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Structural studies on IgG oligosaccharides of patients with primary Sjögren's syndrome

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Sjögren's syndrome (SS) is an autoimmune disease, and some patients have been found to have SS complicated with rheumatoid arthritis (RA), in which IgG is known to carry abnormal *N*-linked oligosaccharides. In order to investigate the relationship between SS and RA, the structures of *N*-linked oligosaccharides of IgG from 12 primary SS patients without RA, 9 RA patients, and 8 healthy individuals were analyzed using reversed-phase high-performance liquid chromatography, in combination with sequential exoglycosidase treatment and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. All of the IgG samples obtained from primary SS patients, RA patients, and healthy individuals contained the same series of biantennary complex-type oligosaccharides, but the ratio of each oligosaccharide differed among these 3 groups. The incidence of galactose-lacking *N*-linked oligosaccharides obtained from the IgG of RA patients was significantly higher than that from healthy individuals, but that from the serum IgG of primary SS patients varied among individuals. The patients with primary SS were classified into two groups based on the galactosylation levels of IgG oligosaccharides; one group exhibits galactosylation levels as low as those of RA patients and another exhibits levels similar to those of healthy individuals. Measurement of levels of rheumatoid factor (RF) revealed that primary SS patients with a high incidence of RF belonged to the low galactosylation group, as did RA patients. These results suggest that appearance of IgG carrying abnormal *N*-linked oligosaccharides in primary SS may be related to future complication with RA.

***Keywords:* N-linked oligosaccharides, IgG, Sjögren's syndrome, rheumatoid arthritis, autoimmune diseases**

***Abbreviations:* ABEE, *p*-amino benzoic acid ethyl ester; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; RA, rheumatoid arthritis; RF, rheumatoid factor; RCA, *Ricinus communis* agglutinin; SS, Sjögren's syndrome.**

Introduction

Human IgG contains an asparagine-linked carbohydrate chain at Asn_{297} in the CH_2 domain in the Fc region of each heavy chain. The oligosaccharide moieties of human IgG consist of a series of biantennary complex-type structures of ±Galβ1-4GlcNAcβ1-2Manα1-6(±GlcNAcβ1-4)(±Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc [1]. It has been demonstrated that some systemic autoimmune diseases are accompanied by alteration of oligosaccharide structures of IgG [2–10]. Particularly, the incidence of *N*-linked oligosaccharides lacking outer-arm galactose is significantly higher in the serum IgG of patients with rheumatoid arthritis (RA[2]), suggesting that galactose-free IgG molecules are associated with RA [5,6,11–13]. However, the detailed structures of IgG oligosaccharides have yet to be analyzed in most autoimmune diseases except for RA. Thus, the structural analysis of oligosaccharides in IgG is critical to understanding respective autoimmune diseases.

Sjögren's syndrome (SS) is an autoimmune inflammatory disease that is characterized by dry mouth and dry eye, the presence of anti-SSA and anti-SSB autoantibodies, and the presence of rheumatoid factor (RF). In addition, some SS patients have been complicated with autoimmune diseases including RA (secondary SS). Although several reports have cited the structural change of IgG oligosaccharides in primary SS patients based on the different reactivities of lectins toward serum IgG [3,4,10] or on the different patterns of elution profiles of the IgG oligosaccharides [7,9], the detailed structures of IgG oligosaccharides in patients with SS have yet to be analyzed. To

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understand the relationship between SS and RA based on the carbohydrate structures of IgG, the structures of IgG oligosaccharides purified from the sera of patients with primary SS without autoimmune rheumatic diseases including RA [14,15] were analyzed and compared to those from patients with RA and healthy individuals in the current research.

Materials and methods

Patients

Sera were obtained from 12 primary SS patients who do not have RA (females with ages of 54.8 ± 12.9 years), from 9 sex and age-matched patients with RA (females with ages of 51.6 ± 12.2 years), and 8 sex and age-matched healthy individuals (females with ages of 49.1 ± 8.3 years). All patients with RA fulfilled the criteria of the American Rheumatism Association [16], and all patients with primary SS fulfilled the criteria from Vitali et al. [17]. RA patients did not have SS and primary SS patients had no other autoimmune rheumatic diseases including RA.

