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Autoantibody Activity of IgG Rheumatoid Factor Increases with Decreasing Levels of Galactosylation and Sialylation[1]

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The occurrence of *N*-linked oligosaccharides lacking galactose is significantly higher than normal in serum IgG of patients with rheumatoid arthritis (RA) in whom rheumatoid factor (RF), an autoantibody against autologous IgG, has been detected. In the present study, IgGs with and without RF activity (IgGRF and non-RF IgG, respectively) were prepared from sera of RA patients, and their oligosaccharide structures were characterized in order to investigate the relationship between RF activity and glycosylation. Three IgGRF fractions and a non-RF IgG fraction were obtained based on their ability to bind to an IgG-Sepharose column. The specific RF activity, as measured by immunoassays, was highest in the IgGRF fraction, which bound most avidly to the IgG-Sepharose. When the oligosaccharides were released by hydrazinolysis, and analyzed by MALDI-TOF mass spectrometry and HPLC, in combination with sequential exoglycosidase treatment, all the IgG samples were found to contain a series of biantennary complex-type oligosaccharides. The incidence of galactose-free oligosaccharides was significantly higher in both IgGRFs and non-RF IgG from RA patients compared with IgG from healthy individuals. In all IgGRFs, the levels of sialylation and galactosylation were lower than those in non-RF IgG from RA patients; the sialylation of non-RF IgG was the same as that of IgG from healthy individuals. In addition, the decreases in galactosylation and sialylation of oligosaccharides in IgGRF correlated well with the increase in RF activity. These findings could contribute to our understanding of the mechanisms of IgG-IgG complex formation and the pathogenicity of these complexes in RA patients.

Key words: autoantibodies, glycosylation, IgG, rheumatoid arthritis, rheumatoid factor.

Human IgG is a glycoprotein composed of two light and two heavy protein chains; each heavy chain contains an *N*-linked oligosaccharide on the CH2 domain. Oligosaccharides of human IgG have been shown to possess a series of complex-type biantennary structures, *i.e.* ±Galβ1-4GlcNAcβ1-2Manα1-6(±GlcNAcβ1-4)±Galβ1-4GlcNAcβ1-2Manα1-3)-Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc with 0–2 sialic acid residues bound to the non-reducing terminal Gal residues (*1*). The carbohydrate moiety of IgG is essential for the so-called effector functions of antibodies such as binding to Fc receptors on macrophages, induction of antibody-dependent cellular cytotoxicity, rapid elimination of antigen-antibody complexes from the circulation, and feedback immunosuppression (*2*, *3*). This glycoprotein is also a target for rheumatoid factor (RF), which is an antibody against autologous IgG and the characteristic autoantibody associated with rheumatoid arthritis (RA). It has been shown that *N*-linked oligosaccharides lacking galactose are significantly increased in total serum IgG of RA patients (*4*, *5*), and that this abnormality can be used as a diagnostic marker for RA (*6*). The same abnormality in IgG oligosaccharide structures also occurs in autoimmune MRL/Mp-*lpr*/*lpr* mice, which show rheumatoid arthritis-like articular changes (*7*). In addition, it has been reported that the high incidence of galactose-free oligosaccharides in serum IgG of RA patients is related to disease activity and progression to erosive articular changes (*8*, *9*). Since IgG from pathological aggregates in synovial fluid of RA patients contains higher levels of galactose-free oligosaccharides than non-aggregated IgG in the same synovial fluid, it has been suggested that galactose-free IgG could be pathogenic (*10*).

However, it remains unknown whether the abnormal glycosylation of IgG in RA occurs in IgG molecules with or without RF activity, or whether it is directly involved in the pathogenesis of this disease. In this study, as a step towards elucidating the role of galactose-free IgG in RA, we

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Abbreviations: RA, rheumatoid arthritis; RF, rheumatoid factor; IgGRF, IgG with RF activity; non-RF IgG, IgG without RF activity; ABEE, *p*-amino benzoic acid ethyl ester; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose. All sugars mentioned in this paper were of the D-configuration except for fucose, which had the L-configuration.

have prepared IgG fractions with and without RF activity (IgGRF and non-RF IgG, respectively) from sera of patients with this disease. We have analyzed the oligosaccharide structures present in each fraction, and describe here the relationship between RF activity and glycosylation of IgG in RA patients.

