Aberrant Lectin-Binding Activity of Immunoglobulin G in Serum from Rheumatoid Arthritis Patients

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Structural studies of oligosaccharide chains of immunoglobulin G (IgG) in serum have revealed a specific galactosylation deficiency associated with rheumatoid arthritis (RA). Using a two-site lectin–immunofluorometric assay, I studied the interaction of IgG with immobilized lectins. Compared with control IgG, IgG purified from RA patients' sera bound up to 40-fold more strongly to immobilized Bandeiraea simplicifolia agglutinin II, a lectin that specifically binds agalacto forms of other glycoproteins. However, inhibition studies and treatment of IgG with glycosidase suggested that only a minor part of this binding was mediated by agalacto oligosaccharides of IgG. Furthermore, these IgG samples bound even more intensively to some other immobilized lectins, including Ricinus communis agglutinin (RCA). The binding to RCA was not inhibited by lactose, a hapten sugar of RCA, whereas other lectin species in solution effectively inhibited it. Compared with intact RA IgG, isolated F(ab')2 fragments displayed only low RCA-binding activity. These results indicate the existence of a carbohydrate-nondependent interaction between RA IgG and different plant lectins. With use of immobilized lectins, the lectin–immunofluorometric assay was rapid and reproducible for measuring the aberrant lectin-binding activity of IgG directly in diluted serum samples.

The etiology of rheumatoid arthritis (RA), a systemic chronic inflammatory disease, is unknown but is thought to have an autoimmune component (1). Autoantibodies reacting with IgG—i.e., "rheumatoid factors"—are a characteristic finding in serum and synovia from RA patients (2, 3). The antigenic site in IgG has been localized to the Fc portion, but there is no evidence of changes in amino acid sequence in this region of IgG in RA. On the other hand, N-linked oligosaccharides of IgG from RA patients differ from those of control IgG with respect to the degree of galactosylation (4, 5). This results in more agalacto oligosaccharides in the Fc region that lack both sialic (neuraminic) acid and galactose and carry terminal N-acetylgalactosamine residues (4). It has been hypothesized that this galactose deficiency is responsible for inducing rheumatoid factors by exposing a structure in the Fc region that either forms a new antigenic determinant or possesses a lectin-like activity, resulting in complex formation and autoaggregation (4). The mechanism behind the galactose deficiency of IgG in RA has been suggested to be a decreased activity of galactosyltransferase (EC 2.4.1.151) in B-cells (6). On the other hand, oxygen-centered free radicals were recently reported to destroy terminal galactose residues on IgG (7), suggesting that the galactose deficiency of IgG may also be a consequence of inflammation-dependent post-synthetic degradation.

The amount of agalacto oligosaccharides in IgG has been measured by detaching chemically N-linked oligosaccharides from isolated IgG and separating the labeled oligosaccharides by gel filtration (8). By this method, the amount of agalacto oligosaccharides in IgG was found to be increased in both adult and juvenile RA and to fluctuate with disease activity (8). Another approach used for determining the degree of galactosylation of IgG was to quantify total galactose content in isolated IgG by gas–liquid chromatography (9). By this method a substantially decreased galactose content of IgG was seen, not only in RA but also in some other chronic inflammatory diseases such as systemic lupus erythematosus and Crohn's disease.

Oksanen and I recently described (10) a lectin–immunochemical assay for determining galactosylation variants of glycoproteins by using a solid-phase-bound Bandeiraea simplicifolia II lectin (BS-II) to capture glycoproteins carrying terminal N-acetylgalactosamine residues and a europium-labeled detecting antibody. Using this lectin–immunofluorometric assay, I have studied the interaction of IgG with immobilized lectins and found that agalacto oligosaccharide chains of IgG are poorly accessible for immobilized BS-II. However, RA IgG, but not control IgG, binds intensively to different immobilized lectins by a mechanism that depends on neither the carbohydrates of IgG nor the antigen-binding Fab fragments. I describe here a rapid, reproducible method for quantifying this aberrant lectin-binding IgG in serum samples.

