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Structural changes in the oligosaccharide moiety of human IgG with aging

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In order to elucidate the relationship between glycosylation of IgG and aging, oligosaccharide structures of human IgG purified from sera of men and women aged 18 to 73 years were investigated. Oligosaccharides were liberated quantitatively from IgG by hydrazinolysis followed by *N*-acetylation and were tagged with *p*-aminobenzoic acid ethyl ester. The oligosaccharide structures were then analyzed by HPLC in conjunction with sequential exoglycosidase digestion. All IgG samples were shown to contain a series of biantennary complex type oligosaccharides which consisted of ±Galβ1-4GlcNAcβ1-2Manα1-6(± GlcNAcβ1-4)(± Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(± Fucα1-6)GlcNAc and their mono- and disialo glycoforms in different ratios. In female IgG samples only, the incidence of non-galactosylated oligosaccharides with non-reducing terminal GlcNAc residues increased with aging ($r > 0.8$), whereas that of digalactosylated oligosaccharides decreased ($r < -0.8$). A weaker correlation was observed between aging and the incidence of neutral and monosialo oligosaccharides in female IgG ($r = 0.461$ and $r = -0.538$, respectively) and between aging and the incidence of oligosaccharides with a bisecting GlcNAc in both male and female IgG samples ($r = 0.566$ and $r = 0.440$, respectively). In addition, a significant change with aging in the galactosylation of IgG oligosaccharides was observed in females in their thirties, fifties, and sixties ($p < 0.02$, $p < 0.01$, and $p < 0.04$, respectively). These findings may contribute to our understanding of autoimmune diseases such as rheumatoid arthritis in which glycosylation is involved.

Keywords: **aging, Asn-linked oligosaccharide, glycosylation, IgG, galactose, *p*-aminobenzoic acid ethyl ester**

Abbreviations: **ABEE, *p*-aminobenzoic acid ethyl ester; IgG, IgA, and IgM, immunoglobulin G, A, and M, respectively; HPLC, high performance liquid chromatography; RA, rheumatoid arthritis**

Introduction

Human immunoglobulin G (IgG) is a glycoprotein that contains asparagine-linked oligosaccharide chains linked to Asn297 on CH_2 domains in the Fc region. The oligosaccharide moiety of human IgG has been shown to consist of a series of biantennary complex-type structures of ± Galβ1-4GlcNAcβ1-2Manα1-6 (± GlcNAcβ1-4)(± Galβ1-4 GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(± Fucα1-6)GlcNAc containing 0–2 sialic acid residues linked to Gal residues [1]. Structural variation among the oligosaccharides arises from modification of the trimannosyl core structure (bisecting *N*-acetylglucosaminylation and fucosylation) and of the outer arms (galactosylation and sialylation). It has been reported [2, 3], that 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.

The structures of IgG oligosaccharides reflect various immunological changes such as monoclonal expansion of B lymphocytes in patients with IgG myeloma [1] and the development of autoimmune diseases including rheumatoid arthritis (RA) [4–7]. The mechanisms that cause disease-related glycosylation changes have not yet been elucidated. In order to better understand these mechanisms, it is also important to define glycosylation changes as they relate to normal immunological processes. Aging is known to influence immunological competence. It has been reported that levels of serum immunoglobulins reactive with several

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autoantigens and resistance to self-tolerance increase with aging [8–10]. Parekh *et al.* [11] reported age-related galactosylation of IgG oligosaccharides in both males and females, which was inconsistent with our recent preliminary observation.

In the present study, we investigated the structures of oligosaccharide chains of IgG purified from sera of both men and women of various ages and demonstrated age-associated changes in the glycosylation of human IgG.

