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Oligosaccharide structures of immunoglobulin G from two patients with carbohydrate-deficient glycoprotein syndrome

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The carbohydrate moiety of immunoglobulin G (IgG) from patients with carbohydrate-deficient glycoprotein (CDG) syndrome was analyzed. Galactosyl species were reduced in the reversed-phase chromatogram of pyridylaminated oligosaccharides as compared with child controls, and the hypogalactosylation was remarkable in a patient with typical manifestations. The abnormality was verified by composition analysis of the hydrolyzed monosaccharides from this patient, but the contents of mannose and *N*-acetylglucosamine were not reduced. Hypogalactosylation is the characteristic feature of IgG molecules in CDG syndrome, in contrast to the oligosaccharide deficiency of transferrin from the same patients. These findings suggest that the molecular phenotypes of different glycoproteins from patients with CDG syndrome are diverse.

Keywords: carbohydrate, carbohydrate-deficient glycoprotein syndrome, hypogalactosylation, IgG

Introduction

Carbohydrate-deficient glycoprotein (CDG) syndrome is a disease involving many systems and whose etiology is unknown [1–3]. It was first identified as a discrete clinical entity by the characteristic pattern of serum glycoproteins on electrophoresis [4, 5]. The distinct abnormality of charge derived from the carbohydrate moiety was first found in transferrin [6], because transferrin is a major glycoprotein in serum and because the *N*-linked oligosaccharide modifying the molecule is rather homogeneous in structure: it is almost completely sialylated at two biantennary oligosaccharides or tetrasialylated. The detection of less negatively charged isoforms, namely disialo- or asialotransferrins, by isoelectric focusing has been used as a diagnostic marker for CDG syndrome.

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The detailed structure of the molecular abnormality of transferrin in the CDG syndrome was first characterized by our group [7] and by Yamashita *et al.* [8] as a glycosylation defect in which one or two oligosaccharide chains are missing from the molecule. The structure suggests that the primary defect involves the earliest steps in the initial glycosylation of glycoproteins in the endoplasmic reticulum.

Immunoglobulin G (IgG) is the most abundant glycoprotein component in blood and its carbohydrate moiety has frequently been a target of research. The IgG molecule contains a single oligosaccharide chain of complex type in each heavy subunit, and has many different glycoforms [9, 10]. It is now well known that increased expression of the agalactosyl IgG glycoforms occurs in patients with various disorders, including rheumatoid arthritis [11], juvenile-onset rheumatoid arthritis [12], tuberculosis [13], Crohn's disease

[14], myeloma [15] and, most recently, myotonic dystrophy [16].

Here we analyze the carbohydrate structures of IgG from two patients with CDG syndrome to determine whether oligosaccharide deficiency occurs, as in the case of transferrin, or whether abnormal glycoform distribution is present as in the disorders described above.

Materials and methods

Patients

Patient 1 was a baby girl showing the typical manifestations of CDG syndrome type I: facial dysmorphism, fat pads over the buttocks, floppiness, failure to thrive, hepatic dysfunction, cardiac failure due to pericardial effusion and cerebellar hypoplasia. Serum IgG concentration at 5 months old was normal (5.24 g/l). A blood sample was obtained at 2 years of age.

Patient 2 was a sister of affected siblings from consanguineous parents [17]. She showed facial dysmorphy, failure to thrive, floppiness and delayed psychomotor development. She had cerebral but no cerebellar atrophy, and experienced tonic–clonic seizures during late infancy. A blood sample was obtained at 4 years of age, when the serum IgG concentration was normal (10.6 g/l).

Isoelectric focusing of serum transferrin from these patients has been reported previously [7]; there was a marked increase in disialo- and asialotransferrins in patient 1, while the abnormal isoform pattern was mild in patient 2. In both cases, oligosaccharide deficiency was identified by mass spectrometry [7, 18].

Purification of IgG

IgG was purified by a protein G affinity column (Protein G Sepharose 4 FF, Pharmacia). Purified IgG was quantified by colorimetry with Coomassie brilliant blue (Bio-Rad protein assay kit) using human IgG (Sigma) as a standard.