Chemicals and enzymes

p-Aminobenzoic acid ethyl ester (ABEE) and sodium cyanoborohydride were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Neutral, monosialo, and disialo biantennary ABEE-oligosaccharide were prepared from oligosaccharides, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, of human fibrinogen [18] via their conversion into ABEE-derivatives as described previously [19,20]. The structures of the authentic standards of ABEE-oligosaccharides 1 to 12 were as follows: (1) Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, (2) Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, (3) GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, (4) GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, (5) Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, (6) Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, (7) GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, (8) GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, (9) Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, (10) GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, (11) Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, and (12) GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE. These standards were prepared from ABEE-oligosaccharides from mouse IgG, human IgG, and human fibrinogen [1,18,21]. *Arthrobacter ureafaciens* neuraminidase was purchased from Nacalai Tesque, Inc. Jack bean meal β-galactosidase and β-*N*-acetylhexosaminidase were purified in accordance with previously reported methods [22,23]. Glycosidase digestion of ABEE-oligosaccharides was performed as described previously [19,24,25].

Separation and purification of IgG from serum

Each serum sample was dialyzed against 15 mM phosphate buffer, pH 8.0, and then subjected to chromatography on a DEAE-cellulose (DE-52 from Whatman International Ltd., Maidstone, UK) column to isolate the IgG fraction [20]. The pass-through fraction was used as the total IgG. The purity of the total IgG thus obtained was confirmed by chromatography on a Superdex 200 HR column (10 × 30 cm, Pharmacia Biotech) equilibrated with 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli system [26], followed by Western blotting analysis using antibodies against IgG, IgM, IgA, and transferrin, respectively.

Liberation and isolation of N-linked oligosaccharides from IgG samples

Each sample of IgG purified from the serum of individuals was dialyzed against distilled water and then subjected to gas-phase hydrazinolysis at 90°C for 3 h followed by N-acetylation to quantitatively liberate N-linked oligosaccharides of IgG, as described previously [1,23,25]. The oligosaccharide mixture was purified with cellulose column chromatography [27] and then converted into ABEE-derivatives by reductive amination [19,20].

Analysis of N-linked oligosaccharides in IgG by HPLC

Analysis of the IgG-derived ABEE-oligosaccharides was performed using ion exchange HPLC with a COSMOGEL DEAE column (7.5 × 75 mm, Nacalai Tesque Inc.) as described previously [19,20]. A desialylated neutral oligosaccharide mixture obtained using exhaustive neuraminidase treatment of the ABEE-oligosaccharide fraction was purified by ion exchange HPLC. Then, the neutral ABEE-oligosaccharide mixture was subjected to HPLC using a Wakosil 5C18-200 column (0.5 × 25 cm, Wako Pure Chemical Industries), as described previously [18,20]. ABEE-oligosaccharides eluted from the column were detected by monitoring absorbance at 304 nm. Mass spectrometry analysis of ABEE-oligosaccharides was performed using a MALDI-TOF mass spectrometer, Model Vision 2000 (ThermoBioAnalysis, Hemel Hempsted, Herts, UK).

Measurement of rheumatoid factors

Measurement of rheumatoid factors (RFs) in each serum sample was performed with lectin enzyme immunoassay using Eitest

CA-RF (Eisai Co., Ltd., Tokyo, Japan). This method was originally developed in the authors' laboratory and detects galactose residues in the oligosaccharides of various classes of rheumatoid factors bound to galactose-free IgG [8]. Briefly, serum samples were diluted 200-fold with the dilution buffer in the kit and then 100 μl of the diluted serum were added to the plates coated with galactose-free IgG. Galactose residues in RFs that bound to the galactose-free IgG were detected using RCA120 conjugated to biotin followed by horseradish peroxidase-conjugated streptavidin. The absorbance was measured at a wavelength of 405 nm with a Model 450 Microplate Reader (Bio-Rad Laboratories, CA) using a reference wavelength of 490 nm.