Materials and methods

Chemicals and Enzymes

p-Aminobenzoic acid ethyl ester (ABEE) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium cyanoborohydride was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Neutral, monosialo, and disialo biantennary ABEE-oligosaccharides were prepared from oligosaccharides, ± NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(± NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, of human fibrinogen [12] by ABEE-derivatization as described below. ABEE-oligosaccharides 1 to 12, with respective structures of Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)-Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)-GlcNAc-ABEE, Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, and GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manα1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE were prepared from oligosaccharides of mouse IgG, human IgG and fibrinogen [1, 12, 13] 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 Inc. Jack bean meal β-galactosidase and β-*N*-acetylhexosaminidase were purified according to a previously reported method [14]. Glycosidase digestion of oligosaccharides was performed as described previously [15, 16].

Purification of IgG from sera

Human IgG was isolated from sera of 43 healthy female individuals whose ages ranged from 18 to 73 years and 37 healthy male individuals with ages of 18 to 71 years who had no family history of autoimmune disease. The age distribution of the blood donors is summarized in Table 1. IgG from RA patients was also purified from sera of the five female patients who were all in their forties (45.0 ± 4.1). Total serum IgG was isolated by ammonium sulphate precipitation, followed by chromatography on a DEAE-cellulose (DE-52 from Whatman International Ltd, Maidstone, UK) column and a Superdex 200 pg column (2.6 × 60 cm, Pharmacia Biotech, Uppsala, Sweden). The purity of the IgG thus obtained was confirmed by FPLC with a Superdex 200 column (1.0 × 30 cm, Pharmacia Biotech), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli system [17], and Western blotting analysis using antibodies against IgG, IgA, IgM, transferrin, and normal human serum. The IgG samples were dialysed against distilled water and then lyophilized.

Liberation and isolation of N-linked oligosaccharides from IgG samples

Purified IgG samples (1–2 mg) were 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, 15, 16]. To isolate the liberated oligosaccharides, the hydrazinolysate was subjected to paper chromatography using 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 distilled water.

Table 1. Age distribution of the blood donors.

Age	*No. of samples (Mean ± SD)*	
	Female	*Male*
10–19	2 (18.5)	2 (18.5)
20–29	8 (24.4 ± 2.5)	6 (24.7 ± 2.8)
30–39	8 (35.4 ± 2.4)	8 (34.6 ± 3.0)
40–49	8 (45.1 ± 3.0)	8 (44.1 ± 3.5)
50–59	8 (54.1 ± 2.9)	6 (56.2 ± 2.5)
60–69	8 (63.9 ± 3.2)	6 (65.0 ± 2.5)
70–79	1 (73.0)	1 (71.0)
Total	43 (44.0 ± 15.5)	37 (43.6 ± 15.6)

Preparation of ABEE-labelled oligosaccharides

Each oligosaccharide fraction obtained from 80 human IgG samples was derivatized with a UV-absorbing compound, ABEE, by reductive amination as follows. The isolated oligosaccharides were dissolved in 10 μl of distilled water. The oligosaccharide solution was mixed with 40 μl of reagent solution, freshly prepared by mixing 35 mg ABEE, 3.5 mg $NaBH_3CN$, 41 μl acetic acid, and 350 μl MeOH, and was then incubated at 80 °C for 1 h. After the reaction, 400 μl of distilled water were added and the excess ABEE was removed by repeating the extraction three times with 400 μl of diethyl ether. The ABEE-labeled oligosaccharides (ABEE-oligosaccharides) recovered in the aqueous layer were purified by column chromatography with a Bond Elute C18 cartridge (Varian, CA) and then with a Bio-Gel P-4 (200–400 mesh, Bio-Rad Laboratories, CA) column (1.6×12 cm) equilibrated with distilled water. ABEE-oligosaccharides eluted from the Bio-Gel P-4 column were detected by monitoring absorbance at 304 nm.

