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Detection of disease-specific augmentation of abnormal immunoglobulin G in sera of patients with rheumatoid arthritis

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Galactose-free immunoglobulin G (IgG), which is known to be higher in the sera of patients with rheumatoid arthritis, was prepared from IgG of healthy volunteers using enzymes. Its reactivity to lectins was analyzed. The galactose-free IgG showed no reactivity to *Ricinus communis* agglutinin 120 but displayed greater reactivity to concanavalin A and *Lens culinaris* lectin than did intact human IgG. Then, IgG in serum samples was bound to protein A immobilized on a nitrocellulose membrane, and its reactivity to biotinylated concanavalin A was measured with streptavidin-conjugated horseradish peroxidase. When the reactivity to concanavalin A of IgG in sera from healthy individuals and patients with rheumatoid arthritis (RA), osteoarthritis, systemic lupus erythematosus, or hepatic disease was compared, higher levels were shown in patients with RA, notably in 60% of the seronegative patients and 80% of the early phase patients. Therefore, it was suggested that augmentation of the abnormal IgG in sera was highly specific to patients with RA and that this novel serum test could be very useful for an accurate diagnosis of this disease.

Keywords: **immunoglobulin G, rheumatoid arthritis, rheumatoid factor**

Abbreviations: **Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; IgG, immunoglobulin G; Man, mannose; PBS, phosphate buffered saline; RA, rheumatoid arthritis; RF, rheumatoid factor**

Introduction

Human immunoglobulin G (IgG) is a glycoprotein composed of two heavy and two light protein chains, and an *N*-linked oligosaccharide chain on the CH2 domain of each heavy chain. This glycoprotein reacts with a rheumatoid factor which is an autoantibody against autologous IgG. It has been shown that *N*-linked oligosaccharides lacking galactose are significantly higher in serum IgG of patients with rheumatoid arthritis (RA) in whom the rheumatoid factor (RF) has been detected [1–3]. It has also been shown that IgG oligosaccharide abnormality in patients with RA occurs in autoimmune MRL/Mp-*lpr/lpr* mice, which produce RF and show RA-like articular change, related to the presence of a certain IgG subpopulation [4]. Therefore, these findings suggest that abnormal glycosylation of serum IgG could be a common feature in animals that develop arthritis with production of the RF regardless of species and that the level of this abnormal IgG may also be higher in sera of patients who do not have RA but in whom the RF has been detected by the conventional serum test. Since complex experimental procedures are required for purifying IgG from sera and for analyzing changes in oligosaccharide structures [4–9], a simple rapid method was needed to examine this abnormal IgG.

In the present study, galactose-free IgG was prepared from IgG of healthy volunteers and its reactivity with lectins was analyzed. This led to the development of a novel serum test for its measurement. Sera then tested were from healthy individuals, patients with RA, and from those with osteoarthritis, systemic lupus erythematosus, and hepatic disease who frequently showed positive results with the conventional serum test for detection of RF in serum. This article demonstrates that an increase in this abnormal IgG

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in serum is specific to patients with RA, and, as a consequence, this novel serum test may be very useful for accurate diagnosis of this disease.

Materials and methods

Materials

Human IgG was purified from pooled serum of healthy individuals by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose and Sephacryl S-300 columns, as described previously [5]. Protein A and biotinylated lectins used in this study were purchased from Honen Corporation (Tokyo, Japan) or Sigma-Aldrich (Tokyo, Japan). Sialidase purified from *Arthrobacter ureafaciens* was purchased from Nacalai Tesque Inc. (Kyoto, Japan) and β-galactosidase purified from *Streptococcus* 6646K was from Seikagaku Corporation (Tokyo, Japan). Protein A-Sepharose CL-4B was from Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose membranes (0.45 μm) were from Schleicher & Schuell (Dassel, Germany).