MATERIALS AND METHODS

Chemicals and Enzymes—Sodium cyanoborohydride was purchased from Nacalai Tesque (Kyoto). *p*-Aminobenzoic acid ethyl ester (ABEE) was purchased from Wako Pure Chemical Industries (Osaka). Neutral, monosialo, and disialo biantennary ABEE-oligosaccharides, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, were prepared from oligosaccharides of human fibrinogen (*11*) by ABEE-derivatization, as described below. ABEE-oligosaccharides 1 to 12, with the same structures as oligosaccharides a to l shown in Fig. 5, respectively, were prepared from oligosaccharides of mouse IgG, human IgG, and human fibrinogen (*1*, *11*, *12*) by ABEE-derivatization. ABEE-oligosaccharides of Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE were prepared from ABEE-oligosaccharides 5 and 1, respectively, by digestion with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase. *Arthrobacter ureafaciens* neuraminidase was purchased from Nacalai Tesque. Jack bean meal β-galactosidase and β-*N*-acetylhexosaminidase were purified according to a previously reported method (*13*). Glycosidase digestion of oligosaccharides was performed as described previously (*1*, *14*, *15*). Galactose-free human IgG was prepared by sequential treatment of intact human IgG with neuraminidase followed by β-galactosidase, as described previously (*6*).

Purification of Total IgG from Sera of RA Patients—Pooled sera from 10 RA patients (females aged 47.2 ± 3.6 years) and from 10 sex- and age-matched healthy individuals (females aged 47.3 ± 3.4 years) were dialyzed against 15 mM phosphate buffer, pH 8.0, prior to chromatography on a DEAE-cellulose column (DE-52 from Whatman International, Maidstone, UK) equilibrated with 15 mM phosphate buffer, pH 8.0. IgG was eluted in the pass-through fraction. The purity of the total IgG was confirmed by FPLC on a Superdex 200 HR column (1 × 30 cm; Pharmacia Biotech, Tokyo) equilibrated with 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl, SDS-PAGE on 10% gels or 5–20% gradient gels with the Laemmli system (*16*), and Western blotting analysis with antibodies against the human immunoglobulin γ-, μ-, and α-chains, and human transferrin.

Preparation of IgGRF and Non-RF IgG—IgGRF and non-RF IgG were prepared from total IgG purified from the sera of RA patients by chromatography on a human IgG-Sepharose column (1.5 × 20 cm; IgG Sepharose 6 Fast Flow from Pharmacia Biotech, Tokyo) as follows. Total IgG was dissolved in 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl and 0.05% Tween20 (TBS-T), applied to the IgG-Sepharose column equilibrated with TBS-T, and then circulated through the column at the flow rate of 1 ml/min at 4°C for 12h. The column was washed with TBS-T until the absorbance of the eluate at 280 nm became zero. The column was then eluted with 0.1 M glycine-HCl buffer, pH 3.0, and 2 ml fractions were collected in tubes containing 70 μl of 1 M Tris-HCl buffer, pH 8.0, for neutralization. Fractions containing RF eluted with the acidic buffer were pooled and designated as IgG(+). The pass-through fraction on the first IgG-Sepharose column chromatography was then subjected to a second cycle of chromatography, using the same IgG-Sepharose column, to obtain an IgG (–)(+) fraction. Finally, a third IgGRF fraction, IgG(–)(–)(+), which bound to the column, and a non-RF IgG fraction, IgG(–)(–)(–), which passed through the column on the third cycle of IgG-Sepharose column chromatography, were obtained. The protein concentration of each sample was determined by means of a standard protein assay (Protein Assay Reagent from Bio-Rad Laboratories, CA) based on the Bradford method with human IgG (Sigma-Aldrich Japan, Tokyo) as a standard protein.

Measurement of RF—Measurement of RF was performed by means of ELISA with an Eitest IgGRF test kit (Eisai, Tokyo) and a lectin enzyme immunoassay. The ELISA detects IgGRF bound to the pre-coated human IgG-Fc region with an enzyme-conjugated antibody against the human IgG-Fd region. Absorbance readings at 405 nm and at the reference wavelength of 490 nm were made with a Model 450 Microplate Reader (Bio-Rad Laboratories, CA). The IgGRF Index was calculated from the absorbance data according to the manufacturer's instructions. The specific RF activity of IgGRF was calculated as the IgGRF Index obtained with 1 μg of purified IgG. The lectin enzyme immunoassay for detection of RF was originally developed in our laboratory and is now available as Eitest CA-RF from Eisai. This method detects galactose residues in the oligosaccharides of various classes of RFs bound to galactose-free IgG. Briefly, diluted serum samples were added to plates coated with galactose-free IgG. The galactose residues in RF bound to the galactose-free IgG were detected using RCA120 conjugated to horseradish peroxidase or to biotin, followed by horseradish peroxidase-conjugated streptavidin. The absorbance at 415 nm was measured with a Model 450 Microplate Reader.