Analyses of IgG N-linked oligosaccharides by HPLC

Analysis of the IgG-derived ABEE-oligosaccharides was performed by ion exchange HPLC with a COSMOGEL DEAE column (7.5×75 mm, Nacalai Tesque Inc.) as described previously [18]. The desialylated neutral oligosaccharide mixture obtained by exhaustive neuraminidase treatment of the ABEE-oligosaccharide fraction derived from each IgG sample was purified by ion exchange HPLC and then analyzed by ODS-HPLC. The neutral ABEE-oligosaccharide mixture was subjected to HPLC using a Wakosil 5C18-200 column (0.5×25 cm, Wako Pure Chemical Industries). A mixture of solvent A (5% acetonitrile in 50 mM acetic acid) and solvent B (15% acetonitrile in 50 mM acetic acid) was used for elution of the column as follows. The column was equilibrated with solvents A and B (60 : 40, v/v). After injection of each sample, a linear gradient elution to a final solvent ratio of 30 : 70 was performed for 60 min at a flow rate of 0.8 ml min^{-1} at 50 °C. ABEE-oligosaccharides eluted from the column were detected monitoring absorbance at 304 nm.

Statistical analyses

Statistical analyses, Student's *t* test and calculation of correlation coefficient (r), were performed by means of a Macintosh computer (Power Macintosh 7600/200 from Apple Japan, Inc., Tokyo, Japan) using Microsoft Excel Version 5.0 for the Power Macintosh as the software. Statistical significance of the correlation coefficient was tested according to the textbook for statistics [19].

Results

Purity of IgG samples

The purity of IgG samples analyzed by SDS-PAGE is shown in Figure 1. All of the purified IgG samples migrated as a single band on SDS-PAGE under non-reducing conditions, and dissociated into heavy and light chains under reducing conditions. In addition, Western blotting analysis using several antibodies indicated that all IgG samples prepared contained solely IgG and were not contaminated by other immunoglobulins. Therefore, all the samples were confirmed to be highly pure and suitable for structural analysis of their oligosaccharides.

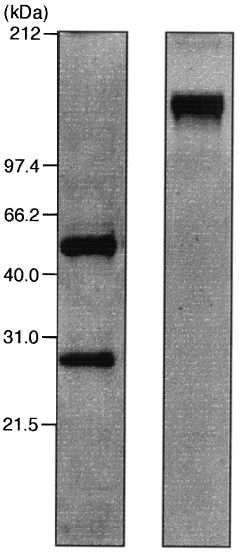

Figure 1. SDS-PAGE of a purified IgG sample. This sample was subjected to SDS-PAGE using a 5–20% gradient gel under reducing (*left*) or non-reducing (*right*) conditions. Proteins were stained with Coomassie Brilliant Blue R-250. The positions and mol. wt (kDa) of protein markers are indicated on the left.

Analysis of sialo oligosaccharides of IgG

In order to determine the charges of IgG oligosaccharides, ABEE-oligosaccharide fractions obtained from all IgG samples were subjected to ion exchange HPLC with a COSMOGEL DEAE column. Each ABEE-oligosaccharide fraction was separated into a neutral fraction (N) and two acidic oligosaccharide fractions (A1 and A2), as shown in Figure 2. Since elution positions of A1 and A2 were the same as those of authentic mono- and di-sialo biantennary ABEE-oligosaccharides, respectively, and since they were converted to neutral oligosaccharides by sialidase treatment (data not shown), A1 and A2 were shown to be mono- and di-sialo ABEE-oligosaccharides, respectively. The molar ratios of N, A1, and A2 for all ABEE-oligosaccharide fractions derived from 80 IgG samples were then calculated on the basis of absorbance at 304 nm.

Structural analysis of desialylated oligosaccharides

In order to analyse the oligosaccharide structures of IgG samples, desialylated neutral ABEE-oligosaccharide

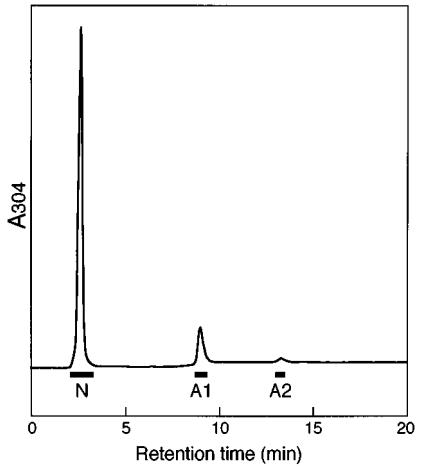

Figure 2. DEAE-HPLC analysis of ABEE-oligosaccharides obtained from human serum IgG. The ABEE-oligosaccharides derived from IgG were applied to a COSMOGEL DEAE column (7.5 × 75 mm).