Materials and Methods

Materials

Lectins from Ricinus communis (120-kDa form), Lotus tetragonolobus, Triticum vulgari, Arachis hypogaea, Da turra stramonium, and Erythrina cristagalli, lectin I from Ulex europaeus, lectin II from Bandeiraea simplicifolia, concanavalin A, human transferrin, Aspergillus niger β-galactosidase (EC 3.2.1.23), and jackbean β-N-acetylgalactosaminidase (EC 3.2.1.30) were from Sigma Chemical Co., St. Louis, MO. Vibrio cholerae neuraminidase (sialidase, EC 3.2.1.18) was from Calbiochem–Behring Diagnostics, La Jolla, CA. GlcNAc-β-O-2-2-carboxymethylethylthio)ethyl, bovine serum albumin was from Carbohydrates International, Arlöv, Sweden. Eu-labeled monoclonal mouse antibody reacting with the Fc region of all human IgG subclasses was kindly provided by Wallac Biochemical Laboratories, Turku, Finland. Polyclonal rabbit antibodies against human IgG (Fc-region-specific) and human transferrin, and goat antibody against rabbit IgG, were from Dako Corp., Glostrup, Denmark. Polyclonal rabbit antihuman Fab fragment antibody was from Cappel–Organon Teknika, Turnhout, Belgium. Isothiocyanatophenylidithi-
ylenetriaminepentaacetic acid complexed with europium (Eu chelate) and "enhancement solution" (containing, per liter, 1 g of Triton X-100 surfactant, 6.8 mmol of potassium hydroxide, 100 mmol of acetic acid, 50 μmol of tri-n-octylphosphine oxide, and 15 μmol of 2-naphthoyltrifluoroacetone) (II) were from Wallac Biochemical Laboratories.

Methods

Labeling of antibodies. I labeled the antibodies with Eu-chelate, essentially as described before (II). In brief: IgG was precipitated with 180 g/L Na2SO4 solution, dissolved in NaHCO3 buffer (0.1 mol/L, pH 9.3), and reacted with a 100-fold molar excess of the Eu chelate overnight at 4 °C. The labeled antibodies were purified by gel filtration on a 0.8 × 20 cm column of Sephacryl S-200 HR (Pharmacia-LKB, Bromma, Sweden) and stored in Tris-buffered saline (per liter, 2 mmol of Tris hydrochloride buffer, pH 7.4, 150 mmol of NaCl, and 5 mmol of Na2SO4) containing bovine serum albumin, 1 g/L.

Serum samples. Serum samples from patients with active seropositive RA fulfilling the criteria of the American Rheumatism Association for definite or classical RA were kindly provided by Robert von Essen, Rheumatism Foundation Hoepital, Heinola, Finland. Control sera were obtained from healthy individuals and 20 patients with acute infections having C-reactive protein concentrations of 50 to 150 mg/L.

Purification of IgG. IgG was purified from diluted serum samples by ion-exchange chromatography on "Quick-Sep" columns (Isolab, Akron, OH) (12). From pooled serum samples, IgG was precipitated with 180 g/L Na2SO4 solution, dialyzed against Tris hydrochloride buffer (20 mmol/L, pH 8.8) containing 30 mmol of NaCl per liter, and further purified on a 3 × 10 cm column of diethylaminoethyl-Trisacryl M (Pharmacia-LKB) eluted with the same buffer. For some experiments, IgG was further fractionated by affinity chromatography on a 0.8 × 2.5 cm column of Protein A-Sepharose (Pharmacia-LKB). After IgG (at most 10 mg) was applied to the column in 2 mL of phosphate buffer (0.1 mol/L, pH 7.0), the column was washed with 10 mL of the same buffer and eluted with glycine hydrochloride buffer (0.1 mol/L, pH 3.0), followed by brief neutralization of the fractions with Tris buffer (1 mol/L, pH 9.0).