Liberation and Isolation of N-Linked Oligosaccharides from IgG—Total IgG purified from the sera of RA patients and healthy individuals, and the IgGRF and non-RF IgG fractions prepared as above were desalted by dialysis against distilled water, lyophilized, and then subjected to gas-phase hydrazinolysis at 90°C for 3 h, followed by *N*-acetylation to quantitatively liberate the *N*-linked oligosaccharides as described previously (*1*, *14*, *15*). To isolate the free oligosaccharides, the hydrazinolysates were subjected to paper chromatography with a solvent system of 1-butanol:ethanol:water (4:1:1, v/v). The area around the origin containing the oligosaccharides was cut out and the oligosaccharides were recovered by elution with water. Each oligosaccharide fraction was then converted into ABEE-derivatives by reductive amination, as described previously (*17*).

Analysis of the Structures of N-Linked Oligosaccharides of IgG—Analysis of the structures of the ABEE-oligosaccharides derived from each IgG sample was performed initially by ion exchange HPLC on a COSMOGEL DEAE column (7.5 × 75 mm; Nacalai Tesque), as described previously (*17*, *18*). Briefly, ABEE-oligosaccharides were applied to the DEAE column equilibrated with 0.5 mM CH_3COO-

Na. Elution was performed at the flow rate of 1 ml/min with a linear gradient of 0.5 to 150 mM CH_3COONa for 30 min. The neutral oligosaccharide mixture obtained on exhaustive neuraminidase treatment of the ABEE-oligosaccharide fraction dervied from each IgG sample was then purified by ion exchange HPLC and analyzed by ODS-HPLC. The neutral ABEE-oligosaccharide mixture was subjected to HPLC on a Wakosil 5C18-200 column (0.5 × 25 cm; Wako Pure Chemical Industries), as described previously (*17*). ABEE-oligosaccharides eluted from the column were detected by monitoring the absorbance at 304 nm. Mass spectrometry analysis of ABEE-oligosaccharides was performed with a MALDI-TOF mass spectrometer, Model Vision 2000 (ThermoBioAnalysis, Hemel Hempsted, Herts, UK).

RESULTS

Purification of IgG with and without RF Activity—Total IgG from pooled sera of RA patients was purified by DEAE-cellulose column chromatography. Upon staining with Coomasie brilliant Blue following SDS-PAGE, the purified IgG was found to have migrated as a single band corresponding to a molecular weight of around 150 kDa under non-reducing conditions, and to have been separated into heavy and light chains with molecular weights of about 50 and 25 kDa, respectively, under reducing conditions (Fig. 1). On Western blotting analysis, the 150 kDa protein band observed under non-reducing conditions and a single protein band corresponding to the heavy chain observed under reducing conditions were detected with the antibody against the human γ-chain. No bands were detected under non-reducing or reducing conditions with antibodies against the human μ-chain or α-chain, or human transferrin (data not shown). Therefore, the total IgG prepared from the pooled RA sera was judged to be highly pure.

IgGRF was then prepared from the total IgG by affinity chromatography on an IgG-Sepharose column, based on the fact that RF is an immunoglobulin which binds to IgG. Three IgGRF fractions which bound to the column on the first, second, and third rounds of chromatography were obtained and designated as IgG(+), IgG(–)(+), and IgG(–)(–)(+), respectively. The pass-through fraction on the third IgG-Sepharose column chromatography, designated as IgG(–)(–)(–), was considered to be a non-RF IgG, since no protein was detected in the IgG(–)(–)(–)(+) fraction obtained on the fourth round of chromatography. Each of the three IgGRF fractions derived from the pooled RA sera was eluted as a single peak at a position corresponding to monomeric human IgG of about 150 kDa on FPLC on a Superdex 200 HR column, suggesting that IgG-IgG self-associating complex formation hardly occurs in the solution used for these IgGRF fractions. The yield of total IgG from 30 ml of pooled sera from RA patients was 410.7 mg. The weight ratio of IgG(+), IgG(–)(+), IgG(–)(–)(+), and IgG(–)(–)(–) was about 1.5:0.9:0.8:96.8. This result indicates that about 3% of total IgG in the sera of RA patients contained RF activity.

IgGRFs and Their RF Activities—In order to analyze the RF activity of the IgG fractions obtained on IgG-Sepharose column chromatography, the levels of their binding to the Fc region of IgG were measured by ELISA. For all three IgGRFs, the specific RF activities, *i.e.* the values obtained for 1 μg of each protein, were significantly higher than those for the non-RF IgG [IgG(–)(–)(–)] and the total IgG. The intensities of the specific RF activities of the IgGRFs were in the order IgG(+) > IgG(–)(+) > IgG(–)(–)(+) (Fig. 2A). Therefore, the behavior of these fractions on the repeated IgG-Sepharose column chromatographies correlated with their specific RF activities. Similarly, when the three IgGRFs were analyzed by means of lectin enzyme immunoassaying (Fig. 2B), the value obtained for 1 μg of each protein was significantly higher than those for the non-RF IgG and the total IgG. However, the values obtained in this assay were in the order IgG(+) < IgG(–)(+) < IgG(–)(–)(+), which is the reverse of the order for specific RF activities. Since the lectin enzyme immunoassay detects galactose residues in the *N*-linked oligosaccharides of the IgGRF bound to immobilized galactose-free IgG and the ELISA measures the specific RF activity of the IgG fractions, these results suggest that the IgGRFs with the lowest levels of galactosylation of *N*-linked oligosaccharides exhibit the highest specific RF activity for binding to the Fc region of IgG.