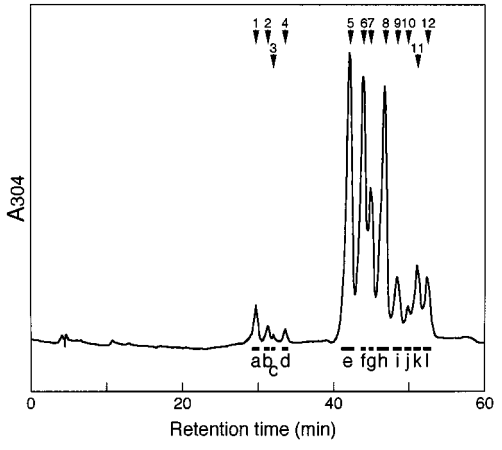

Figure 3. ODS-HPLC analysis of the neutral ABEE-oligosaccharide mixture obtained from human serum IgG. This mixture was desialylated and subjected to HPLC using a Wakosil 5C18-200 column (0.4 × 25 cm). Arrowheads 1–12 indicate elution positions of authentic biantennary complex-type oligosaccharides 1–12, respectively. Structures of these authentic oligosaccharides are described in Materials and methods.

mixtures were subjected to ODS-HPLC with a Wakosil 5C18-200 column (Figure 3). All mixtures were separated into 12 oligosaccharide fractions (a to l) in different ratios. Fractions a to 1 were eluted at the same positions as those of a series of authentic biantennary complex-type oligosaccharides numbered 1 to 12 with structures of ± Galβ1-4GlcNAcβ1-2Manα1-6(± GlcNAcβ1-4)(± Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(± Fucα1-6)GlcNAc-ABEE, respectively, as shown in Figure 3. Four ABEE-oligosaccharides with a bisecting GlcNAc, no fucose, and 0-2 galactose were not detected as distinct oligosaccharide peaks.

The structures of respective IgG-derived ABEE-oligosaccharides were analyzed by sequential exoglycosidase digestion with jack bean β-galactosidase and β-N-acetylhexosaminidase followed by ODS-HPLC. Conversion of the 12 ABEE-oligosaccharide fractions (a to l) into three oligosaccharide fractions (d, h, and l) was observed on incubation with jack bean β-galactosidase (data not shown). Upon sequential digestion with β-galactosidase and β-*N*-acetylhexosaminidase, followed by ODS-HPLC of the reaction products, the 12 fractions were converted into two oligosaccharide fractions which were eluted at the same positions as the authentic oligosaccharides, 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 oligosaccharide fractions a to 1 obtained with the ODS-HPLC column and the results of sequential exoglycosidase digestion, it was suggested that structures of the ABEE-oligosaccharides a to l derived from each IgG sample were as shown in Figure 4. This was also confirmed by MALDI-TOF MS analyses of these twelve oligosaccharides (data not shown). The molar ratios of oligosaccharides a to l for all ABEE-oligosaccharide fractions derived from the 80 IgG samples were then calculated on the basis of absorbance at 304 nm.

Structural changes of IgG oligosaccharides accompanying aging

The amount of each oligosaccharide structure obtained from all IgG samples by ion exchange HPLC and ODS-HPLC was statistically examined for evidence of correlation between glycosylation and aging. Calculated correlation coefficients are summarized in Table 2. It is obvious that the incidence of non- and digalactosylated oligosaccharides in female IgG samples varied with aging ($r = 0.816$ and -0.814, respectively), while that of male IgG hardly showed a correlation with aging ($r = 0.332$ and -0.275). The incidence of neutral and monosialo oligosaccharides in female IgG samples had a weaker correlation with aging ($r = 0.461$ and $r = -0.538$, respectively), as did that of oligosaccharides with a bisecting GlcNAc in both male and female IgG ($r = 0.566$ and $r = 0.440$, respectively). On the other hand, there was no correlation between aging and disialo, monogalactosylated, or fucosylated oligosaccharides in either male or female IgG ($|r| < 0.21$).