Results

Purification of IgG from sera of primary SS patients

In order to analyze detailed structure of oligosaccharides on IgG of primary SS patients, IgG samples were purified from sera obtained from primary SS patients. In addition, IgG samples were also purified from sera obtained from RA patients and healthy individuals with sexes and ages matched among the groups to compare the IgG oligosaccharides of these groups to those of primary SS patients. Each purified IgG was eluted as a single peak at the position of authentic human IgG in chromatography with a Superdex 200 HR column, migrated as a single band with a molecular mass around 150 kDa under non-reducing conditions, and separated into heavy and light chains with molecular masses of about 50 kDa and 25 kDa under reducing conditions, respectively, in SDS-PAGE (Figure 1). In Western blotting analysis, a single protein band corresponding to the heavy chain observed under reducing conditions was detected with the antibody against the human γ-chain. No bands were detected under reducing conditions using antibodies against the human

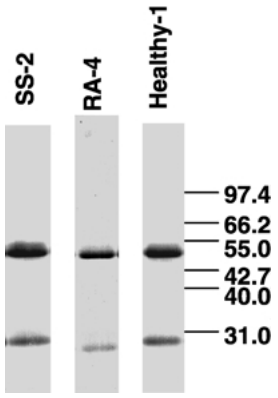

Figure 1. SDS-PAGE analysis of the total IgG isolated from sera of SS patients, RA patients, and healthy individuals. Each IgG sample isolated from primary SS patients, RA patients, and healthy individuals was subjected to SDS-PAGE under reducing conditions using a 10% gel. Proteins were stained with Coomassie Brilliant Blue R-250. The positions of protein markers are indicated on the right of the panel.

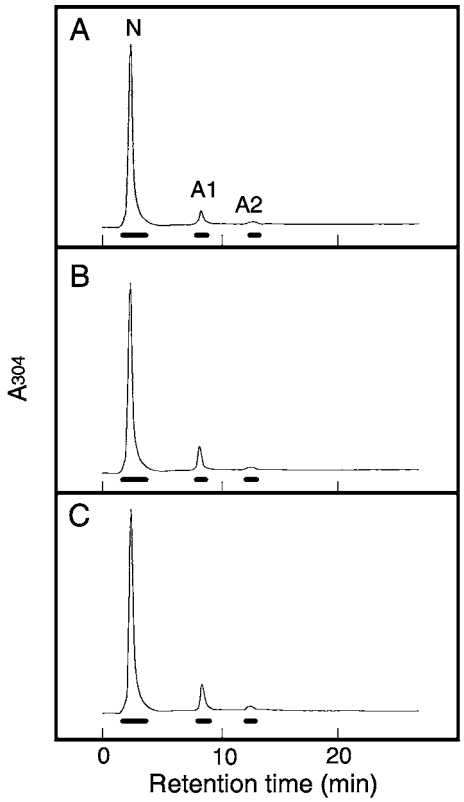

Figure 2. Ion-exchange HPLC analysis of ABEE-oligosaccharides. The oligosaccharides on each IgG were liberated with hydrazinolysis and converted into ABEE-derivatives as described in "Materials and Methods." The ABEE-oligosaccharides derived from each IgG sample were then subjected to DEAE-HPLC with a COSMOGEL DEAE column (7.5 $\times$ 75 mm). Panels A, B, and C are the typical elution profiles of IgG oligosaccharides derived from an RA patient (RA-1), a healthy individual (Healthy-5), and a patient with primary SS (SS-9). N, A1, and A2 indicate the neutral, monosialylated, and disialylated oligosaccharides, respectively.

μ-chain, α-chain, or transferrin. Therefore, the total IgG prepared from individual serum was considered to be extremely pure.

