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STRUCTURE AND LOCATION OF ASPARAGINE-LINKED OLIGOSACCHARIDES IN THE Fc REGION OF A HUMAN IMMUNOGLOBULIN D

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Seven kinds of asparagine-linked oligosaccharides were bound to the Fc region of a human immunoglobulin D(NIG-65). The oligosaccharides quantitatively released from four species of glycopeptides by digestion with almond glycopeptidase, were separated by Bio-Gel p-4 column chromatography and were purified further by thin-layer chromatography. The sugars were identified with GC-MS following the permethylation of respective oligosaccharide. To Asn-68(NIG-65 Fc numbering (1)), two kinds of high-mannose-type oligosaccharides were bonded. To Asn-159, a kind of hybride-type and two kinds of bisected complex-type oligosaccharides were attached. From Asn-210, four kinds of bisected complex-type oligosaccharides were isolated.

Although different classes of human immunoglobulins differ considerably in the content, the kinds and the number of oligosaccharides, the combining sites are often homologous and are related to the basic immunoglobulin domain structure. The carbohydrates are also thought to play significant roles in biological functions of immunoglobulins. In the previous paper, Shinoda et al. (1) have reported amino acid sequence of the entire Fc region of a human IgD(NIG-65) and have also shown unique evolutionary trees for the middle and the last domains of IgD. Since the kinds and the location of three glucosamine-containing oligosaccharides have been described by Takayasu et al.(2) for the Fc region of

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IgD, elucidation of their structures will be needed to insight further into their biological significances.

For the oligosaccharide analysis, we used almond glycopeptidase(3-5), which released quantitatively all kinds of desialylated asparagine-linked oligosaccharide chains from the IgD glycopeptides. In this communication, we demonstrate the precise location of the N-glycosidically linked oligosaccharides in the Fc region of IgD(NIG-65).

MATERIALS AND METHODS

Materials: Glycopeptides from the Fc region of IgD(NIG-65) were prepared and purified in the same manners as described by Shinoda et al.(1) and Takayasu et al.(2). Designations and sequences of these glycopeptides are as follows: GlcN-1(linked to Asn-68), Arg-His-Ser-Asn(CHO)-Gly-Ser-Gln-Ser-Gln; GlcN-2(linked to Asn-159), Glu-Val-Asn(CHO)-Thr-Ser-Gly-Phe; and GlcN-3(linked to Asn-210), Asp-Ser-Arg-Thr-Leu-Leu-Asn(CHO)-Ala-Ser-Arg-Ser-Leu-Glu. Since glycopeptide GlcN-3 has been separated into two fractions, though their amino acid sequences have been shown to be identical(2), by column chromatography with DEAE-Sephadex A-25, they were separately pooled and designated as GlcN-3A for an acidic one, and GlcN-3N for a neutral one, respectively.

Almond glycopeptidase was prepared from almond nuts as described previously(5) with a slight modification. The enzyme preparation used was free of α-mannosidase, β-galactosidase and β-N-acetylglucosaminidase. Bio-Gel p-4(200-400 mesh) column chromatography was performed with a column(0.5 X 90 cm) at 55°C. Thin-layer chromatography was performed on a silica gel 60 plate (Merck Art. 5553) with n-propanol/acetic acid/water(3:3:2, v/v) as a solvent. The color producing reagent on a silica gel plate was orcinol-H_2SO_4 reagent(6).

Preparation and analyses of oligosaccharides from glycopeptides: Each 200-300 nmol of the four kinds of IgD glycopeptides (GlcN-1, GlcN-2, GlcN-3A and GlcN-3N) was dissolved in 200 µl of 0.01M HCl(pH 2) and was heated at 90°C for 1 h to eliminate sialic acids in the carbohydrate moieties. The incubation mixture was evaporated to dryness. The residue was dissolved in 20 µl of 1M citrate-phosphate buffer(pH 5.0), and incubated with 5 µl (0.5 munit) of almond glycopeptidase at 37°C for 20 h. The reaction mixture was applied on a column of Bio-Gel p-4. The column was eluted with water. Oligosaccharide fractions were pooled and evaporated to dryness. For desalting, the oligosaccharide mixture was successively passed through columns of 0.5 ml each of Amberlite IR-120(H^+) and Amberlite IRA-400(CO_3^{2-}). Oligosaccharide fractions were further separated by TLC. Each oligosaccharide separated on a silica gel plate was extracted with water. $NaBH_4$-reduced oligosaccharide alcohols were dissolved in 0.2 ml of dimethylsulfoxide and methylated by the method of Hakomori(7). The permethylated products were purified on a silica gel column(0.58 X 5 cm) (8). Analysis of alditol acetates obtained from hydrolysates of permethylated oligosaccharides was performed with a Hewlett-Packard 5710A gas chromatograph combined with a double-focussing mass spectrometer(JMS D-300; JEOL). The data were stored and processed by a JMA 2000 data system of JEOL. The gas chromatograph was equipped with a 30 m X 0.25mm I.D. OV-101 open-tubular glass capillary column and a splitless injector.