Structural Analysis of Oligosaccharides of IgGRFs and Non-RF IgG from RA Patients—In order to confirm the

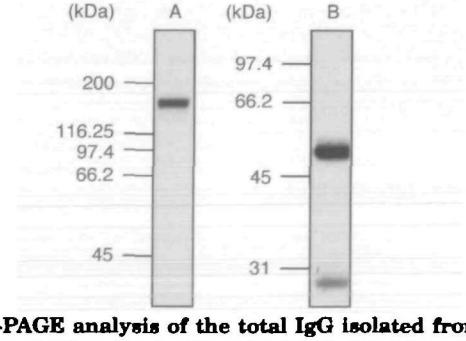

Fig. 1. **SDS-PAGE analysis of the total IgG isolated from the pooled sera of RA patients.** The total IgG isolated from RA patients was subjected to SDS-PAGE under non-reducing (A) and reducing conditions (B) on a 5–20% gradient gel (A) or a 10% gel (B). Proteins were stained with Coomassie Brilliant Blue R-250. The positions of protein markers are indicated on the left of each panel.

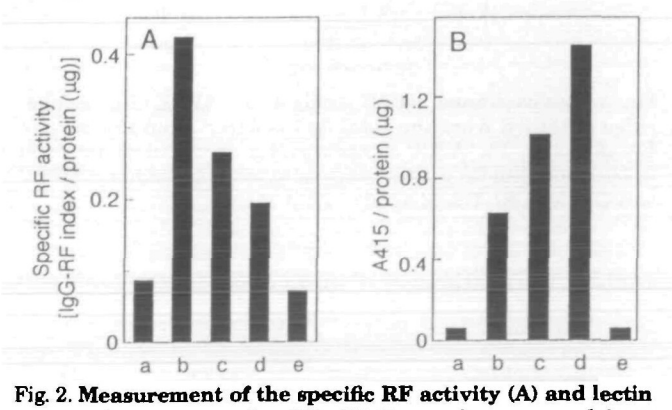

Fig. 2. **Measurement of the specific RF activity (A) and lectin enzyme immunoassaying (B) of IgG samples prepared from the sera of RA patients.** Measurement of the specific RF activity and lectin enzyme immunoassaying of IgG samples were performed as described under "MATERIALS AND METHODS." a, total IgG; b, c, and d, IgGRFs IgG(+), IgG(–)(+), and IgG(–)(–)(+), respectively; e, non-RF IgG [IgG(–)(–)(–)].

reverse correlation between the specific RF activity and the galactosylation of oligosaccharides in the IgGRF from RA patients, the structures of the oligosaccharide moieties of the three IgGRFs, non-RF IgG, and total IgG from pooled sera of RA patients, and total IgG from pooled sera of healthy individuals were analyzed.

Oligosaccharides of all IgG samples were quantitatively liberated from the polypeptide portion on hydrazinolysis and then labeled with ABEE. In order to determine the charge of the oligosaccharides, the ABEE-labeled fractions obtained from each IgG sample were subjected to ion exchange HPLC. Each ABEE-oligosaccharide sample was separated into a neutral fraction (N) and two acidic fractions (A1 and A2), as shown in Fig. 3. The acidic oligosaccharides, A1 and A2, were eluted at the same positions as authentic mono- and di-sialo biantennary oligosaccharides, respectively; in addition they were converted to neutral oligosaccharides on neuraminidase treatment (data not shown), indicating that A1 and A2 were mono- and di-sialo oligosaccharides, respectively. The molar ratios of N, A1, and A2 for the ABEE-oligosaccharide fractions derived from the IgG samples were then calculated on the basis of the absorbance at 304 nm (Table I).