Anion-exchange chromatography of IgG oligosaccharides obtained from SS patients

The oligosaccharides of each IgG sample were quantitatively liberated from the polypeptide portion by hydrazinolysis and converted into ABEE-derivatives. Then the ABEE-labeled oligosaccharides obtained from each IgG sample were subjected to ion exchange HPLC. As shown in Figure 2, all of the ABEE-oligosaccharide samples derived from the IgG samples of primary SS patients as well as those from healthy individuals and RA patients were separated into a neutral fraction (N) and two acidic fractions (A1 and A2). The acidic oligosaccharides, A1 and A2, eluted at the same positions as authentic mono and di-sialo biantennary oligosaccharides, respectively, and were

converted into neutral oligosaccharides by neuraminidase treatment, 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 each IgG sample were then calculated on the basis of the absorbance at 304 nm. The mean molar ratios of neutral (N), mono-sialo (A1), and di-sialo oligosaccharides (A2) in IgG derived from the primary SS patients, RA patients, and healthy individuals were 83.0 ± 2.7:14.6 ± 2.7:2.4 ± 0.4, 85.4 ± 1.7:12.9 ± 1.5:1.7 ± 0.3, and 82.7 ± 2.6:15.2 ± 2.3:2.1 ± 0.5, respectively. The molar ratios of N, A1, and A2 in IgG derived from the primary SS patients did not significantly differ from the molar ratios of healthy individuals.

Structural analysis of IgG oligosaccharides obtained from SS patients

The mixture of ABEE-oligosaccharides in each IgG sample was then treated with neuraminidase, and the resulting neutral oligosaccharides were subjected to reversed-phase HPLC. Each ABEE-labeled mixture was separated into 12 oligosaccharide fractions (a to l) as shown in Figure 3, although the ratios of the fractions differed among the samples. The ratio of the fractions differed substantially with respect to IgG samples obtained from both an RA patient and a healthy individual (Figure 3A and B). This difference is caused by a decrease in galactose residues of the oligosaccharides on IgG from RA patients [6,11–13]. Results of reversed-phase HPLC using IgG samples obtained from primary SS patients are noteworthy since the pattern of ABEE-oligosaccharides of one SS patient appeared similar to patterns in RA patients, while that of another SS patient was similar to the patterns in healthy individuals (Figure 3C and D).

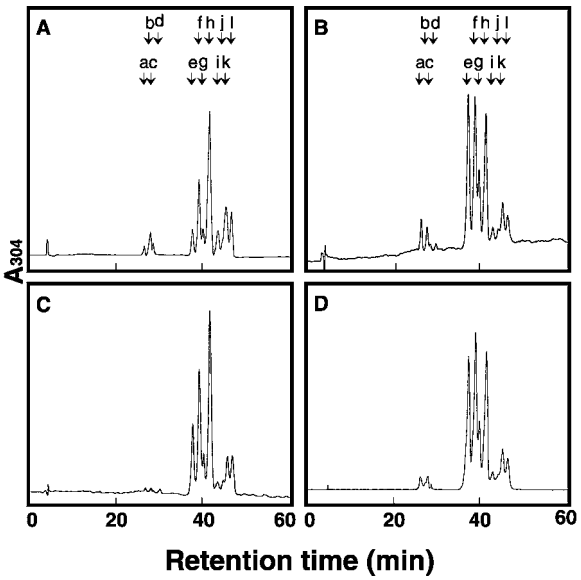

Figure 3. Reversed-phase HPLC analysis of the neutral ABEE-oligosaccharides obtained from each IgG sample. The ABEE-oligosaccharides of each IgG sample were treated with neuraminidase, and the resulting neutral oligosaccharide mixture was subjected to ODS-HPLC using a Wakosil 5C18-200 column (0.4 × 25 cm). Panels A and B are the typical elution profiles of the neutral ABEE-oligosaccharides obtained from IgG in sera of an RA patient (RA-1) and a healthy individual (Healthy-3), respectively. Panels C and D are the two distinct elution profiles of the neutral ABEE-oligosaccharides obtained from IgG in sera of patients with primary SS (SS-9 and SS-5). The neutral ABEE-oligosaccharides derived from all IgG samples yielded twelve peaks indicated as peaks a to l.