Preparation of pyridylaminated (PA) oligosaccharides and high-performance liquid chromatographic (HPLC) analysis

One milligram (6.7 nmol) of IgG was subjected to hydrazinolysis as previously described [19], and the resulting free oligosaccharides were then reductively modified with a fluorescent reagent, 2-aminopyridine (Wako, Japan) [20, 21], in the presence of dimethylamine borane (Aldrich) [22]. Pyridylaminated oligosaccharides were purified by gel filtration with Toyopearl HW-40F (Tosoh, Japan) [20, 21]. To overcome the complexity resulting from variable sialylation, pyridylaminated oligosaccharides were desialylated with neuraminidase (Seikagaku-kogyo, Japan) before HPLC analysis. HPLC was carried out on a ODS-80TM reversed-phase column (4.6 × 150 mm, Tosoh, Japan) equilibrated with 20 mM ammonium acetate, pH 4.0. Elution was performed using a gradient of 1-butanol at 55°C, and monitored by an F1000 fluorescent spectrophotometer (Hitachi, Japan) with excitation and emission wavelengths of 320 and 400 nm respectively [23].

Determination of carbohydrate composition

IgG was hydrolyzed with a mixture of 2 M HCl and 2 M trifluoroacetic acid according to the procedure of Suzuki *et al.* [24]. The hydrolysate was automatically pyridylaminated by Glyco-TAG (Takara Shuzo, Japan), and PA monosaccharides were then subjected to chromatography on a PALPAK Type A anion-exchange column (4.6 × 150 mm, Takara Shuzo) and monitored with a fluorescence spectrophotometer RF-550 (Shimadzu, Japan) with excitation and emission wavelengths of 310 and 380 nm respectively [24]. The elution solvent was a mixture of one part acetonitrile and nine parts 0.7 M borate buffer, pH 9.0. The flow rate was 0.3 ml/min and the column temperature was 65°C. A mixture of 100 pmol each of several monosaccharides (GalNAc, Xyl, GlcNAc, Glc, Man, Fuc and Gal) as standards was subjected to the same analysis in parallel. Ribose was used as an internal standard.

Assay for serum galactosyltransferase (GalTase) activity

The PA derivative of agalactosyl biantennary oligosaccharide (structure A in Figure 1) was employed as a substrate [25]. The reaction solution contained 12.5 μM substrate and 250 μM uridine-5′-diphosphogalactose (UDP-Gal) in 100 mM HEPES buffer, pH 7.2, 20 mM $MnCl_2$, 60 mM NaCl and 5 mM KCl. The reaction was started by adding 10 μl of serum to 40 μl of the reaction solution, and the solution was kept at 37°C for 2 h before heating to 100°C. The reaction products were analyzed by HPLC as described for the oligosaccharide analysis. The amount of product was estimated by the fluorescence intensity using pyridylaminated *N*-acetylglucosamine as a standard [26]. Specific activity of GalTase was expressed as nmol of Gal transferred/ml serum/h.