Preparation and characterization of galactose-free IgG

Human IgG was digested with 50 milliunits of sialidase for 24 h followed by 15 milliunits of β-galactosidase for 48 h. The digestion was performed at 37°C under a toluene atmosphere, as described previously [6]. After incubation, IgG was purified from the reaction mixture by protein A-Sepharose column chromatography. The galactose-free IgG thus obtained was dialyzed against phosphate buffered saline (PBS). Purity of the galactose-free IgG was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of β-mercaptoethanol and Ouchterlony's double immunodiffusion assay. Structural analysis of oligosaccharide chains of the galactose-free IgG was performed after quantitative liberation of the oligosaccharide chains by hydrazinolysis, as described previously [4–9].

Reactivity of the IgGs to lectins

Reactivity of the galactose-free IgG and the intact IgG to biotinylated lectins was analyzed as follows. Twenty μl of protein A in PBS (6.25 μg/μl) was dot-blotted onto a nitrocellulose membrane. The membrane was air dried for 15 min and soaked for 2 h in gelatin buffer (2.5% gelatin, 0.05% NP-40, 50 mM Tris-HCl, pH 7.4, and 0.15 M NaCl). The membrane was incubated with the galactose-free IgG or the intact IgG solution (140 μg of IgG/ml of PBS) for 1 h, washed with gelatin buffer, and incubated with biotinylated lectin solution (10 μg/ml of the gelatin buffer) for 1 h. The membrane was washed and incubated with streptavidin-conjugated horseradish peroxidase in the gelatin buffer for 1 h. After washing, the dot on the membrane was visualized in 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 4-chloronaphthol (0.5 mg/ml), and 0.05% H_2O_2. Intensity of the colored dot on the membrane was counted at 540 nm with a dual wave-length flying-spot scanner, Model CS-9000 from Shimadzu Corporation (Kyoto, Japan). Scores were graded as follows: − for no count, ± for 1–100 counts, + for 101–1000 counts, ++ for 1001–10,000 counts, and +++ for more than 10,000 counts. All procedures were performed at room temperature.

Detection of the abnormal serum IgG by concanavalin A

Sera from healthy individuals (n=92) and patients with RA (n=60), osteoarthritis (n=19), systemic lupus erythematosus (n=12), and liver disease (n=29) were blinded so that the diagnosis was unknown prior to assay, and then analyzed. Patients with RA were diagnosed according to the ARA criteria [10]. Concentration of IgG in serum samples ranged from 740 to 1650 mg/dl for healthy individuals, 850 to 2,980 mg/dl for patients with RA, 970 to 2760 mg/dl for those with systemic lupus erythematosus, and 930 to 3770 mg/dl for those with liver disease. The range for patients with osteoarthritis was within the variation of healthy individuals. Each serum was diluted 50 times with the gelatin buffer, and reactivity of the IgG in each serum sample to biotinylated concanavalin A was analyzed as described above. A positive ratio for detection was obtained based on the value (mean + 2 × s.d.) calculated from the data of healthy individuals. Serum that showed higher counts than the above value was evaluated as the positive. The same serum samples, when available, were also blinded and then analyzed by the conventional RA test which detects RF in serum by IgG-coated latex-bead agglutination. Serum that agglutinated the beads was evaluated as the positive.

Results

Galactose-free IgG

Galactose-free IgG prepared using enzymes as described in Materials and Methods and intact healthy human IgG were subjected to SDS-PAGE followed by electroblotting onto a nitrocellulose membrane. The membrane was stained with amido black, anti-human IgG antibody, and horseradish peroxidase-conjugated *Ricinus communis* agglutinin 120 (RCA120 lectin), which binds to the nonreducing terminal β-galactosyl residue of the oligosaccharides. Both IgG samples migrated to the same position as a single band stained by amido black and the antibody. Intact IgG was detected at the same position by RCA120 lectin-staining, but galactose-free IgG was not (data not shown). Therefore, it was shown that no proteolytic degradation occurred during the enzyme treatment of healthy human IgG and that the treatment completely deprived intact IgG of binding ability to the RCA120 lectin.

