBS under vacuum (approximately 100 mbar). After washing 2x with 200 μl water, immunoglobulins (IgG1, IgG2, and IgG4) were eluted with 100 μl of 100 mM formic acid (p. a. for mass spectrometry; Merck, Darmstadt, Germany) into a V-bottom microtiter plate (Nunc, Roskilde, Denmark). Samples were dried by vacuum centrifugation.

Purification of anti-citrullinated peptide antibodies (ACP A)

Sera for the isolation of ACPA were chosen based on their reactivity against a second generation citrullinated peptide antigen (CCP2) in a commercially available ELISA system (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). For purification, sera of ACPA-positive and ACPA-negative RA-patients were diluted 1:10 in dilution buffer (provided with the ELISA kit) and incubated in CCP2-coated ELISA plates for 1 hour at 37°C. Supernatants were discarded and plates were washed 2x with PBS followed by 2x washing with 25 mM ammonium bicarbonate (Merck, Darmstadt, Germany). ACPA were then eluted using three different approaches:

1. Elution with sodium-isothiocyanate: After washing, samples were incubated with 100 μl 5M sodium-isothiocyanate for 15 min at room temperature. Eluates were collected from the ELISA plate and immediately dialyzed (dialysis membranes with molecular weight cut-off 12-14 kD, cat.-no. 551300; Thermo Fisher Scientific, Waltham, MA) over night at 4°C against PBS. ACPA IgG was purified from PBS using Prot A beads (see 2.1) and subjected to trypsin treatment (2.4) and Fc-glycosylation analysis (2.5).

2. In-plate tryptic digest: After washing, 30 μl of 25 mM ammonium bicarbonate buffer containing 200 ng of sequencing grade modified trypsin (Promega, Leiden, The Netherlands) was added to each well of the ELISA-plate. Plates were incubated for
30 min on a shaker at room temperature followed by overnight incubation at 37°C. Samples were subjected to Fc-glycosylation analysis (2.5).

3. Elution with 100 mM formic acid: After washing, 50 µl 100 mM formic acid was added to each well to achieve antibody elution. Plates were incubated for 15 min at room temperature. Eluates were transferred to 96 well V-bottom microtiter plates, dried in a vacuum centrifuge, and subjected to trypsin treatment (2.4) and Fc-glycosylation analysis (2.5).

For comparison of the methods, the isolation procedures 2 (in-plate tryptic digest) and 3 (formic acid elution) were performed in parallel on one plate in order to avoid inter-assay variation.

**Enzyme-linked immunosorbant assay (ELISA)**

Detection of human IgG: Human IgG was detected using an antibody-based sandwich ELISA system. In brief, 96 well flat-bottom microtitration plates (Maxisorb; Nunc, Copenhagen, Denmark) were coated with polyclonal rabbit anti-human IgG (Dako A0423; Glostrup, Denmark) diluted 1:5000 in coating buffer (50mM sodium bicarbonate buffer pH 9.6) for 2 hours at 37°C. Plates were blocked with PBS containing 1%BSA for 30 min at 37°C. Samples and standard (pooled normal human serum) were added to the wells diluted in PBS containing 1%BSA and 0.05% Tween and incubated for 30 min at 37°C. Biotinylated goat F(ab’)2 anti-human IgG diluted 1:40000 (Biosource International AHI1309, Camarillo, CA) in PBS containing 1%BSA and 0.05% Tween was used for detection. Plates were subsequently incubated with poly-horse radish peroxidase labeled with streptavidine (diluted 1:750 in PBS containing 1%BSA and 0.05% Tween) (Sanquin M2051, Amsterdam, The Netherlands) and bound IgG was visualized using 2,2’-azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma). Color signal was measured at an optical density of 415nm using a conventional ELISA reader. Plates were vigorously washed between each step with PBS containing 0.05% Tween 20. Antibody dilutions were optimized by serial titrations for detection of the standard IgG prior to performance of the assay.

Detection of ACPA: ACPA were detected using a second generation ELISA-kit (Immunoscan RA Mark 2; Euro-Diagnostica) according to the manufacturer’s instructions.