In order to further analyze the oligosaccharide structures, the neutral oligosaccharide mixture obtained on neuraminidase treatment of the ABEE-oligosaccharides from each IgG sample was subjected to ODS-HPLC. Each ABEE-labeled mixture was separated into 12 oligosaccharide fractions (a to l), as shown in Fig. 4, although the ratios of the fractions differed among the IgG samples. Oligosaccharide fractions a to l were eluted at the same positions as those of a series of authentic biantennary oligosaccharides, 1 to 12, mentioned under "MATERIALS AND METHODS" with the structures of ±Galβ1-4GlcNAcβ1-2Manα1-6(±GlcNAcβ1-4)(±Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-ABEE. The structure of each ABEE-oligosaccharide fraction was then analyzed by sequential exoglycosidase treatment with jack bean β-galactosidase and β-*N*-acetylhexosaminidase, followed by ODS-HPLC. Upon incubation with jack bean β-galactosidase, the 12 ABEE-oligosaccharide fractions (a to l) were converted into three oligosaccharide fractions (d, h, and l) eluted at the same positions as authentic biantennary agalacto-oligosaccharides 4, 8, and 12, respectively (data not shown). Upon sequential incubation with β-galactosidase and β-*N*-acetylhexosaminidase, the 12 ABEE-oligosaccharide fractions were converted into two oligosaccharide fractions eluted at the same positions as authentic Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE (data not shown). From the behavior of the oligosaccharides in fractions a to l on the ODS-HPLC column and

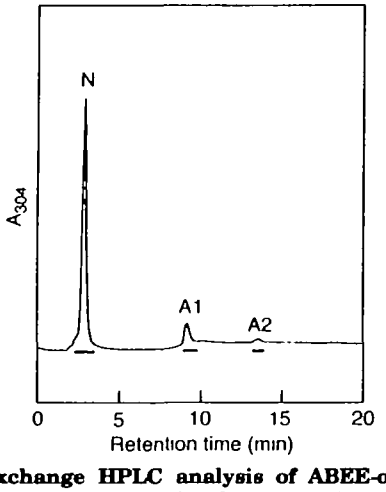

Fig. 3. **Ion-exchange HPLC analysis of ABEE-oligosaccharides obtained from the total IgG isolated from the sera of RA patients.** The ABEE-oligosaccharides derived from the total IgG isolated from RA patients were subjected to ion exchange HPLC on a COSMOGEL DEAE column (7.5 × 75 mm).

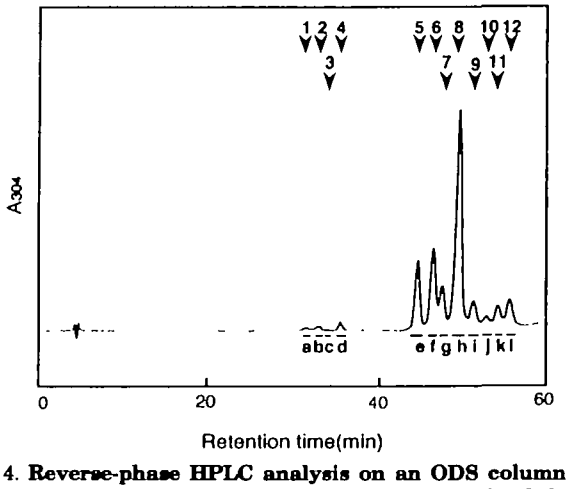

Fig. 4. **Reverse-phase HPLC analysis on an ODS column of the neutral ABEE-oligosaccharide mixture obtained from the total IgG isolated from the sera of RA patients.** The ABEE-oligosaccharide mixtures obtained from the total IgG of RA patients were desialylated and then subjected to reverse-phase HPLC on a Wakosil 5C18-200 column (0.4 × 25 cm), peaks a to l being eluted. Arrowheads 1–12 indicate the elution positions of authentic biantennary complex-type oligosaccharides 1–12, respectively. The structures of these authentic oligosaccharides are given under "MATERIALS AND METHODS."

TABLE I. **The distribution of oligosaccharide structures present in various IgG samples.**

IgG sample	Incidence (%)[a]							
	N	A1	A2	G0	G1	G2	F	Bi
Healthy total IgG	83.04	13.73	3.23	27.18	39.55	33.27	98.12	16.85
RA total IgG	84.40	12.70	2.90	50.01	25.38	24.61	97.80	15.43
IgG(+)	93.04	5.93	1.03	58.73	25.80	15.47	97.32	15.33
IgG(−)(+)	88.52	9.68	1.80	54.79	28.76	16.45	97.40	15.51
IgG(−)(−)(+)	86.52	10.86	2.62	52.14	29.79	18.07	97.29	17.08
IgG(−)(−)(−)	82.79	13.92	3.29	49.59	26.14	24.27	96.78	15.32

[a]Percent incidences of oligosaccharides with 0, 1, or 2 sialic acid residues (N, A1, and A2, respectively), with 0, 1, or 2 galactose residues (G0, G1, and G2, respectively), with a core fucose (F), and with a bisecting GlcNAc (Bi).

the results of sequential exoglycosidase treatment, it was suggested that the structures of the ABEE-oligosaccharides in fractions a to l derived from the IgG samples are as shown in Fig. 5. This was also supported by MALDI-TOF mass spectrometry analyses, in which the 12 oligosaccharide fractions exclusively gave mass peaks corresponding to the mass numbers calculated from the proposed structures with an accuracy of error below 1 Da. The molar ratios of oligosaccharides a to l in each IgG sample were then calculated on the basis of their absorbance at 304 nm (Fig. 5). The percent incidences of oligosaccharides with 0–2 sialic acid residues, 0–2 galactose residues, a core fucose residue, and a bisecting GlcNAc residue are shown in Table I.