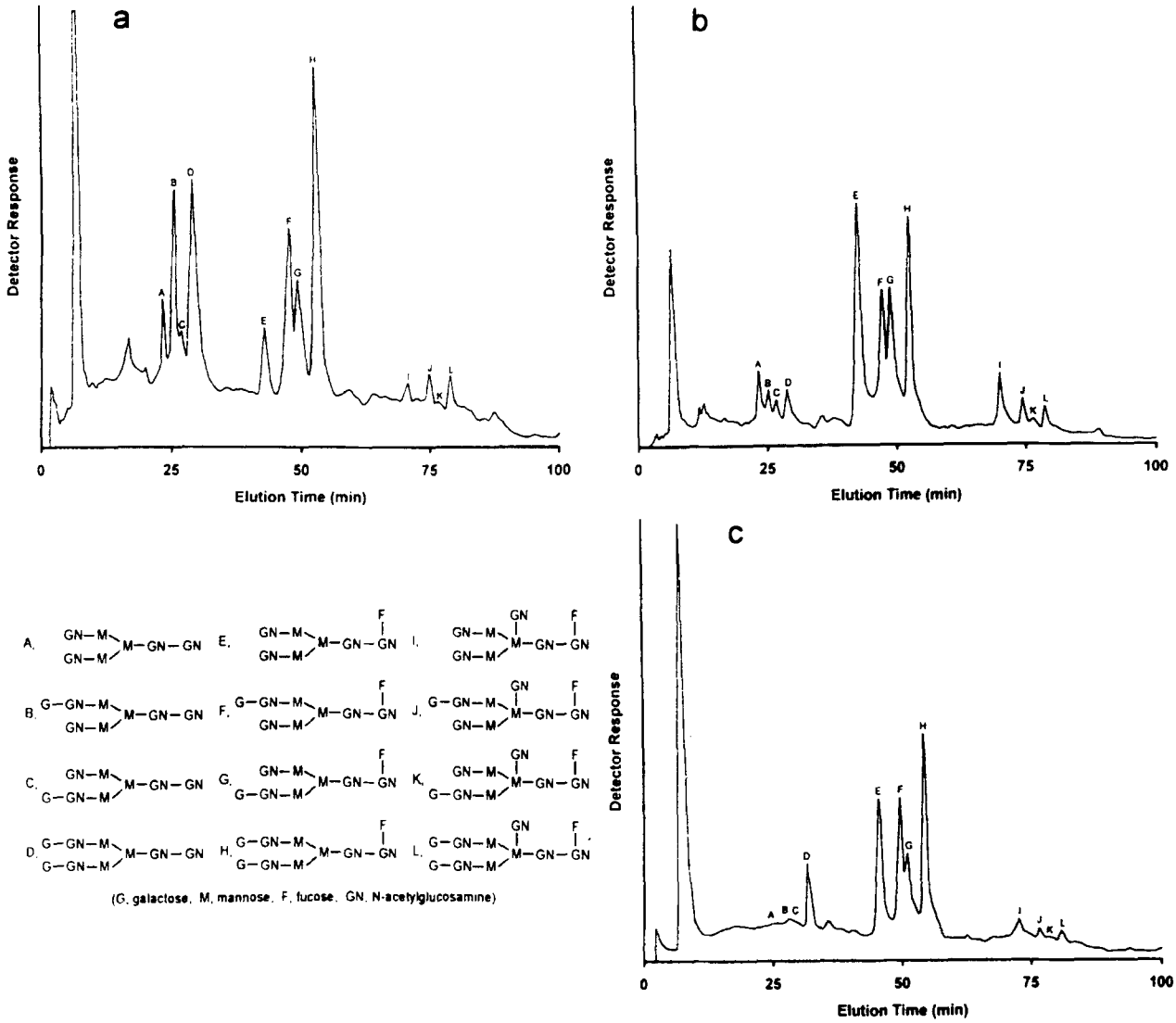

Figure 1. HPLC profiles of PA oligosaccharides from IgG. (a) Child control, 3 years old; (b) patient 1; (c) patient 2. The heterogeneous pattern can be divided in three groups: afucosyl biantennary (A–D), fucosyl biantennary (E–H) and fucosyl bisecting (I–L) oligosaccharides. Peaks A, E and I are agalactosyl, peaks B, C, F, G, J and K are monogalactosyl, and peaks D, H and L are digalactosyl species. All agalactosyl species (A, E and I) were increased in patient 1, and agalactosyl, fucosyl oligosaccharide was slightly increased in patient 2.

Results

HPLC profiles of pyridylaminated oligosaccharides of IgG

A heterogeneous mixture of oligosaccharides from IgG was separated by HPLC and was classified into three groups – afucosyl biantennary (A–D), fucosyl biantennary (E–H) and fucosyl bisecting (I–L) oligosaccharides (Figure 1a) – which have previously been characterized by ^{1}H-NMR [23]. In each group, oligosaccharides were separated by galactosylation at two non-reducing ends as follows: agalactosyl (A, E and I) or G(0), monogalactosyl (B, C, F, G, J and K) or G(1), and digalactosyl (D, H and L) or G(2) species. In child controls, the fucosyl biantennary group (E–H in Figure 1a) was predominant, and digalactosyl oligosaccharides were the major species in each group.