**IgG digestion with trypsin**

A 20 µg aliquot of trypsin (sequencing grade; Promega, Leiden, The Netherlands) was dissolved in 4 ml of 25 mM ammonium hydrogencarbonate. Within 1 min after preparation, 40 µl of this mixture was added per well to the dried purified antibodies. Samples were shaken (1 min), incubated overnight at 37°C, and stored at -20°C until usage.
Nano-LC-ESI-ion trap-MS

Prior to analysis, microtitration plates containing tryptic digests of IgG were subjected to centrifugation (10 min at 3000 g). 0.2 μl aliquots of trypsinized Protein A eluates (corresponding to IgG1, IgG2, and IgG4 from 10 nl of serum) and 2 μl aliquots of trypsinized samples obtained from the various ACPA purification procedures were applied to a reverse-phase column (C\textsubscript{18} PepMap 100Å, 3 μm, 75 μm x 150 mm; Dionex/LC Packings, Amsterdam, the Netherlands) using an Ultimate nano-LC, a Famos autosampler, and a Switchos trap-column system (Dionex/LC Packings). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water and 0.4% acetonitrile) at a flow rate of 150 nL/min. After injection of the samples, a gradient was applied to 25% eluent B (95% acetonitrile, 5% water containing 0.1% formic acid) in 15 min and 70% eluent B at 25 min followed by an isocratic elution with 70% eluent B for 5 min. The eluate was monitored by UV absorption at 215 nm.

The LC system was coupled via an online nanospray source to an Esquire HCT ultra ESI-IT-MS (Bruker Daltonics, Bremen, Germany) equipped with an electron transfer dissociation module (PTM Discovery System™) and was operated in the positive ion mode. For electrospray (1100-1250 V), capillaries (360 μm OD, 20 μm ID with 10 μm opening) from New Objective (Cambridge, MA, USA) were used. The solvent was evaporated at 165°C employing a nitrogen stream of 7 L/min. Ions from \textit{m/z} 600 to \textit{m/z} 1800 were registered. For glycosylation profiling, the mass spectrometer was used in the MS mode. The HPLC method resulted in a resolution of the glycopeptides based on the peptide moiety with IgG1 glycopeptides eluting first, followed by IgG4 and IgG2 glycopeptides. Moreover, glycopeptides with neutral glycan moieties tended to elute earlier than glycopeptides with antennae sialylation, as described before\textsuperscript{12}. For both the neutral and the acidic glycopeptides of each IgG subclass, average mass spectra were generated over a 1 min elution range.

RESULTS

Purification of anti-citrullinated peptide antibodies (ACPA) was achieved using commercially available ELISA plates which contain immobilized citrullinated peptide antigens. Sera from rheumatoid arthritis patients that were previously tested as ACPA-positive or ACPA-negative (controls) were diluted in PBS and added to the ACPA ELISA plate wells in order to allow binding to the immobilized citrullinated peptide antigens. Unbound serum IgG as well as other serum components were washed away, and IgGs bound to the ACPA plates were analyzed using three different procedures, as schematically presented in Figure 1.
In a first attempt, antibodies bound to the ACPA plates were eluted using sodium isothiocyanate. For this experiment sera were used from four ACPA-positive rheumatoid arthritis patients and four ACPA-negative rheumatoid arthritis patients, as determined by ELISA (Fig. 2A). Sodium isothiocyanate was removed by dialysis against PBS, and the amount of captured IgG was quantified for the 8 sera using an IgG-ELISA (Fig. 2B). The results showed the presence of captured IgG for the 4 ACPA-positive RA patients. For the ACPA-negative RA-patients no IgG was detected after dialysis, i.e., no unspecific co-purification of non-ACPA IgGs was detected. These results indicate the successful purification of ACPA with high specificity using an affinity capturing approach at the ELISA plate format. Virtually no background level of unspecifically bound IgG was detected.