The oligosaccharide fractions a to l in IgG samples obtained from primary SS patients as well as those obtained both from RA patients and healthy individuals eluted at the same positions as those of a series of authentic biantennary oligosaccharides 1 to 12 as described in Materials and Methods. Furthermore, the 12 ABEE-oligosaccharide fractions in IgG from primary SS patients were converted by treatment of the oligosaccharides with jack bean β-galactosidase (data not shown) into three oligosaccharide fractions (d, h, and l) eluted at the same positions as authentic biantennary agalacto-oligosaccharides 4, 8, and 12, respectively. Sequential incubation with β-galactosidase and β-N-acetylhexosaminidase converted the 12 ABEE-oligosaccharide fractions in IgG obtained from SS patients into two oligosaccharide fractions eluted at the same positions as authentic Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE (data not shown). MALDI-TOF mass spectrometry analysis of the fractions indicated that the oligosaccharide fractions, a to l, derived from each IgG sample of a primary SS patient yielded a mass number corresponding to the theoretical mass number of the proposed oligosaccharide structures with a degree of error below 1 Da (Figure 4). The behavior of oligosaccharides in fractions a to l on the reversed-phase HPLC column and the results of sequential exoglycosidase digestion as well as MALDI-TOF mass spectrometry analysis strongly indicated that the ABEE-oligosaccharides in fractions a to l derived from each IgG sample of the primary SS patients were as shown in Figure 4. These result indicate that the IgG molecule derived from primary SS patients contains the same series of biantennary complex-type oligosaccharides as do IgG molecules of healthy individuals and RA patients [1,6,11–13], but the incidence of each oligosaccharide in the IgG molecule of primary SS patients differs from that of healthy individuals and RA patients. The molar ratios of oligosaccharides a to l in each IgG sample were then calculated on the basis of their absorbance at 304 nm. The percent incidence of galactose-free oligosaccharides (G0; peaks d, h, and l in Figure 3), monogalactosylated oligosaccharides (G1; peaks b, c, f, g, j, and k), and digalactosylated oligosaccharides (G2; peaks a, e, and i), and the percent incidence of oligosaccharides with a core fucose residue (F; peaks from e to l) and with a bisecting GlcNAc residue (Bi; peaks from i to l) are summarized in Table 1.

ODS-fractions	Identified oligosaccharide structures	Mass numbers [M + Na]+	
		Observed	Theoretical
a:	Galβ1→4GlcNAc β1→2Man α1↘6 / Galβ1→4GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1812.6	1812.7
b:	Galβ1→4GlcNAc β1→2Man α1↘6 / GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1650.6	1650.6
c:	GlcNAc β1→2Man α1↘6 / Galβ1→4GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1650.6	1650.6
d:	GlcNAc β1→2Man α1↘6 / GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1488.4	1488.6
e:	Fucα1↓6 Galβ1→4GlcNAc β1→2Man α1↘6 / Galβ1→4GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1958.7	1958.7
f:	Fucα1↓6 Galβ1→4GlcNAc β1→2Man α1↘6 / GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1797.0	1796.7
g:	Fucα1↓6 GlcNAc β1→2Man α1↘6 / Galβ1→4GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1796.8	1796.7
h:	Fucα1↓6 GlcNAc β1→2Man α1↘6 / GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1634.7	1634.6
i:	GlcNAc β1↓4 Fucα1↓6 Galβ1→4GlcNAc β1→2Man α1↘6 / Galβ1→4GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	2161.8	2161.8
j:	GlcNAc β1↓4 Fucα1↓6 GlcNAc β1→2Man α1↘6 / Galβ1→4GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1999.7	1999.8
k:	GlcNAc β1↓4 Fucα1↓6 Galβ1→4GlcNAc β1→2Man α1↘6 / GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1999.7	1999.8
l:	GlcNAc β1↓4 Fucα1↓6 GlcNAc β1→2Man α1↘6 / GlcNAc β1→2Man α1↗3 Manβ1→4GlcNAc β1→4GlcNAc-ABEE	1837.8	1837.7

Figure 4. Structure of desialylated ABEE-oligosaccharides a-l derived from human IgG samples. The mass number of each oligosaccharide given by MALDI-TOF mass spectrometry analysis and the theoretical mass number calculated from each proposed oligosaccharide structure were also indicated.