Relationship between the RF Activity and the Oligosaccharide Structures in IgGRF from RA Patients—In order to elucidate the relationship between RF activity and glycosylation in the IgGRF from RA patients, the distribution of oligosaccharide structures in each IgG sample was compared. The level of sialylation in each sample was calculated as the sum of the percentage of monosialo oligosaccharides (A1) plus twice the percentage of disialo oligosaccharides (A2), as shown in Fig. 6A. In all three IgGRFs, both the incidence of monosialo and disialo oligosaccharides (Table I), and the levels of sialylation (Fig. 6A) were significantly lower than those in the non-RF IgG [IgG(–)(–)(–)] and the total IgG, and were in the order IgG(+) < IgG(–)(–)(+) < IgG(–)(–)(+). In contrast, the level of sialylation of oligosaccharides in the non-RF IgG from RA patients was almost the same as that in total IgG from healthy individuals (Fig. 6A). Since the specific RF activities of the three IgGRFs were in the order IgG(+) > IgG (–)(+) > IgG(–)(–)(+) (Fig. 2A), these results suggest that an increase in RF activity is correlated with a decrease in sialylation of the oligosaccharides in the IgGRF from RA patients, and that the IgGRF in RA patients could be characterized by this decreased level of sialylation.

When the distributions of the twelve neutral oligosaccharide structures were compared, there were no significant

Fractions	Structures of Oligosaccharides	Incidence of oligosaccharides (%)					
		healthy total IgG	RA total IgG	IgG(+)	IgG(-)(+)	IgG(-)(-)(+)	IgG(-)(-)(-)
a.	Galβ1→4GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	1.14	0.40	0.32	0.26	0 46	0.69
b.	Galβ1→4GlcNAcβ1→2Manα1↘6 / GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	0 38	0.58	0.33	0 46	0 87	0 99
c.	GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	0.12	0.05	0.29	0 23	0 16	0.07
d.	GlcNAcβ1→2Manα1↘6 / GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	0 24	1.17	1.74	1.65	1.22	1.47
e.	Fucα1↓6 Galβ1→4GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	26.85	19 50	12.85	13.92	14 79	19.69
f.	Fucα1↓6 Galβ1→4GlcNAcβ1→2Manα1↘6 / GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	23.16	17.30	17.51	18.50	16 45	17.66
g.	Fucα1↓6 GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	9 18	4 07	3.63	4.72	7.06	4.03
h.	Fucα1↓6 GlcNAcβ1→2Manα1↘6 / GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	22.08	41.50	48.00	44.75	41.91	40.08
i.	GlcNAcβ1↓4 Galβ1→4GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Fucα1↓6 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	5 28	4 71	2.30	2.27	2.82	3.89
j	GlcNAcβ1↓4 GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Fucα1↓6 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	1.54	0.53	0.59	0.43	1.07	0 57
k.	GlcNAcβ1↓4 Galβ1→4GlcNAcβ1→2Manα1↘6 / GlcNAcβ1→2Manα1↗3 Fucα1↓6 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	5.17	2 85	3 45	4.42	4.18	2.82
l.	GlcNAcβ1↓4 GlcNAcβ1→2Manα1↘6 / GlcNAcβ1→2Manα1↗3 Fucα1↓6 Manβ1→4GlcNAcβ1→4GlcNAc-ABEE	4.86	7.34	8.99	8.39	9.01	8.04

Fig. 5. Structures of desialylated ABEE-oligosaccharides a–l derived from human IgG samples.

differences in the occurrence of oligosaccharides with a core fucose and those with a bisecting GlcNAc among all the IgG samples derived from both RA patients and healthy individuals (Table I). However, the incidence of oligosaccharides terminating with galactose did vary (Table I); the proportion of galactose-free oligosaccharides in the total IgG from RA patients was significantly higher than in that from healthy individuals, as reported previously (*4*, *5*, *17*). This reduced galactosylation occurred in all the samples isolated from RA sera, indicating that the abnormal galactosylation of IgG oligosaccharides found in RA patients occurs in both IgGRF molecules and non-RF IgG molecules. The level of galactosylation in each IgG sample (Fig 6B) was calculated as the sum of the percentage of monogalactosylated oligosaccharides, G1 (b, c, f, g, j, and k in Fig. 5), plus twice the percentage of digalactosylated oligosaccharides, G2 (a, e, and i in Fig. 5). When these levels were compared among the three IgGRFs and non-RF IgG from RA patients, they showed the order IgG(+) < IgG(–)(+) < IgG(–)(–)(+) < IgG(–)(–)(–), which is the reverse of that for the specific RF activity (Fig. 2A). Therefore, on the basis of the oligosaccharide structures of IgG in sera of RA patients, it was confirmed that the lower the level of galactosylation of oligosaccharides of IgGRF, the higher the RF activity.