The obtained amounts of ACPA were sufficient for Fc-glycosylation analysis by nano-LC-ion trap-MS at the tryptic glycopeptide level (Fig. 3B) using a recently described method\(^\text{12}\). The results obtained for patient RA10 are shown in Fig. 3B. Next to ACPA IgG1 glycosylation profiles, the Fc-glycosylation profiles of IgG1 from total serum were registered after ProtA capturing and trypsin treatment (Fig. 3B). IgG1 Fc glycopeptides were identified based on their specific tryptic peptide moiety (E\(_{293}\)EQYNSTYR\(_{301}\)\(\text{G}^\text{G0}\)), which allows to distinguish them from glycopeptides of other IgG subclasses based on the resulting glycopeptide masses and elution positions\(^\text{12}\). Interestingly, the obtained glycosylation profile of ACPA IgG1 differed from the profile obtained for total IgG1 of the same patient (Fig. 3A): whilst total IgG1 exhibited a typical rheumatoid arthritis glycosylation profile with a low degree of galactosylation (high G0; glycoform at \(m/z\) 878.7), ACPA of RA10 showed a much higher degree of galactosylation, with G1 as the major glycoform (\(m/z\) 932.8). Moreover, sialylation was slightly higher in ACPA

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**Figure 1:** Schematic representation of the three strategies which were evaluated for purification of anti-citrullinated peptide antibodies (ACPA).
compared to total serum IgG, with a relatively higher signal for the core-fucosylated biantennary monosialylated glycoform (triple protonated species at $m/z$ 1083.8).

In a next step we wished to confirm that the glycosylation profiles obtained from the eluates of the capturing plate were reflecting total ACPA. We wanted to be sure that specific ACPA glycosylation features were not missed due to the possible depletion for high-affinity ACPA which might not be eluted from the capturing plate with the applied sodium isothiocyanate treatment. We addressed this point by comparing the elution profiles obtained by strategy 1 (Fig. 3B) with ACPA glycosylation profiles obtained by tryptic digest of the captured IgG1 directly on the capturing plate (strategy 2). This comparison was performed for 4 sera from ACPA-positive RA patients and for 2 sera from ACPA-negative RA patients. No IgG glycopeptides were registered for the ACPA-negative RA patients, whilst the Fc-glycosylation profiles of the ACPA-positive patients obtained by strategy 2 (on-plate tryptic digest; shown for patient RA10 in Fig. 3C) were very similar to those obtained by strategy 1 (sodium isothiocyanate elution; shown for patient RA10

Figure 2: Demonstration of ACPA purification by ELISA.

Eight sera from rheumatoid arthritis (RA) patients were assayed for ACPA by ELISA, which revealed 4 ACPA-positive and 4 ACPA-negative RA patients (A). ACPA were purified following strategy 1 (Fig. 1), followed by the measurement of IgG in the eluates by ELISA (B). The results show that ACPA were successfully purified, and no background levels of non-ACPA IgG were detected.

In a next step we wished to confirm that the glycosylation profiles obtained from the eluates of the capturing plate were reflecting total ACPA. We wanted to be sure that specific ACPA glycosylation features were not missed due to the possible depletion for high-affinity ACPA which might not be eluted from the capturing plate with the applied sodium isothiocyanate treatment. We addressed this point by comparing the elution profiles obtained by strategy 1 (Fig. 3B) with ACPA glycosylation profiles obtained by tryptic digest of the captured IgG1 directly on the capturing plate (strategy 2). This comparison was performed for 4 sera from ACPA-positive RA patients and for 2 sera from ACPA-negative RA patients. No IgG glycopeptides were registered for the ACPA-negative RA patients, whilst the Fc-glycosylation profiles of the ACPA-positive patients obtained by strategy 2 (on-plate tryptic digest; shown for patient RA10 in Fig. 3C) were very similar to those obtained by strategy 1 (sodium isothiocyanate elution; shown for patient RA10.
Figure 3: IgG Fc-glycosylation analysis of total serum IgG1 of patient RA10 (A) and of ACPA of the same patient purified following strategy 1 (B), strategy 2 (C), and strategy 3 (D). Nano-LC-MS signals are shown for the glycopeptides with neutral N-glycan chains (left panels) and acidic N-glycan chains (right panels). Triple protonated signals were registered throughout. Signals representing a fucosylated glycoform are labeled with filled triangles, whilst signals of non-corefucosylated glycoforms are labeled with open triangles. *, non-glycopeptide signal or irrelevant adduct.
Figure 4: IgG Fc-glycosylation analysis of total serum IgG1 and ACPA IgG1 of patients RA9 (A), RA14 (B), RA18 (C), and RA30 (D). Nano-LC-MS signals are shown for the glycopeptides with neutral N-glycan chains (left panels) and acidic N-glycan chains (right panels). Triple protonated signals were registered throughout. Signals representing a fucosylated glycoform are labeled with filled triangles, whilst signals of non-corefucosylated glycoforms are labeled with open triangles. *, non-glycopeptide signal or irrelevant adduct.
in Fig. 3B). The rationale for using strategy 2 was that all ACPA adsorbed to the capturing plate would be hydrolyzed by trypsin, and the observed glycosylation profiles would reflect with great certainty total ACPA Fc-glycosylation. Because the obtained ACPA Fc-glycosylation profiles were virtually identical, both on-plate tryptic digest and sodium isothiocyanate elution are suitable for ACPA Fc-glycosylation profiling.
While the direct tryptic treatment on the ACPA capturing plate provided a sensitive and rapid manner for ACPA Fc-glycosylation profiling, these samples showed a rather high level of background signals. Therefore, we decided to evaluate another, simple method, which involves ACPA elution with 100 mM formic acid, drying of the samples in a vacuum centrifuge, and tryptic digestion of ACPA (strategy 3, Fig. 3D). The obtained ACPA Fc-glycosylation profiles were again very similar to the one obtained following strategies 1 and 2 (shown for patient RA10 in Fig. 3D). Due to its simplicity and the good quality of the data, strategy 3 was selected for further analysis of ACPA Fc-glycosylation.