The level of galactosylation of IgG from SS patients

The distribution of the twelve neutral oligosaccharide structures in each IgG sample was compared among primary SS patients, RA patients, and healthy individuals. Among IgG samples derived from primary SS patients, RA patients, and healthy individuals, there was no statistical difference in the occurrence of oligosaccharides with a core fucose ($P = 0.11$ for SS vs. Healthy and $P = 0.29$ for SS vs. RA) and those with a bisecting GlcNAc ($P = 0.23$ for SS vs. Healthy and $P = 0.13$ for SS vs. RA) (Table 1). The incidence of galactose-free oligosaccharides (G0) in the IgG samples derived from RA patients ($45.8 \pm 5.7\%$) was significantly higher ($P < 0.0001$) than the incidence in IgG samples derived from healthy individuals ($28.5 \pm 4.5\%$), as reported previously [5,6,11–13]. In the case of primary SS patients, however, the incidence of G0 in IgG samples did vary among individuals; IgG derived from 4 (SS-6, 9, 11, and 12) of the 12 patients contained galactose-free oligosaccharides with an incidence of more than 40% but IgG from 2 (SS-5 and 10) had an incidence of less than 30%, and IgG from other patients had an incidence of oligosaccharides of about 30–40%. This data strongly indicated that abnormal galactosylation of oligosaccharides occurred in IgG of some primary SS patients without RA.

In order to elucidate the relationship between glycosylation in IgG and the type of disease, the level of galactosylation in

Table 1. The distribution of neutral oligosaccharide structures present in various samples of IgG

		Incidence (%)[1]				
	IgG	*Bi*	*F*	*G2*	*G1*	*G0*
SS patients	1	26.4	95.2	16.3	44.5	39.2
	2	20.2	96.0	18.4	43.6	38.0
	3	20.0	96.4	25.1	38.8	36.1
	4	18.2	97.1	27.4	37.9	34.7
	5	19.3	95.6	25.7	45.8	28.5
	6	15.0	94.6	21.5	36.8	41.7
	7	23.0	96.9	22.4	40.8	36.8
	8	20.1	97.4	24.5	45.2	30.3
	9	31.2	91.6	15.7	39.9	44.4
	10	8.6	94.2	32.1	43.4	24.5
	11	14.5	97.2	16.5	31.1	52.4
	12	10.1	97.4	21.4	36.4	42.2
RA patients	1	15.1	98.0	12.2	39.0	48.8
	2	15.2	95.7	16.7	43.0	40.3
	3	14.7	92.5	13.0	42.5	44.5
	4	16.9	98.5	14.2	36.6	49.2
	5	9.1	94.2	19.4	41.7	38.9
	6	22.4	97.0	12.7	32.5	54.8
	7	15.6	96.5	17.3	42.1	40.6
	8	14.6	99.3	16.5	40.6	42.9
	9	15.9	99.8	14.3	33.5	52.2
Healthy individuals	1	12.9	97.7	32.9	47.0	20.1
	2	19.4	96.7	27.9	40.8	31.3
	3	16.8	95.6	27.4	43.7	28.9
	4	12.1	96.9	26.3	44.5	29.2
	5	17.8	98.3	23.7	49.9	26.4
	6	16.5	99.6	29.5	45.2	25.3
	7	20.2	96.6	18.7	47.5	33.8
	8	15.1	94.7	25.7	41.3	33.0

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

each IgG sample derived from RA patients, primary SS patients, and healthy individuals was then calculated as the sum of the percentage of monogalactosylated oligosaccharides plus twice the percentage of digalactosylated oligosaccharide (G1 + 2 × G2) for comparison of the samples (Figure 5). The galactosylation levels of IgG derived from all RA patients (69.3 ± 7.8%) were distributed under the level of the mean -2SD of galactosylation levels of IgG derived from healthy individuals (98.1 ± 8.1%). In contrast, the galactosylation levels of IgG derived from primary SS patents were widely distributed (84.9 ± 12.1%) in comparison to the levels of IgG derived from RA patients and those from healthy individuals. The levels of 6 of the 12 SS patients (SS-1, 2, 6, 9, 11, and 12) were distributed under the value of mean - 2SD of galactosylation levels of IgG derived from healthy individuals,