The oligosaccharide chains were quantitatively liberated by hydrazinolysis from the galactose-free IgG prepared as

described in Materials and Methods and radiolabeled with NaB^3H_4. Structures of the radioactive oligosaccharides thus obtained were examined using several methods, including high voltage paper electrophoresis and enzymatic microsequencing combined with Bio-Gel P-4 HPLC, as described previously [4–9]. All oligosaccharides derived from the galactose-free IgG were neutral and deficient in galactose (data not shown). In addition, the oligosaccharide structures of the galactose-free IgG were demonstrated as shown in Figure 1. Therefore, it was concluded that sialic acid and galactose in the intact IgG were completely removed by the enzyme treatment without damaging the protein component.

Galactose-free IgG and intact IgG were bound to protein A, which was immobilized on a nitrocellulose membrane, and reactivity to various biotinylated lectins was examined. As summarized in Table 1, reactivity to the mannose-binding lectins, concanavalin A and *Lens culinaris* lectin, was greatly enhanced by the enzyme treatment which had removed sialic acid and galactose from the intact IgG, although reactivity to RCA120 lectin, a galactose-binding lectin, disappeared with the treatment. No significant change in reactivity to the following lectins was observed: *Phytolacca americana, Bandeiraea simplicifolia* II, *Triticum vulgaris*, and *Lycopersicon esculentum*, lectins that have an affinity to *N*-acetylglucosamine; and *Arachis hypogaea*, *Dolichos biflorus*, and *Phaseolus vulgaris*-E, lectins that show an affinity to galactose, *N*-acetylgalactosamine, and Galβ1-4GlcNAcβ1-2Man, respectively (Table 1). From these results, it is suggested that concanavalin A and *Lens culinaris* lectin, a mannose-binding lectin, are suitable for distinguishing galactose-free IgG from intact,