The age-related variations in the galactosylation of IgG oligosaccharides are shown in Figure 5. In female IgG samples, the incidence of oligosaccharides with no galactose but with terminal GlcNAc residues increased with aging (Figure 5A), whereas that of the digalactosylated oligosaccharides decreased (Figure 5c). In contrast, this change in galactosylation was not observed significantly in male IgG samples (Figure 5D and F).

The differences in the incidence of non-galactosylated oligosaccharides with each advancing decade are shown in

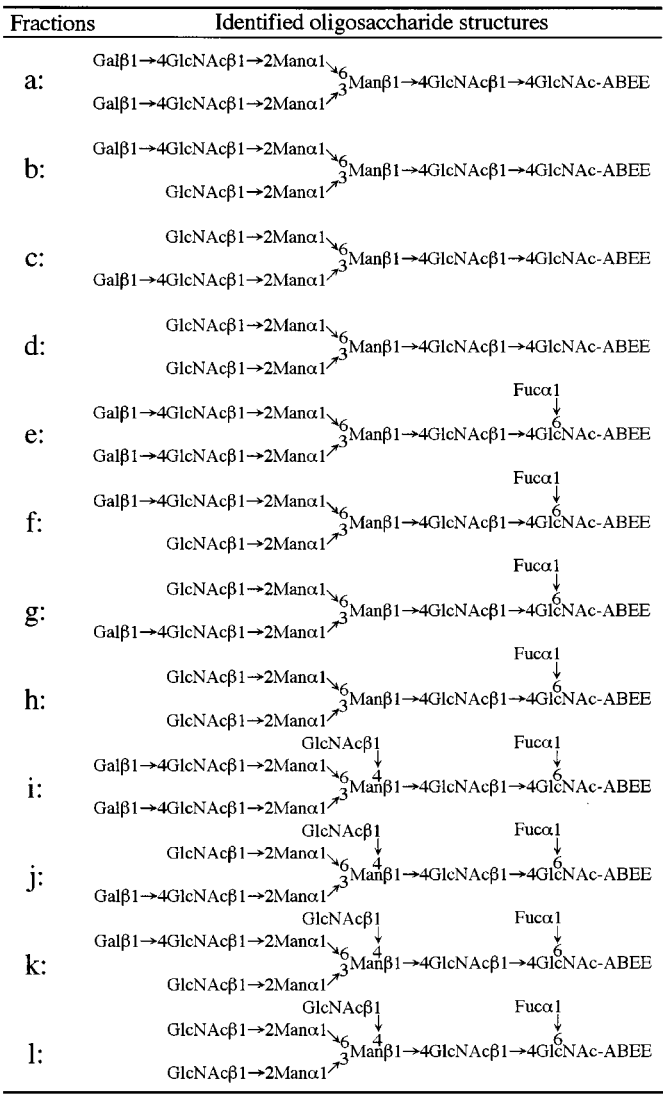

Figure 4. Identified structures of desialylated ABEE-oligosaccharide fractions a–1 of human IgG.

Table 2. Correlations between oligosaccharide structures of serum IgG and aging.

Oligosaccharides	*Correlation coefficient (r =)*	
	Female	*Male*
Neutral	0.461*	− 0.108
Mono-sialylated	− 0.538**	0.072
Di-sialylated	0.024	0.209
Non-galactosylated	0.816**	0.332
Mono-galactosylated	− 0.075	− 0.167
Di-galactosylated	− 0.814**	− 0.275
Bisecting GlcNAc-containing	0.440*	0.566**
Fucosylated	0.133	0.067

Statistical significance of *, $p < 0.01$; **, $p < 0.001$.