The patients with CDG syndrome showed different distributions from the child controls. In both patients, there was an increase in agalactosyl components (A, E and I in patient 1 and E and I in patient 2) and a corresponding decrease in digalac-

tosyl ones. The abnormal distribution was pronounced in patient 1. The average Gal content in a biantennary chain was low, 0.90 ($0.01 > P > 0.001$) and 1.10 ($0.5 > P > 0.1$) mol for patients 1 and 2 respectively, compared with the controls (1.25 ± 0.08 mol) (Table 1).

Composition analysis of monosaccharides

To verify the hypogalactosylation in patient 1, composition analysis of monosaccharides from oligosaccharide chains was performed. As shown in Table 2, the content of Gal in 1 mol IgG was low (0.99 mol) compared with child controls (1.55 ± 0.21 mol), confirming the result described above. On the other hand, neither GlcNAc nor Man was decreased. This indicated that the oligosaccharide chain was not deficient in the IgG from the patient, since GlcNAc and Man constitute the core structure of *N*-linked oligosaccharide. Fucose was decreased in this analysis.

Table 1. Galactosylation levels of IgG oligosaccharides from patients with CDG syndrome and child controls

	Age (years)	G(0)	G(1)	G(2)	x[a] (mol)
Control					
1	2	0.13	0.45	0.42	1.29
2	3	0.13	0.42	0.45	1.32
3	3	0.23	0.38	0.39	1.16
4	3	0.09	0.47	0.44	1.34
5	7	0.14	0.44	0.42	1.28
6	9	0.23	0.40	0.37	1.15
7	10	0.22	0.34	0.44	1.23
Patient					
1	2	0.35	0.40	0.25	0.90**
2	4	0.25	0.39	0.36	1.11*

The relative abundances of agalactosyl G(0), monogalactosyl G(1) and digalactosyl G(2) oligosaccharides were calculated by summing the concentrations of constituent species in Figure 1, A + E + I, B + C + F + G + J + K and D + H + L respectively.

[a]Average galactose content in a biantennary oligosaccharide calculated by an equation, $x = G(1) + G(2) \times 2$. It was 1.25 (mean) ± 0.08 (SD) mol for child controls.

*$P < 0.5$, **$P < 0.01$.

Table 2. Monosaccharide composition of IgG from a patient with typical CDG syndrome and two child controls (values are expressed in mol of carbohydrate per mol of IgG)

	Age (years)	GlcNAc	Man	Fuc	Gal
Control 1	2	7.03	4.61	0.75	1.51
Control 2	3	7.14	4.52	0.91	1.78
Patient 1	2	7.08	4.45	0.44	0.99

Serum GalTase activities

GalTase was assayed by the transfer of Gal from UDP-Gal to the pyridylaminated agalactosyl biantennary oligosaccharide as a substrate. Galactosylated products (peaks B, C and D) were determined by HPLC as typically shown in Figure 1. In this analytical system, GalTase activities in the serum of the patients with CDG syndrome seemed low compared with child controls (Table 3).

Discussion

IgG is normally a polyclonal product and has very diverse amino acid sequences. This heterogeneity is a serious impediment in probing carbohydrate abnormalities by isoelectric focusing, as was carried out for transferrin, and thus there have been no reports regarding the molecular pathology of IgG molecules in patients with CDG syndrome.

In this study, we analyzed IgGs from two CDG patients. Patient 1 has typical manifestations of type I CDG [2], whereas patient 2 has a variant form showing different clinical pictures and a less remarkable disialo- and asialotransferrin pattern of isoelectric focusing [7]. The molecular abnormality of transferrin was more pronounced in patient 1 than in patient 2 [18]. The carbohydrate changes in IgG delineated in this study were apparently different from those found in transferrin with regard to the following two points.