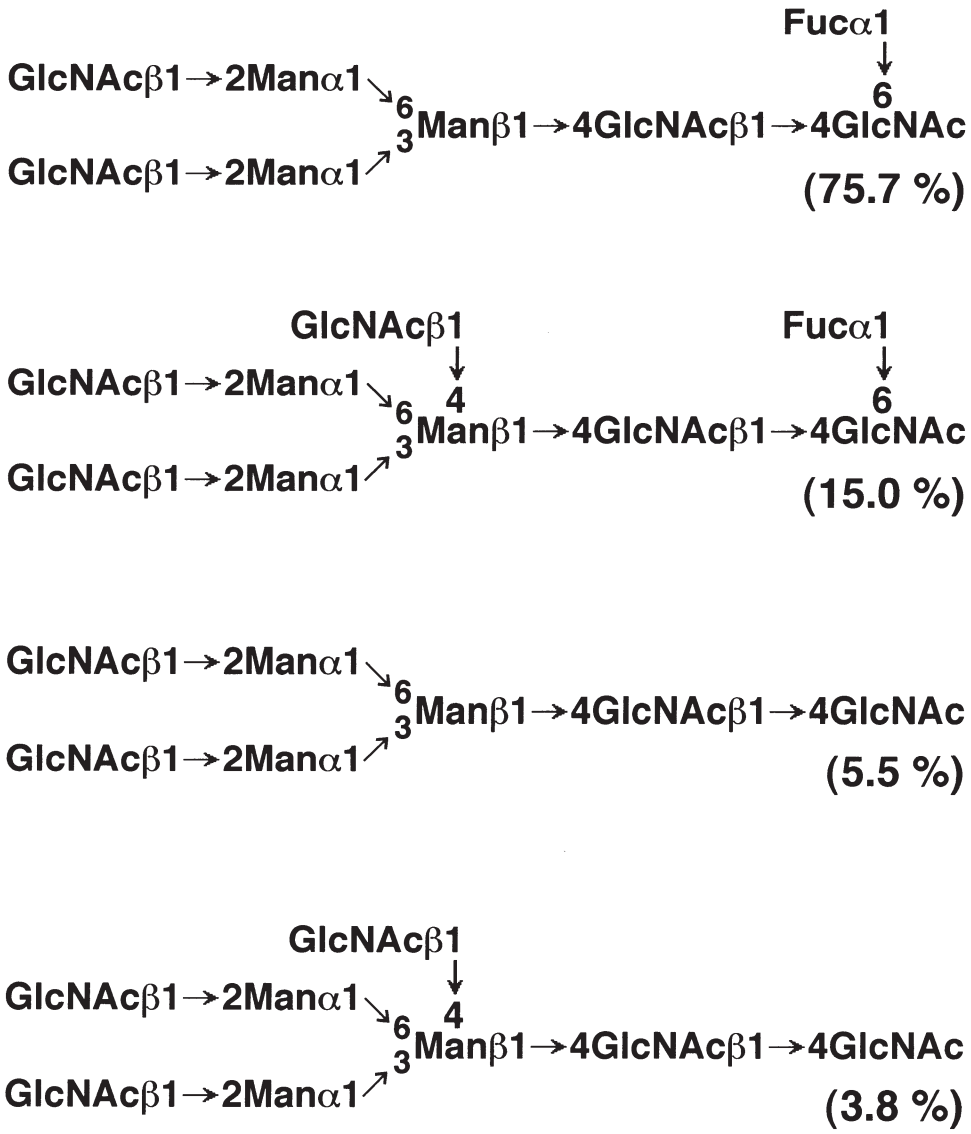

Figure 1. Oligosaccharide structures of galactose-free IgG prepared from healthy human IgG.

Table 1. Reactivity of the galactose-free IgG and intact IgG to lectins

	Reactivity	
Lectins	*Galactose-free IgG*	*Intact IgG*
Concanavalin A	+++	+
Phytolacca americana	±	−
Dolichos biflorus	−	−
Bandeiraea simplicifolia II	+	−
Phaseolus vulgaris-E	+++	+++
Lens culinaris	+++	+
Triticum vulgaris	−	−
Arachis hypogaea	−	±
Lycopersicon esculentum	−	−
Ricinus communis (RCA120)	−	+++

Scores of reactivity are based on the intensity of the colored dot: −, no count; ±, 1–100 counts; +, 101–1000 counts; +++, more than 10,000 counts.

healthy human IgG and could be useful tools for detection of the abnormal IgG that occurs in sera from patients with RA.

Disease-specific augmentation of abnormal IgG in sera from patients with RA

Since it was suggested that the abnormal IgG might be higher in sera of patients who produce RF but do not have RA, sera from healthy individuals (n=92) and certain groups of patients (n=120) were blinded so that the diagnosis was not known prior to assay, and then analyzed by a novel serum test using concanavalin A. The patients tested included those with osteoarthritis (n=19), systemic lupus erythematosus (n=12), and liver disease (n=29) who had frequently shown positive results in the conventional serum test for detecting RF in sera, as well as patients with rheumatoid arthritis (n=60). The 60 patients with RA included 30 early phase patients whose disease onset was within one year before testing and 16 seronegative patients who gave negative results in the RA test, the conventional serum test for detecting RF in serum by agglutination of latex-beads coated with IgG. All 14 remaining patients were positive using the RA test. Serum samples from patients with systemic lupus erythematosus were collected only from those who gave negative results in the RA test and were not diagnosed as RA according to the ARA criteria [10] to avoid the possibility that these patients also had RA. Of the 29 patients with liver disease who did not have RA, 7 had chronic hepatitis, 9 had cirrhosis, 2 had hepatocellular carcinoma, and 11 had cirrhosis and hepatocellular carcinoma. All 19 patients with osteoarthritis did not have RA.