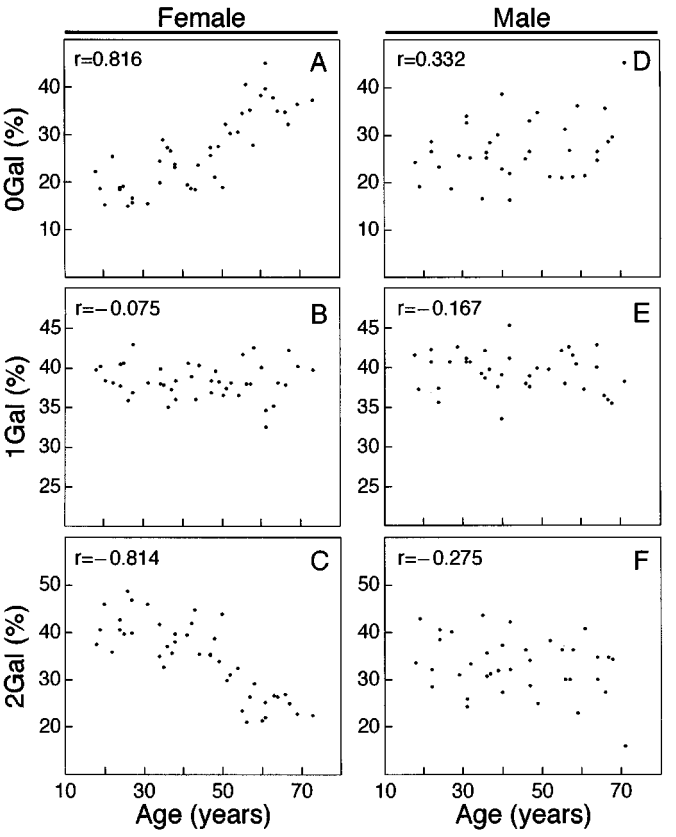

Figure 5. Relationship between age and galactosylation of human serum IgG. 0Gal, non-galactosylated oligosaccharides; 1Gal, mono-galactosylated oligosaccharides; 2Gal, digalactosylated oligosaccharides.

Figure 6. In terms of female IgG samples, in contrast to male samples, there were statistically significant differences in the incidence of non-galactosylated oligosaccharides between their twenties and thirties ($p < 0.02$), their forties and fifties ($p < 0.01$), and their fifties and sixties ($p < 0.04$). When IgG samples from female RA patients in their forties were analyzed (Figure 6), it was shown that the incidence of non-galactosylated oligosaccharides increased significantly compared with healthy females of the same age ($p < 0.0000002$) as well as healthy females in their sixties ($p < 0.0003$).

Discussion

In the present study, it was shown that the level of non-galactosylated oligosaccharides in female IgG increases with aging ($|r| = 0.816$) and that this change is caused by a decrease in digalactosylated oligosaccharides ($|r| = 0.814$) but not monogalactosylated oligosaccharides ($|r| = 0.075$) (Table 2 and Figure 5). The negative correlation ($r = -0.538$) between aging and the incidence of monosialo oligosaccharides in female IgG (Table 2) can be attributed