Quantification of IgG. IgG was measured nephelometrically (Turbox; Oriola, Helsinki, Finland). The protein concentration of isolated IgG fragments was determined with a Coomassie Brilliant Blue G-250 dye-binding assay (Bio-Rad Labs., Cambridge, MA).

Preparation of proteolytic fragments of IgG. Papain treatment of a Protein A-binding IgG fraction was carried out by incubating, for 4 h at 37 °C, 10 mg of IgG in 1 mL of phosphate buffer (0.1 mol/L, pH 7.0) containing 1 mg of cysteine hydrochloride, 0.5 mg of EDTA, and 0.1 mg of papain (EC 3.4.22.2) per milliliter (13). The reaction mixture was diluted with 1 mL of the phosphate buffer, and Fab and Fc fragments were separated by affinity chromatography on the Protein A-Sepharose column as described above, the Fab fragments being eluted with the phosphate buffer and the Fc fragments with the glycine buffer. After concentration (Speed Vac Concentrator; Savant Instruments, Hicksville, NY), the fragments were further purified by gel filtration on a 1 × 40 cm column of Sephacryl S-200 HR eluted with Tris-buffered saline at a flow rate of 25 mL/h.

F(ab')2 fragments were prepared from the same IgG preparation by incubating, for 6 h at 37 °C, 5 mg of IgG in 1 mL of Na acetate buffer (0.1 mol/L, pH 4.4) containing 100 μg of pepsin (EC 3.4.23.1) per milliliter. The fragments were purified from the reaction mixture by gel filtration on the Sephacryl S-200 HR column. The purified fragments migrated as single bands in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Phastgel, Pharmacia-LKB) under nonreducing conditions.

Glycosidase treatments and reduction of IgG. IgG was treated for 24 h at 37 °C with sialidase (0.05 kU/L), β-galactosidase (0.1 kU/L), or β-N-acetylgalactosaminidase (0.1 kU/L) in sodium acetate buffer (50 mmol/L, pH 4.5) containing 1 mmol of CaCl2, 1 mmol of MnCl2, and 5 mmol of NaN3 per liter. The IgG was then reduced in Tris hydrochloride buffer (0.2 mol/L, pH 8.2) containing 20 mmol of diethiothreitol per liter, for 1 h at room temperature. Then the pH was lowered to 7.5 with Tris hydrochloride buffer (0.2 mol/L, pH 7.0), and the IgG was alkylated with 0.1 mol/L iodoacetamide for 1 h at room temperature. These treatments broke the disulfide bonds of IgG but did not dissociate the heavy and light chains, as confirmed by native and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Phastgel).

Lectin-immunofluorometric assay. Coat polystyrene microtiter well strips (Titertek; Labeyystems, Helsinki, Finland) overnight at 4 °C with lectins or reference proteins, 10 mg/L in NaHCO3 buffer (0.1 mol/L, pH 9.0). Wash the wells twice with washing solution (per liter, 9 g of NaCl, 0.2 g of Tween 20, and 0.5 g of NaN3) and incubate for 3 h at room temperature with Tris-buffered saline containing 1 g of bovine serum albumin per liter. To 200 μL of the assay buffer (50 mmol/L Tris hydrochloride buffer, pH 7.7, containing, per liter, 9 g of NaCl, 0.5 g of NaN3, 5 g of bovine serum albumin, 0.5 g of bovine immunoglobulin, and 0.1 g of Tween 40) add 25 μL of purified IgG or serum samples diluted 40-fold in the same buffer. (With immobilized BS-II, use an assay buffer containing in addition 0.1 mmol of CaCl2 and 0.1 mmol of MnCl2 per liter.) Incubate the wells, in duplicate, for 2 h at room temperature, with IgG terminal shaking. Wash the wells twice and incubate with the Eu-labeled antibodies—25 ng of the monoclonal antibody per well, or about 100 ng of IgG per well for the polyclonal antibodies—in assay buffer for 1 h at room temperature, with continuous shaking. Wash the wells four times, add 200 μL of the enhancement solution, and 20 min later measure the fluorescence (I used an LKB 1230 Arcus fluorometer, Wallac Biochemical Laboratories).