Since galactosylation is essential for the sialylation of IgG oligosaccharides, the relationship between galactosylation and sialylation was compared among IgG samples. In the non-RF IgG, the level of sialylation was almost the same as that in total IgG from healthy individuals, although the level of galactosylation was significantly decreased (Table I and Fig. 6). This suggests that the decrease in galactosylation of non-RF IgG of RA patients occurs only in the neutral oligosaccharides. In all three IgGRFs, the level of galactosylation was greatly decreased to 56.7–65.9%, as opposed to 106.1% in the total IgG of healthy individuals, while the level of sialylation was decreased to 8–16.1% as opposed to 20.2% in healthy controls (Table I and Fig. 6). The decreases in both galactosylation and sialylation were very similar between the three IgGRFs [4 and 5% for galactosylation, and 3 and 5% for sialylation between IgG(–)(–)(+) and IgG(–)(+), and IgG(–)(+) and IgG(+), respectively]. This suggests that the decrease in galactosylation of IgGRFs of RA patients occurs in the sialylated oligosaccharides in addition to the neutral oligosaccharides, and that the decrease in sialylated oligosaccharides found specifically in IgGRFs can be ascribed to this decrease in galactosylation. It was also suggested that the increase in specific RF activity of IgGRF of RA patients may be mainly caused by the loss of galactose residues from sialylated oligosaccharides in these IgGRFs.

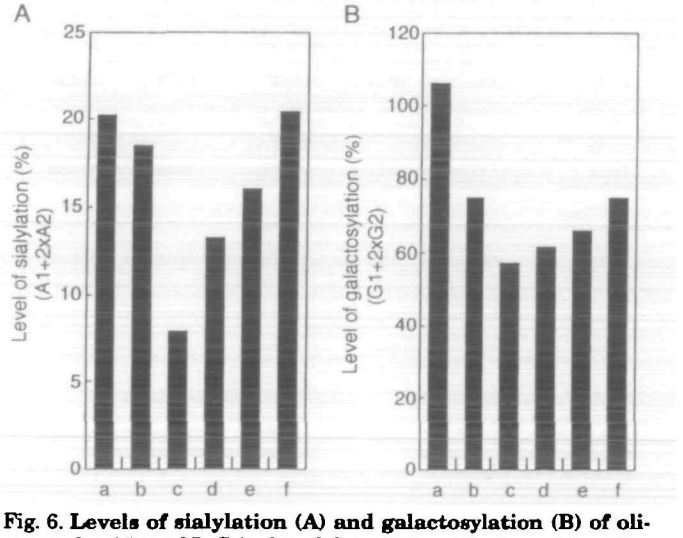

Fig. 6. **Levels of sialylation (A) and galactosylation (B) of oligosaccharides of IgG isolated from RA patients and healthy controls.** The level of sialylation (A1 + 2 × A2) was calculated as the sum of the incidence (%) of monosialo oligosaccharides (A1) plus twice the incidence (%) of disialo oligosaccharides (A2). The level of galactosylation (G1 + 2 × G2) was calculated as the sum of the incidence (%) of monogalactosylated oligosaccharides (G1) plus twice the incidence (%) of digalactosylated oligosaccharides (G2). a, total IgG from healthy individuals; b, total IgG from RA patients; c, d, and e, IgGRFs from RA patients, IgG(+), IgG(–)(+), and IgG(–)(–)(+), respectively; f, non-RF IgG from RA patients [IgG(–)(–)(–)].