In addition, the Fc-glycosylation profiles of total IgG1 (upper panels) and ACPA IgG1 (lower panels) were analyzed for 4 additional ACPA-positive RA patients (Figure 4), which are patients RA9, RA14, and RA30 (all included in Fig. 2), as well as patient

![Graph](https://example.com/graph.png)

**Figure 5:** Relative expression levels of glycoforms of IgG1 from total serum and ACPA for 5 RA patients.

Relative intensities are given for the triple-charged ions of the various glycoforms (Fig. 3A and B). Total serum IgG1 and ACPA IgG1 for patient RA10 were measured three times and seven times, respectively, and average relative intensities as well as standard deviations are given for the fourteen glycoforms which were registered (A).
IgG1 Fc-glycosylation profiling

RA18 (not included in Fig. 2). The glycosylation data for these four patients are represented in histograms (Fig. 5B-E), together with the data for patient RA10 (Fig. 5A). A comparison of the total IgG1 Fc-glycosylation profiles for the five patients showed marked differences. The major differences were in the degree of galactosylation (upper panels of Fig. 4A-D). Moreover, some differences in sialylation were observed. Notably, the ACPA Fc-glycosylation profiles observed for the 4 RA patients (lower panels Fig. 4A-D) differed from the profiles obtained for total IgG1. RA10 showed a much higher degree of galactosylation for ACPA compared to total IgG1 (Fig. 3; Fig. 5A). RA9 and RA14 likewise showed a higher degree of galactosylation, though less prominent. RA30, in contrast, showed a rather high degree of galactosylation for total IgG1 and a much lower galactosylation of ACPA. ACPA of RA18 likewise showed a lower degree of galactosylation than total serum IgG1 of RA18. Next to the differences in galactosylation, some small variations in sialylation were observed between ACPA and total serum IgG1.

In conclusion, ACPA Fc-glycosylation profiles can be monitored efficiently with the protocol established in this study. The observed glycosylation patterns show differences in galactosylation and sialylation between individual RA patients. Moreover, these first analyses indicate that ACPA glycosylation may be very different from total IgG1 glycosylation.