Results

Interaction of IgG with BS-II

To study the interaction of RA-associated IgG, reported to carry terminal N-acetylgalactosamine residues (4), with the N-acetylgalactosamine-specific BS-II lectin, I incubated, in BS-II-coated microtiter wells, IgG samples purified from serum of RA patients and healthy controls. I measured the amount of the bound IgG by time-resolved immunofluorescence, using a Eu-labeled monoclonal antibody reacting with the Fc region of IgG. The binding of IgG from RA patients was highly variable, some samples displaying up to 40-fold greater binding than IgG from control sera (Figure 1). When serum samples were incubated in the lectin-coated microwells without a prior purification, dose-response curves for the binding of IgG were similar to those observed with the corresponding purified IgG samples.
Fig. 1. Binding of IgG from rheumatoid arthritis patients to microtiter wells coated with BS-II

Purified IgG (---) or diluted serum (----) from two patients with RA (Δ, ○) and healthy control (□) were incubated in the wells at different IgG concentrations. The bound IgG was detected by time-resolved immunofluorometry with use of an Eu-labeled monoclonal antibody reacting with the Fc portion of IgG. Background signal, i.e., the binding of the detecting antibody to the BS-II-coated wells (about 4 x 10^4 counts), not subtracted.

This indicated that the increased binding was not due to denaturation of IgG during purification. However, the binding of IgG to BS-II was only partly inhibited by N-acetylgalactosamine at 0.1 mol/L—at most by 30% (Figure 2)—even though this monosaccharide totally inhibits the binding of agalacto forms of transferrin and α1-acid glycoproteins to immobilized BS-II (10). Bovine serum albumin, modified to contain on average 21 covalently linked N-acetylgalactosamine residues per molecule, induced only a similar inhibition as free N-acetylgalactosamine (Figure 2) and β-N-acetylgalactosaminidase treatment of IgG (data not shown) decreased the binding only slightly. These findings suggested that the increased binding of IgG from RA patients to the immobilized BS-II was only partly mediated by terminal N-acetylgalactosamine residues of IgG.

Specificity of the Aberrant Lectin-Binding Activity of IgG

To gain insight into the specificity of the N-acetylgalactosamine-resistant interaction of IgG with BS-II, I compared IgG samples purified from a pooled specimen of serum from RA patients showing increased binding to BS-II (RA IgG) with those of healthy controls (control IgG) for binding to different lectins and various other proteins immobilized onto microtiter wells. RA IgG displayed about 30- to 40-fold more binding than did control IgG to four of the lectins studied, i.e., UEA, LTA, RCA, and ECA (Figure 3). Two other lectins, DSA and BS-II, bound about 20-fold more RA IgG than did control IgG, whereas WGA, PNA, and ConA exhibited only low binding of both IgG samples. Wells coated with albumin only or with various other proteins representing extracellular matrix proteins (collagens, laminin, fibronectin), human serum proteins (transferrin), or glycoproteins exhibiting different types of oligosaccharide chains (fetuin and ovalbumin) bound both RA and control IgG to a similar extent (Figure 3). As Figure 4 shows, control IgG also bound to the immobilized lectins, but for a similar response with RA IgG a concentration of control IgG about 50-fold higher was required. Figure 4 also demonstrates the lack of binding of the IgG samples to wells coated with bovine serum albumin only.

The binding of RA IgG to immobilized RCA was not affected by lactose at the 0.1 mol/L concentration that inhibits the binding of asialo-glycoproteins to RCA-Sephrose (14) (data not shown). Correspondingly, the hapten sugar of LTA, fucose (15), was ineffective in inhibiting the binding of RA IgG to the immobilized LTA. High salt concentrations (1.5 mol/L NaCl) and polyacrylamides (dextran sulfate and heparin at a concentration of 1 g/L) were also without effect, indicating that the interaction was not ascribable to a charge effect. Neither EDTA nor CaCl2 or

**Fig. 2.** Effect of N-acetylgalactosamine on the binding of IgG from a pool of RA sera to the immobilized BS-II lectin

Purified IgG was incubated in the lectin-coated microtiter wells at concentrations indicated, without inhibitor (○) or in the presence of N-acetylgalactosamine, 0.1 mol/L (Δ), bovine serum albumin, 2 g/L (●), or bovine serum albumin modified to carry 21 N-acetylgalactosamine residues per molecule, 2 g/L (□). The bound IgG was detected by time-resolved immunofluorometry. y-axis is fluorescence, counts per second.