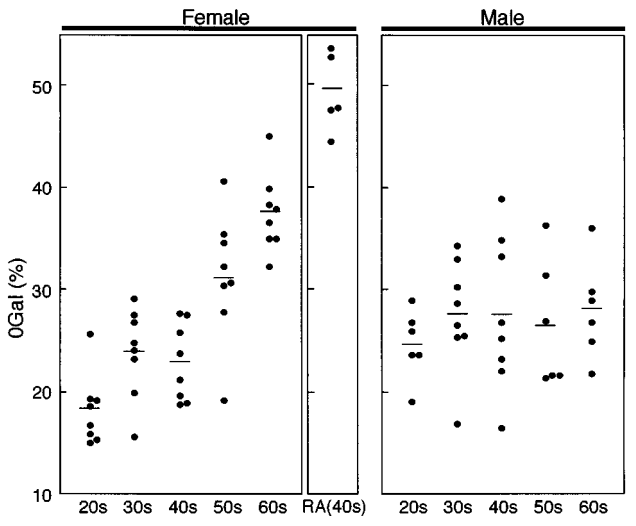

Figure 6. Differences in the incidence of non-galactosylated oligosaccharides with each subsequent decade. Bars indicate the mean value. Results of Student's *t* test analysis: healthy females in their 20s vs 30s, *p* was < 0.02, 30s vs 40s ($p = 0.650$), 40s vs 50s ($p < 0.01$), 50s vs 60s ($p < 0.04$); healthy males in their 20s vs 30s, *p* was 0.376, 30s vs 40s ($p = 0.994$), 40s vs 50s ($p = 0.788$), 50s vs 60s ($p = 0.650$); female RA patients in their 40s vs healthy females in their 20s, 30s, 40s, 50s, and 60 s, values were $p < 0.00000002$, $p < 0.0000005$, $p < 0.0000002$, $p < 0.0002$, and $p < 0.0003$, respectively.

to a low level of galactosylation, which is essential for sialylation of IgG oligosaccharides. In contrast, these age-related changes were not so clear in male IgG samples ($|r| < 0.332$) (Table 2 and Figure 5). Therefore, it was suggested that the decrease in galactosylation of IgG oligosaccharides with aging is a female-specific phenomenon.

Age-related galactosylation of human serum IgG was reported previously by Parekh *et al.* [11]. These investigators reported that the incidence of non-galactosylated oligosaccharides in human serum IgG increased with age (25 to 70 years) in both males and females. Our results disagree strikingly with their observations in terms of sex specificity. In addition, they did not mention anything similar to our finding that the incidence of oligosaccharides with a bisecting GlcNAc increased with age in both male and female IgG (Table 2). This discrepancy might have resulted from a difference in the race of the blood donors or from the methods employed in structural analysis of the oligosaccharides. Although we can not resolve this discrepancy at the present time, the female-specific change in galactosylation of IgG oligosaccharides may provide greater understanding of certain diseases such as rheumatoid arthritis (RA).

Augmentation of non-galactosylated oligosaccharides with non-reducing terminal GlcNAc residues has been demonstrated in IgG of RA patients [4]. It was suggested that agalactosyl IgG autoantibody could indirectly cause type-II collagen-induced arthritis [20]. Furthermore, it has also been proposed that agalactosyl IgG contributes to the development of lesions by activating complement via interaction with a serum lectin [21], mannose-binding protein (MBP), which is capable of binding to agalactosyl IgG preferentially by interacting with the non-reducing terminal *N*-acetylglucosamine [22].

In addition to the increase in RA patient IgG of non-galactosylated oligosaccharides, it is interesting that the great majority of patients with RA are female. This female specificity corresponds to the present observation. Furthermore, the significant change in the level of non-galactosylated IgG oligosaccharides between females in their twenties and thirties and between those in their forties and fifties ($p < 0.02$ and $p < 0.01$, respectively), as shown in Figure 6, may be related to the high incidence of RA in these age ranges. However, the extent of decreased galactosylation in IgG of female RA patients was greater than not only that of healthy females of the same age ($p < 0.0000002$) but also that of healthy older females in their sixties ($p < 0.0003$) (Figure 6). Therefore, the drastic increase in non-galactosylated oligosaccharides in IgG of RA patients could be due to unknown pathological mechanisms in addition to the age-related changes in galactosylation noted in healthy women.

Finally, some consideration should be given to the factors responsible for the age-related changes in galactosylation and bisecting *N*-acetylglucosaminylation of serum IgG molecules. At present, it is difficult to envisage the regulatory mechanisms underlying these changes. However, there have been several reports concerning the regulation of IgG galactosylation. It was reported that β1-4 galactosyltransferase activity is lower in peripheral blood B-cells of RA patients [23, 24]. In addition, it has also been reported that the population of $CD5^+$ B-cells and subclass distribution of IgG are altered in RA patients [25–27]. Therefore, the variation in IgG galactosylation with aging could be a reflection of age-related expansion of spacific B-cell populations which produce agalactosyl IgG.

Although further studies are necessary to clarify the role of age-related glycosylation of IgG, the present findings should contribute to our understanding of not only age-related physiological changes but also autoimmune diseases such as RA in which glycosylation is involved.

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