DISCUSSION

Rheumatoid factor (RF) is a characteristic autoantibody associated with rheumatoid arthritis (RA). Various immunoglobulin classes of RFs have been detected in the sera of RA patients on ELISA, and the levels of RFs are generally expressed as titers. It should be noted that the titers of RF activity are poorly related to the actual concentrations of RF in the sera. In order to understand the involvement of RF in the pathogenesis of RA, it is important to determine the actual amounts of RF in the sera, especially the IgG class of RF, which is thought to be involved in the pathogenesis of this disease (*8–10*). In the present study, we prepared a highly purified total IgG fraction from pooled sera of RA patients. IgG fractions with and without RF activity were then prepared by IgG-Sepharose column chromatography, based on the well-known fact that RFs bind to the Fc region of IgG. Three IgGRF fractions were obtained according to their affinity for the IgG-Sepharose column, and it was shown that about 3% of the total serum IgG from RA patients contained RF activity and bound to IgG-Sepharose. The results of gel filtration analysis of these IgGRFs showed that IgGRFs from RA patients apparently fail to efficiently form IgG-IgG self-associating complexes in a solution, while they avidly bind to IgG or Fc immobilized on a solid phase. IgG, which did not bind to the column on the third cycle of IgG-Sepharose column chromatography, was also obtained as non-RF IgG.

Comparisons of the specific RF activities, *i.e.* the binding to IgG-Fc per μg of protein, of the three IgGRFs and non-RF IgG showed that their different binding affinities for the column correlated with the intensities of their specific RF activities. In contrast, the values obtained with the lectin enzyme immunoassay, in which galactose residues of RF were detected with the RCA120 lectin, were the reverse of those for the specific RF activity. Therefore, it was suggested that the higher the RF activity, the lower the level of galactosylation of IgGRF oligosaccharides, and this was confirmed by structural analysis of the oligosaccharides present in both IgGRF and non-RF IgG.

Structural studies of the oligosaccharides of the three IgGRFs, non-RF IgG, and total IgG from RA patients, and total IgG from healthy individuals have shown that they contain a series of biantennary complex type oligosaccharides, ±Galβ1-4GlcNAcβ1-2Manα1-6(±GlcNAcβ1-4)(±Gal-

β1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc with 0–2 sialic acid residues bound to Gal residues in different molar ratios. The incidence of galactose-free oligosaccharides, G0 (d, h, and l in Fig. 5), in the total IgG from RA patients was significantly higher than that in healthy individuals, which corresponds to earlier observations (*4*, *5*, *17*). One question to be resolved was whether the abnormal galactosylation of IgG oligosaccharides found in RA patients occurs in IgG with or without RF activity (IgGRF or non-RF IgG, respectively). The present study has shown that this abnormality occurs in both IgGRF and non-RF IgG, indicating that the abnormality of oligosaccharides in IgG is not directly associated with RF activity. Indeed, both galactose-free IgG enzymatically prepared from total IgG of healthy individuals (*6*) and the intact IgG showed no binding to IgG-Sepharose (data not shown). However, the abnormality of galactosylation was more pronounced in the three IgGRFs than in the non-RF IgG. Our results also suggest that the decrease in galactosylation of IgGRF oligosaccharides is caused by a loss of galactose from both neutral and sialylated oligosaccharides, while the decrease in galactosylation of non-RF IgG only occurs in the neutral oligosaccharides.

It should be noted that in this study the IgGRF with the highest specific RF activity showed the lowest levels of galactosylation and sialylation (Figs. 2 and 6, and Table I). These results suggest the possibility that the lower levels of galactosylation and sialylation in IgGRF molecules may be related with the strength of the RF reactivity of IgGRF. It was previously observed that the levels of IgG with galactose-free oligosaccharides are higher in IgG aggregates in synovial fluid of RA patients than in non-aggregated IgG in the same synovial fluid (*10*). Indeed, the high specific RF activity may cause the formation of these IgG aggregates, which are considered to be involved in the prominent features of RA (*10*, *19–23*). Therefore, within the IgG class of RF autoantibodies, molecules containing higher levels of galactose-free and less-sialylated oligosaccharides may show an increase in pathogenicity.

One possible mechanism of IgG-IgG complex formation has been suggested to involve Fab oligosaccharides, which may be inserted into the vacant pocket of the Fc of IgG formed by oligosaccharides lacking galactose (*24*, *25*). Thus, IgG-containing immune complexes (IgG-IgG complexes) are formed without an antigen-antibody reaction. However, the present study has shown that oligosaccharides of both IgGRF and non-RF IgG from RA patients exhibit reduced galactosylation compared with those of IgG from healthy individuals (Table I and Fig. 6B), and that IgGRFs, especially those exhibiting the lowest galactosylation, bind to IgG-Fc lacking Fab with high affinity (Figs. 2 and 6B, and Table I). This indicates that the mechanisms of immune-complex formation are not so simple. It has also been reported that the constant region of human IgG_4RF exhibits Fc-binding activity, suggesting that the IgG class of RF may not be classical antibodies (*26*). It remains unclear whether or not IgGRFs with higher levels of abnormal glycoylation preferentially recognize galactose-free IgG rather than normal IgG. The findings in the present study may now contribute to our understanding of the mechanisms of IgG-IgG complex formation and the pathogenicity of these complexes in RA.

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