**Fig. 3.** Binding of IgG purified from pooled serum from RA patients (gray columns) or healthy controls (black columns) to different immobilized proteins

IgGs were incubated at 20 mg/L in microtiter wells coated with the proteins indicated. Col, collagen. The bound IgG was detected by time-resolved immunofluorometry. The error bars indicate the ranges of duplicate determinations.
MnCl₂ affected the binding, indicating that divalent cations were not involved. To investigate whether the binding of IgG to the different lectins was mediated by a similar mechanism, I incubated RA IgG with lectins in the assay buffer in RCA-coated wells (Figure 5). LTA and UEA effectively inhibited the binding, i.e., by about 70% at 10 mg/L. RCA and ECA were somewhat less effective inhibitors, and DSA and BS-II caused only a slight inhibition. ConA, PNA, and WGA were ineffective at the highest concentration tested, 10 mg/L.

To study which part of IgG mediates the lectin-binding activity, I prepared Fc, Fab, and F(\(\text{ab}'\))₂ fragments from RA IgG and incubated the fragments and intact RA IgG in RCA-coated wells at a molar concentration of about 0.3 µmol/L. When the binding was detected by a polyclonal anti-Fc fragment antibody and an Eu-labeled second antibody, the signal obtained with the Fc fragment averaged 30% of that obtained with the intact IgG. When assessed by a polyclonal anti-Fab fragment antibody and the Eu-labeled second antibody, the binding of the F(\(\text{ab}'\))₂ fragment averaged 20% and that of the Fab' fragment <10% of the binding of the intact IgG (data not shown).

The RCA-binding activity was co-eluted with monomeric IgG from the Sephacryl S-200 column, indicating that the interaction was not ascribable to IgG aggregates (data not shown). Fractionation of RA IgG by affinity chromatography on a column of Protein A-Sepharose indicated that the RCA-binding activity was associated with IgG that bound to Protein A-Sepharose and was eluted from the column at pH 3, whereas Protein A-nonbinding IgG displayed no binding to RCA-coated wells (data not shown). Further, to exclude the possible involvement of IgM rheumatoid factor in the interaction, I reduced and alkylated a sample of RA IgG, using conditions under which the 19S IgM rheumatoid factor is destroyed (3). This treatment did not significantly change the binding of RA IgG to RCA-coated wells (data not shown).

Studies with Serum Samples

To test the ability of RCA coated onto microtiter wells to bind glycoproteins carrying terminal β-galactoside residues (14), I incubated native and neuraminidase-treated transferrin in the wells and measured the bound transferrin by immunofluorometry, using an Eu-labeled transferrin-specific antibody. Compared with native transferrin, alcalo transferrin exhibited only about twice stronger binding to RCA-coated wells (data not shown), suggesting that the immobilized RCA had only weak β-galactoside-specific binding activity. I therefore used RCA to study the carbohydrate-nondependent binding activity of IgG in unpurified serum samples, because only low carbohydrate-mediated binding of serum glycoproteins to the solid phase was proposed. The standard curve for the binding of purified RA IgG to the immobilized RCA was nearly linear from 100 µg/L up to the highest concentration of standard studied, 100 mg/L (Figure 6A). The standard curve obtained with diluted serum from a patient with active RA (final dilution 1:360) present paralleled that obtained with only the assay buffer as diluent (Figure 6B). Analytical recovery of the RA IgG added to the diluted serum ranged from 91% to 102% at IgG concentrations >3 mg/L. At lower concentrations the recovery was more variable. The within-run CV (n = 10) was 4.5% for purified RA IgG, 5.7% for a serum with low RCA binding of IgG, and 6.4% for a serum with high RCA binding of IgG. The interassay CV (n = 8) was 21% for the former serum, 12% for the latter. At a final 360-fold dilution, serum samples from patients with active RA displayed clearly higher RCA binding of IgG in the assay system that did serum samples from age-matched healthy controls or patients with acute inflammation (Figure 7).