Injection temperature was 250°C. The column temperature was programmed from 150°C to 260°C at 3°C/min. Electron-impact ionization mass spectra were recorded at an ionizing energy of 22 eV, an ionization current of 300 μA, a separator temperature of 220°C and an accelerating voltage of 3 kV.

RESULTS AND DISCUSSION

Oligosaccharides Profile: The four kinds of desialyzed glycopeptides from human IgD were treated with almond glycopeptidase and the released oligosaccharides were separated by chromatography on Bio-Gel p-4(data not shown). No smaller oligosaccharides or monosaccharides as by-products were detected. Each oligosaccharide fraction corresponded to the eluting positions of standard

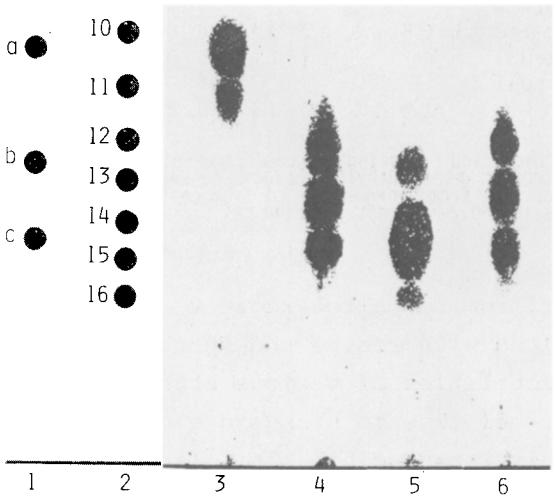

Fig. 1 Thin-layer chromatogram of the oligosaccharides released from the glycopeptides with almond glycopeptidase. Silica gel plate was developed at 25°C for 17 h. Oligosaccharides were visualized with orcinol-H_2SO_4 reagent. 1, Standard oligosaccharides:

a, $(Man1\xrightarrow{\alpha}2)_3$ $\left\{\begin{array}{l} Man1\xrightarrow{\alpha}6 \\ Man1\xrightarrow{\alpha}3 \end{array}\right.$ $Man1\xrightarrow{\alpha}6\,Man1\xrightarrow{\beta}4GlcNAc1\xrightarrow{\beta}4GlcNAc$: from quail ovalbumin ;

b, $Gal1\xrightarrow{\beta}4GlcNAc1\xrightarrow{\beta}2Man1\xrightarrow{\alpha}6$ $\left\{\begin{array}{l} \\ Gal1\xrightarrow{\beta}4GlcNAc1\xrightarrow{\beta}2Man1\xrightarrow{\alpha}3 \end{array}\right.$ $Man1\xrightarrow{\beta}4GlcNAc1\xrightarrow{\beta}4GlcNAc$: from human fibrinogen ;

c, $\left\{\begin{array}{l} Man1\xrightarrow{\alpha}6 \\ Man1\xrightarrow{\alpha}3 \end{array}\right.$ $Man1\xrightarrow{\alpha}6\,Man1\xrightarrow{\beta}4GlcNAc1\xrightarrow{\beta}4GlcNAc$: from hen ovalbumin.

$Gal1\xrightarrow{\beta}4GlcNAc1\xrightarrow{\beta}4$ $\left\{\begin{array}{l} \alpha\,3\,Man1 \\ GlcNAc1\xrightarrow{\beta}2 \end{array}\right.$

2, Mixture of standard glucose oligomers prepared by acid hydrolysis of dextran. Vertical numbers indicate the glucose units. 3, From GlcN-1. 4, From GlcN-2. 5, From GlcN-3A. 6, From GlcN-3N.

Table 1. Methylation analysis of the oligosaccharides from the Fc region of human immunoglobulin D.