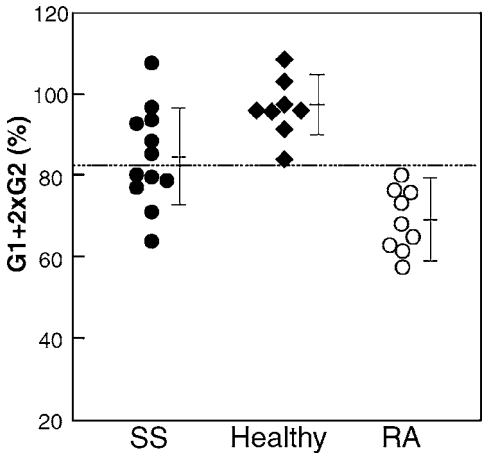

Figure 5. Levels of galactosylation of IgG oligosaccharides derived from SS patients, RA patients, and healthy individuals. The level of galactosylation (G1 + 2 × G2) was calculated as the sum of the percentage of monogalactosylated oligosaccharides (G1) plus twice the percentage of digalactosylated oligosaccharides (G2). The dotted line indicates the mean – 2 SD of the galactosylation level of oligosaccharides in IgG obtained from healthy individuals. Means and means ± SD of the galactosylation levels of oligosaccharides in IgG obtained from SS patients, RA patients, and healthy individuals are also indicated as bars.

while levels of the other 6 patients were distributed over the level.

Relationship between abnormality of IgG oligosaccharides and RF reactivity in sera of patients with primary SS

RA is known to be associated with increased levels of galactose-free oligosaccharides in IgG and with production of RF. In addition, the sera of some patients with primary SS yielded a high incidence of RF similar to the sera of patients with RA [28]. As described above, this data indicated that abnormal galactosylation of oligosaccharides occurred in IgG of some primary SS patients without RA. Therefore, reactivity of RF in the serum samples was measured using lectin enzyme immunoassay [8] to ascertain the relationship between abnormality of IgG oligosaccharides and occurrence of RF in primary SS patients. As shown in Figure 6, all of the sera obtained from RA patients exhibited a high level of absorbance (over 3.0), and all of the sera obtained from healthy individuals exhibited a very low level of absorbance (less than 0.5) at 405 nm. Primary SS patients, in contrast, had differing levels of absorbance; samples from 4 of the 12 patients (SS-1, 9, 11, and 12) exhibited an extremely high level of absorbance as seen with RA patients, and 4 (SS-3, 4, 8, and 10) had medium levels (about 1.5) of absorbance, but other sera exhibited only low levels of absorbance (less than 1.0) as was seen with healthy individuals. An important point to note is that all 4 patients with primary SS whose sera contained a high incidence of RF as with RA patients (SS-1, 9, 11, and 12) belonged to the low galactosylation group, like RA patients,

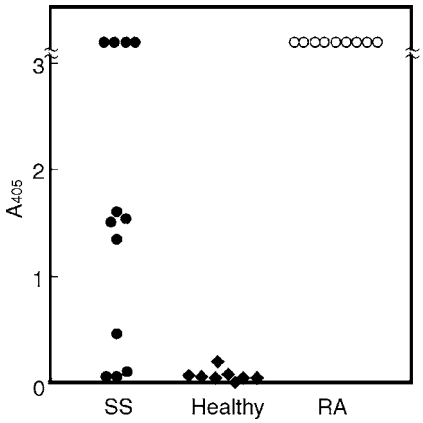

Figure 6. RF reactivity in sera of SS patients, RA patients, and healthy individuals. RF reactivity in each serum sample was determined by means of lectin enzyme immunoassay using Eitest CA-RF. Briefly, serum samples were diluted 200-fold and then 100 μl of the diluted serum were added to wells coated with galactose-free IgG, and the galactose residues in rheumatoid factors that bound to the galactose-free IgG were detected using RCA120 conjugated to biotin followed by horseradish peroxidase-conjugated streptavidin.

although these primary SS patients have not displayed clinical signs and symptoms of RA.