Discussion

My aim here was to measure galactosylation variants of IgG by a two-site lectin–immunofluorometric assay that has recently been shown to measure agalacto forms of other serum glycoproteins specifically (10). IgG purified from sera of RA patients displayed a highly variable binding to several lectins, which was up to 50-fold greater than the binding of control IgG. However, inhibition studies with free and polyvalent saccharides and treatments of IgG with...
glycosidase indicated that only a minor proportion of this lectin-binding activity of IgG was mediated by its glycan chains. Compared with intact RA IgG, the purified Fab and F(ab')2 fragments exhibited only low binding to immobilized RCA, suggesting the aberrant lectin-binding activity of IgG was not an antigen-binding interaction. Gel filtration and reduction and alkylation experiments confirmed that the binding was mediated by monomeric IgG and that 19S IgM rheumatoid factor was not involved. These results thus indicated the existence of a novel type of binding activity in IgG that was exceptionally great in serum of some RA patients.

The ability of the different lectins in solution to inhibit the binding of IgG to immobilized RCA suggested that the binding site of IgG is similar in all of these lectins. The carbohydrate-binding specificities of the IgG-binding lectins are diverse (15) and, for example, LTA and UEA recognize such fucose residues that have not been observed in IgG (16). This further suggests that the interaction of these lectins with IgG is not mediated by their carbohydrate-binding site. On the other hand, most of the IgG-binding lectins are legume lectins that contain highly conserved structures such as a hydrophobic cavity and a binding site for divalent cations (17). The hydrophobic binding site of several lectins has been shown to mediate their interaction with noncarbohydrate ligands independent of the carbohydrate-binding site, suggesting that lectins may possess biologically important functions other than carbohydrate binding (18). The use of lectins as carbohydrate-specific reagents in binding assays must therefore be strictly controlled, as is indicated also by the results of the present study.

Binding of IgG to various lectins has also been described in earlier studies, but these interactions have been carbohydrate-specific. IgG3, unlike other IgG subclasses, was shown to bind to RCA–Sepharose in a lactose-sensitive manner (14). The finding of the present study that Protein A–nonbinding IgG, which corresponds to IgG3 (19), did not bind to RCA-coated microwells probably reflects the poor expression of carbohydrate-binding activity in RCA after its attachment to polystyrene. This was also suggested by the low binding of asialo transferrin to immobilized RCA.

In another previous study, RA IgG was found to bind more strongly than control IgG to immobilized ConA, and this binding could be inhibited with α-methyl-mannoside (20). In the present study, differences between the binding of IgG samples to ConA were also observed, but they were an order of magnitude smaller than those observed in the binding of IgG to RCA and some other lectins.

The clinical significance, if any, of the increased lectin-binding activity of RA-associated IgG is still unknown. Theoretically, it might result from an increased concentration of a minor IgG variant in serum, an altered post-translational modification of IgG, and/or post-synthetic changes in IgG while it is in the circulation. Both an altered post-translational modification in the form of decreased B-cell galactosyltransferase activity (6) and post-synthetic inflammation-dependent changes caused by oxy-
gen-centered free radicals (7, 21) have been described for RA-associated IgG. Their relation to the aberrant lectin-binding activity of RA IgG reported here is currently not known.

This lectin–immunofluorometric assay involving use of immobilized RCA proved to be reproducible and accurate for the measurement of the carbohydrate-nondependent lectin-binding activity of IgG in serum samples. Thus it provides a practicable method for seeing whether the aberrant lectin-binding activity of IgG is specific for RA and whether it has diagnostic or pathophysiological significance.

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References