DISCUSSION

In several autoimmune disorders autoantibodies are thought to be of crucial pathogenetic relevance. Understanding their mode of action might greatly enhance our perception of disease pathogenesis. We here describe an assay which allows the detailed analysis of IgG Fc-glycosylation at the level of autoantigen-specific IgG subpopulations from human serum. The assay makes use of a commercially available ELISA plate with covalently attached citrullinated peptide antigens, which allows the capturing of anti-citrullinated peptide antibodies (ACPA). ACPA are auto-antibodies that are often of the IgG1 subclass and are highly specific for rheumatoid arthritis (RA)\(^{22-24}\). ACPA are continuously produced by B-cells, and have been implicated in disease pathogenesis\(^{25, 26}\). Antibodies are eluted from the capturing plate using a low pH step, followed by trypsin cleavage and profiling of the Fc glycopeptides by nano-LC-ESI-ion trap-MS. This method was found to be highly specific, as no background IgG signals could be detected for RA-patients which were judged to be ACPA-negative on the basis of ELISA results. First results obtained for a limited set of ACPA-positive RA patients indicate differences between ACPA IgG1 Fc-glycosylation and total IgG1 Fc-glycosylation. In particular, ACPA IgG1 appear to be remarkably different from total serum IgG1 with respect to galactosylation, with some patients showing higher galactosylation for ACPA than for
total serum IgG1 and other patients showing higher galactosylation on serum IgG1 than on ACPA. Moreover, some difference between ACPA and total serum IgG1 was found in the degree of sialylation. The latter difference may possibly represent a secondary effect: in the IgG glycosylation process in the Golgi, the attachment of less galactoses means less acceptor structures for sialyl transferases, which may explain the reduced sialylation to a large extent. Notably, for fucose and sialic acid, which are known to exhibit acid-labile glycosidic bonds, no hydrolysis was observed with the applied elution conditions: for a monoclonal antibody, no differences were observed in the degree of Fc glycopeptide sialylation and fucosylation with and without treatment with 100 mM formic acid followed by vacuum centrifugation (data not shown). Hence, the registered values for fucosylation and sialylation are not expected to be influenced by hydrolysis during sample preparation.

ACPA Fc-glycosylation does not appear to feature a particularly high percentage of G0 glycoforms. Hence, the high degree of G0 found for IgGs of many RA patients does not seem to be caused by a very high degree of G0 on ACPA. It may be speculated, therefore, that the low degree of Fc-galactosylation in IgG of RA patients may occur rather on a broad population of IgGs with various antigen specificities and might not be the result of particularly high G0 of RA-associated autoantibodies. However, to be able to draw firm conclusions on the specific features of ACPA glycosylation and its biological and clinical information content, ACPA Fc-glycosylation will have to be analyzed in future studies within large clinical cohorts.

The establishment of a sensitive method for the analysis of ACPA Fc-glycosylation is an important step towards analyzing and understanding IgG glycosylation at an antigen-specific level. With the large variation shown in these first analyses, ACPA fulfill an important prerequisite for a biomarker candidate (large spreading of values which reflects information content). Analyses in various clinical RA cohorts will be performed to evaluate the diagnostic potential of ACPA glycosylation and reveal whether it indeed represents a marker for disease activity or disease progression / prognosis.

Recently Metha et al. have analyzed IgG heavy chain glycosylation of anti-alpha-Gal IgGs from serum of hepatitis C virus-infected persons with fibrosis and cirrhosis and have found increased levels of galactose-deficient glycoforms (IgG-G0) compared to anti-alpha Gal IgGs from healthy controls. Anti-Gal IgGs and ACPA are hitherto the only antigen-specific IgGs studied for Fc-glycosylation. Efforts should be made to analyze the glycosylation of IgGs directed against other auto-antigens, xeno-antigens, vaccines, and infectious agents. For this purpose more assays for the Fc-glycosylation analysis of other antigen-specific IgG subpopulations will have to be established in the future. The application of these assays to large study populations should provide a deeper insight into the complexity and regulatory potential of IgG glycosylation at the antigen-specific level, which may represent important, hitherto neglected, immunological parameters. Based on
the results of Kaneko et al.\textsuperscript{16}, it will be interesting to compare Fc-glycosylation profiles for different phases of antibody responses and B-cell development. Next to population studies, more functional studies in animal models will be necessary to understand the regulatory events which make B-cells produce antibodies with a certain Fc-glycosylation profile. In addition, the effects of the differences in IgG Fc-glycosylation on ADCC and complement-mediated cytotoxicity need further attention. These combined efforts should provide us with a deeper understanding of antibody-mediated immunological effects and may reveal new rationales for modulating the immune response in various diseases.
REFERENCES


IgG1 Fc-glycosylation profiling