Discussion

The detailed structures of IgG oligosaccharides in patients with SS have yet to be analyzed, although the structural changes in IgG oligosaccharides in SS patients have been indicated by the different reactivities of lectins toward serum IgG [3,4,10] or by the different patterns of elution profiles of the IgG oligosaccharides [9]. This study involved the structural analysis of IgG oligosaccharides in the sera of patients with primary Sjögren's syndrome (SS) and the comparison of the structures among primary SS patients, RA patients, and healthy individuals to ascertain the relationship between SS and RA based on the carbohydrate structures of IgG. The structural analysis of the oligosaccharides of respective IgGs isolated from primary SS patients, RA patients, and healthy individuals has indicated that they contain the same series of biantennary complex-type oligosaccharides of ±Galβ1-4GlcNAcβ1-2Manα1-6(±GlcNAcβ1-4)(±Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc with 0-2 sialic acid residues bound to galactose residues in different molar ratios.

Previous research has reported that, based on the reactivity of serum IgG against a specific lectin, the desialylated glycoform of IgG and the level of bisecting GlcNAc in IgG oligosaccharides increased in patients with primary SS [3,4,10]. However, anion-exchange HPLC of the oligosaccharides separated from purified IgG revealed that the molar ratios of desialylated (neutral) oligosaccharides in IgG derived from all of the primary SS patients did not differ significantly from the molar ratios of healthy individuals ($P > 0.5$). In addition, the incidence of oligosaccharides with a core fucose and those with a bisecting GlcNAc in IgG from the primary SS patients did not differ significantly from that in healthy individuals. The incidence of galactose-free oligosaccharides G0 in IgG derived from each RA patient was significantly higher than that from healthy individuals, as described in previous studies [6,11–13]. In contrast to RA patients, the incidence of galactose-free oligosaccharides in IgGs derived from primary SS patients varies widely depending on the individual. The IgGs derived from some of the patients displayed an incidence of galactose-free oligosaccharides similar to that in RA patients, while those from other patients displayed an incidence similar to that of healthy individuals. Parekh et al. [7] previously analyzed the incidence of G0 oligosaccharides in IgGs derived from 8 patients with primary SS, and concluded that the incidence of G0 oligosaccharides in IgG of primary SS patients did not differ statistically from that of healthy individuals, although they observed the elevation of the incidence of G0 oligosaccharides in IgG of 2 patients with primary SS.

When the abnormality of oligosaccharides in each IgG was estimated as the level of galactosylation (sum of the percentage of monogalactosylated oligosaccharides, G1, plus twice the percentage of digalactosylated oligosaccharide, G2, in Table 1), RA patients appeared distinct from healthy individuals based on the galactosylation levels of the oligosaccharides in IgGs (Figure 5), i.e., the galactosylation levels of IgGs derived from all of the RA patients were distributed under the level of the mean - 2SD of galactosylation calculated from healthy individuals. In contrast, the galactosylation levels of oligosaccharides in IgG derived from primary SS patients were widely distributed between levels in healthy individuals and in RA patients. The galactosylation levels of IgGs derived from 6 (SS-1, 2, 6, 9, 11, and 12) of 12 patients were lower than the level of the mean - 2SD of galactosylation calculated from healthy individuals, as with RA patients, and levels from the remaining patients were higher than the level, as with healthy individuals. The possibility of the effect of medication on the galactosylation levels of IgG may be negligible since the patients with primary SS whose sera were used in this study were treated with nosotropic medication, and treatment with steroids has been shown to not affect the galactosylation levels of IgG in patients with several autoimmune diseases [29]. Therefore, patients with primary SS may be classified into two groups based on the levels of galactosylation of IgG oligosaccharides: a group with the same levels of galactosylation as RA patients and another with the same levels of galactosylation as healthy individuals. Interestingly, all 4 patients with primary SS whose sera contained a high incidence of RF as seen in RA patients (SS-1, 9, 11, and 12 in Figure 6) belonged to the low galactosylation group, although the patients have not displayed the symptoms of RA. A long-term follow-up study of primary SS patients indicated that some RF-positive patients with primary SS were given revised

diagnoses including RA over the follow-up period [30]. Since the levels of galactosylation of IgG in RA patients have been shown to reflect the condition of the disease [6,12], primary SS patients that, with respect to the level of galactosylation, belong to the same group as RA patients may have complications with RA in the future. A long-term follow-up of patients with SS is necessary to clarify this hypothesis.

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