IgG in the serum samples of all subjects was bound to protein A on a nitrocellulose membrane, and its reactivity to biotinylated concanavalin A was measured by the activity of the bound streptavidin-conjugated horseradish peroxidase, as described in Materials and Methods. As shown in Figure 2, the reactivity of IgG in serum from most of the patients with RA was higher than that from the healthy individuals ($P < .00001$). This was observed not only in RA test-positive patients but also in seronegative (RA test-negative) patients and early phase patients. In contrast,

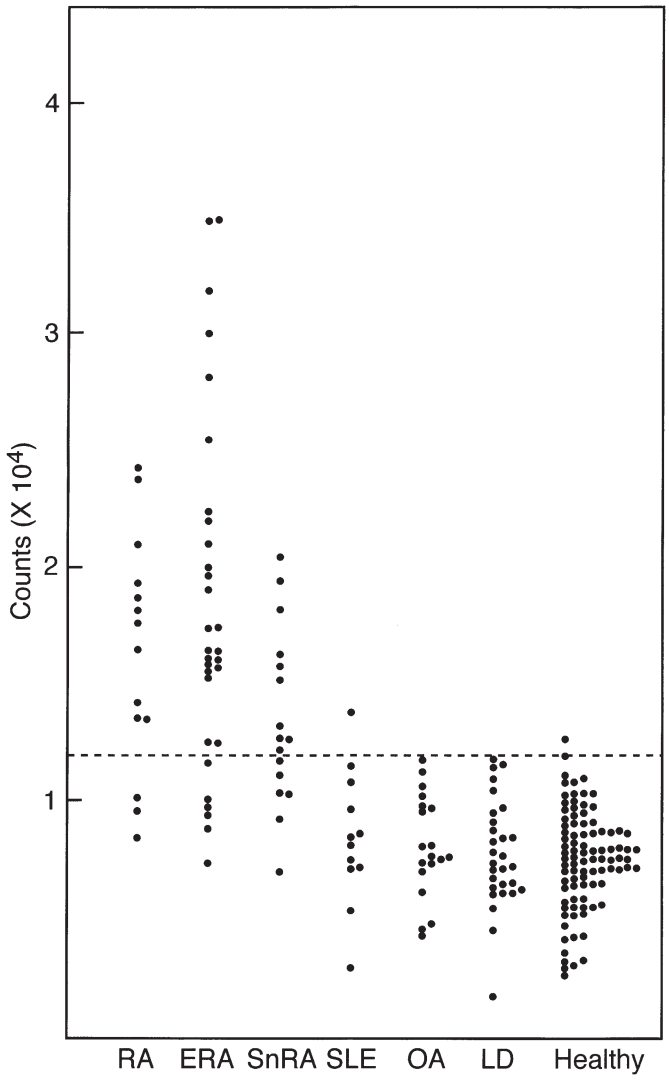

Figure 2. Reactivity of IgG in serum samples to concanavalin A. Serum from healthy individuals (Healthy) and patients were analyzed with the novel serum test. Patients consisted of those with rheumatoid arthritis (RA = RA test-positive patients, ERA = early phase patients, SnRA = seronegative patients), systemic lupus erythematosus (SLE), osteoarthritis (OA), and liver disease (LD). The dashed line indicates the position of the value (mean + 2 × s.d.) calculated from data of healthy individuals. Results of Student's *t*-test analysis: healthy individuals (Healthy) vs patients with RA, $P < .00001$; ERA, $P < .00001$; SnRA, $P < .00001$; SLE, $P > .27$; OA, $P > .44$; and LD, $P > .79$.

serum from patients with osteoarthritis, systemic lupus erythematosus, and liver disease, who had frequently produced RF detected by the conventional serum test, showed reactivity of IgG within the normal range for healthy individuals (Figure 2).

Percentages of patients and healthy individuals who were positive with this novel serum test and those positive with the conventional RA test using the same serum samples are summarized in Table 2. The percentages of patients with RA testing positive for abnormal IgG in serum indicate that this test may be useful for specific detection of this disease. It is noteworthy that 62.5% of the seronegative patients who were negative with the conventional test for detecting RF were positive with the novel test and that the number of positives among the early phase patients reached 80% although half were negative with the conventional test. In addition, all patients with osteoarthritis (19/19), liver disease (29/29), and 11 out of 12 with systemic lupus erythematosus gave negative results with the novel test although some were positive with the conventional RA test (Table 2).