Gal is deficient in IgG oligosaccharide

Hypogalactosylation was demonstrated in this study by two different methods, oligosaccharide profiling (Figure 1 and Table 1) and mono-

Table 3. Serum galactosyltransferase activity

	Age (years)	Activity[a]
Control		
1	2	55
2	3	42
3	3	37
4	7	70
Patient		
1	2	32
2	4	34

[a]Values are expressed as nmol of galactose transferred to PA substrate per ml of serum per h.

saccharide composition analysis (Table 2). We evaluated the data by comparison with child controls, because an age-dependent variation in IgG galactosylation has been reported [27]. As summarized in Table 1, the deficiency was more pronounced in patient 1 with the typical syndrome than in patient 2, correlating with the content of oligosaccharide-deficient transferrins in their blood. The decreased Gal content of IgG should have been suggested from the data of Stibler *et al.* [28]. They analyzed monosaccharide concentrations in serum glycoproteins and demonstrated a 40% reduction in Gal. Since IgG constitutes the great majority of serum glycoproteins, the reduction that they found could be ascribed to IgG abnormality. We calculated a 30% or 40% decrease in oligosaccharide (Table 1) and monosaccharide (Table 2), respectively, in patient 1.

Hypogalactosylation of IgG in pathological conditions was first reported in rheumatoid arthritis (RA) [11] and other inflammatory diseases [12–14]. In RA, an approximately 45% decrease in GalTase activity in B-lymphocytes has been demonstrated [29]. We analyzed GalTase activity in serum but not in B-lymphocytes because it proved to be difficult to obtain enough cells for analysis. GalTase activity was low, though not significantly so, in both patients. Stibler *et al.* [28] reported normal activities in both serum and cultured fibroblasts.

Different mechanisms may operate to give similar molecular phenotypes in CDG syndrome and RA, because in CDG a normal or slightly decreased concentration of serum IgG is present, in contrast with the usually elevated levels in RA. On the other hand, myotonic dystrophy combines hypogammaglobulinemia with marked hypogalactosylation of IgG [16], but the molecular link between defective myotonin protein kinase [30] and the carbohydrate abnormality has not been studied.

With regard to the biological activity of agalactosyl IgG, reduced binding to C1q or Fc receptor [31] and an ability to initiate synovitis [32] have been reported, but the immunological functions of CDG patients have not been studied in detail.

Oligosaccharide chain is not deficient in IgG

Monosaccharide analysis suggested that glycosylation of IgG was normal in patient 1. In this analysis, the molar content of Man should be 6, because IgG molecule contains two oligosaccharide chains of complex type. Smaller values, obtained in controls as well as in a patient, may be due to a loss during sample preparation. However, we believe that the findings concerning the relative contents of each monosaccharide among individuals, and especially the normal Man content for the patient, are reliable, because the decreased Gal content accorded with the result from oligosaccharide analysis. Furthermore, the normal Man content of IgG in CDG syndrome was in agreement with the result of monosaccharide analysis of total serum glycoproteins by Stibler *et al.* [28]. We cannot explain the low fucose content in our monosaccharide analysis.

The finding of normal glycosylation is quite different from the transferrin oligosaccharide deficiency observed in the same patient. Lack of an oligosaccharide chain of transferrin was clearly identified by mass spectrometry in the previous study [7] and has also been demonstrated in other Japanese patients by Yamashita *et al.* [8]. In the two-dimensional electrophoresis carried out by Harrison *et al.* [33], a gross molecular defect which would indicate the absence of oligosaccharide chains was found in other glycoproteins, *e.g.* in ceruloplasmin and α_1-acid glycoprotein. On the other hand, a relative increase in agalactosyl glycoforms or an aberrant distribution of oligosaccharides has not been demonstrated in transferrin [7] or in other glycoproteins. The diverse nature of this phenomenon might be due to the different modes of production: B lymphocytes for immunoglobulins versus hepatocytes for most other glycoproteins.

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