Discussion

A simple rapid method was established for detecting galactose-free IgG which is augmented in patients with RA. Assays of serum samples from a large number of patients and healthy individuals were performed using this novel serum test, and it was shown that abnormal IgG was specifically higher in the sera of patients with RA ($P < .00001$), not only in sera that tested positive with the conventional RA test but also in sera of seronegative (RA test-negative) patients and early phase patients. Patients who did not have RA, even those producing RF detected by the RA test, did not show an increase in levels. These results correspond with our previous findings obtained in studies on oligosaccharide structures of IgG which were highly purified from sera of patients [2, 11, 12]. Therefore, it is suggested that augmentation of the abnormal IgG in serum is highly specific to RA, but is not related to the production of RF. However, the possibility cannot be excluded that RF produced in patients with RA is an antibody against IgG, whereas that detected with the conventional test in patients lacking this disease is not. Future studies are required to clarify the relationship between the increase in galactose-free IgG in sera and production of RF, which is an autoantibody against autologous IgG. The biological significance of the higher levels of galactose-free IgG in patients with RA must also be elucidated. Several kinds of experiments are now in progress to clarify them.

Table 2. Percentage of patients testing positive with the novel serum test and the conventional RA test

	Test-Positive (%)			
Sera	*Novel test*		*RA test*	
Rheumatoid arthritis (RA)				
RA test-positive	78.6	(11/14)	100	(14/14)
Early phase	80.0	(24/30)	53.8	(14/26)
Seronegative	62.5	(10/16)	0	(0/16)
Osteoarthritis	0	(0/19)	21.1	(4/19)
Systemic lupus erythematosus	8.3	(1/12)	0	(0/12)
Liver disease	0	(0/29)	21.4	(6/28)
Healthy individuals	2.2	(2/92)	7.1	(1/14)

Values indicate percentage of patients testing positive and the number of cases.

In the present study, galactose-free IgG completely deficient in sialic acid and galactose was prepared from healthy human IgG by enzyme treatment without damaging the protein portion. It was shown that the removal of sialic acid and galactose renders the IgG molecule more reactive to mannose-binding lectins. The enhanced binding of concanavalin A, the mannose-binding lectin, to the IgG molecule after the enzyme treatment can be explained as follows: Baenziger and Fiete [13] have shown that the association constant of concanavalin A for ^{125}I-labeled IgG glycopeptides is increased more than 2.4 times by the removal of sialic acid and galactose residues and that glycopeptides bind to concanavalin A-Sepharose column. The galactose-free IgG we prepared, however, did not bind to concanavalin A-Sepharose column (data not shown). It is thus possible that galactose-free IgG oligosaccharide structures were exposed by binding of the Fc region of galactose-free IgG to immobilized protein A, to which biotinylated concanavalin A bound at a higher affinity, and that the binding was amplified by streptavidin-conjugated horseradish peroxidase.

Since the mannan/mannose-binding protein, which can activate the complement system, occurs in serum [14, 15], and since macrophages express mannose receptor proteins on their cell surfaces [16, 17], it is suggested that the galactose-free IgG present at higher levels in patients with RA could be recognized by these proteins and that they may play a role in the progress of the disease [18, 19]. In this context, galactose-free IgG prepared in the present study may be a useful tool for elucidation of the physiological function.

This novel serum test using concanavalin A is a simple, rapid method that can indentify patients with RA, including seronegative patients and early phase patients who are difficult to detect with the conventional serum assay, although this novel assay was not positive for 20% and 37% of patients with early phase RA and seronegative RA, respectively. Furthermore, false-positive results were markedly decreased for patients with other diseases as frequently occurs with the conventional test. Since this novel

assay is superior to the conventional serum test (RA test) in terms of accuracy for detection of patients with RA and the effective detection of seronegative and early phase patients, and since the significance of early treatment of RA patients has been recognized quite recently, it may replace the conventional method in the future.

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