Methylated sugar	GlcN-1		GlcN-2			GlcN-3A			GlcN-3N		
	U[a]	L[c]	U	C[b]	L	U	C	L	U	C	L
Mannitol											
2,3,4,6-tetra-o-methyl	3.2	3.3	1.0	+	0.7	1.1	+	+	1.2	+	+
3,4,6-tri-o-methyl	3.0	4.0	1.0	2.0	1.0	1.0	2.0	1.0	1.0	2.0	2.0
2,4,6-tri-o-methyl	-	-	-	-	0.4	-	-	-	-	-	-
2,3,4-tri-o-methyl	-	-	-	+	0.4	-	-	-	-	-	-
3,6-di-o-methyl	-	-	-	-	0.5	+	-	0.5	-	-	-
2,4-di-o-methyl	1.8	1.9	-	-	+	-	-	+	-	-	-
2-mono-O-methyl	-	-	0.5	0.7	0.6	0.5	0.6	0.7	0.5	0.6	0.8
Galactitol											
2,3,4,6-tetra-o-methyl	-	-	0.9	1.2	0.9	1.2	1.9	1.7	1.1	1.2	1.9
Fucitol											
2,3,4-tri-o-methyl	-	-	+	+	-	-	-	-	-	-	-
2-N-Methylacetamido-2-deoxyglucitol											
1,3,5,6-tetra-o-methyl	0.6	0.6	0.7	0.5	0.8	0.6	0.9	0.7	0.6	0.5	0.9
3,4,6-tri-o-methyl	-	-	1.1	1.6	1.5	0.6	1.1	1.5	0.8	1.6	0.9
1,3,5-tri-o-methyl	-	-	+	+	-	-	-	-	-	-	-
3,6-di-o-methyl	1.0	1.0	2.0	2.0	2.0	2.0	3.0	3.0	2.0	2.0	3.0

Numbers were calculated by making the underline values as integers number for neutral sugar, and 2-N-methylacetamido-2-deoxy-3,6-di-o-methylglucitol for amino sugar. a) from upper spot. b) from center spot. c) from lower spot. +, less than 0.2. -, not detected.

glucose oligomer(from isomaltohexaose to isomaltooctadecaose) was pooled and desalted with resins and further separated by TLC (Fig. 1). The mobilities of various standard oligosaccharides (Fig. 1 lane 1) relative to standard glucose oligomers(Fig. 1 lane 2) are highly reproducible. The oligosaccharide derived from GlcN-1(Fig.1 lane 3) corresponded to isomaltodecaose-isomaltoundecaose, the one from GlcN-2(Fig.1 lane 4) corresponded to isomaltododecaose-isomaltopentadecaose, the one from GlcN-3A(Fig. 1 lane 5) corresponded to isomaltododecaose-isomaltohexadecaose and the one from GlcN-3N(Fig. 1 lane 6) corresponded to isomaltododecaose-isomaltopentadecaose.

Structures of Oligosaccharides: Table 1 summarizes the results of methylation analysis of the oligosaccharides. Based on the data, together with those shown in Fig. 1, the structures of seven species of oligosaccharides are proposed for the Fc region of IgD (Table 2). These are characterized as follows: (1) two species of high-mannose type oligosaccharides are linked to Asn-68, (2) a hybrid-type and two species of bisected complex-type(9,10) oligosaccharides are linked to Asn-159, (3) four species of bisected complex-type oligosaccharides are bonded to Asn-210. From the

Table 2. proposed structures of oligosaccharides species present in the Fc region of IgD. a) from upper spot. b) from center spot. c) from lower spot.

$$R_1 \rightarrow \left[\begin{matrix} Man1\rightarrow 6 \\ Man1\rightarrow 3 \end{matrix}\right\rangle Man1\rightarrow 4GlcNAc1\rightarrow 4GlcNAc \quad (R_2 \downarrow 2)$$

R_1	R_2	Found in			
		GlcN-1	GlcN-2	GlcN-3A	GlcN-3N
$(Man1\rightarrow 2)_3$ [Man1→6, Man1→3]	-	U[a]			
$(Man1\rightarrow 2)_4$ [Man1→6, Man1→3]	-	L[c]			
Gal1→4GlcNAc1→2	GlcNAc1→4		U	U	U
Gal1→4 [GlcNAc1→2, GlcNAc1→2]	GlcNAc1→4		C[b]		C
Gal1→4GlcNAc1→2, Gal1→4GlcNAc1→2	GlcNAc1→4			C	L
Man1→2Man1→3 or 6, Gal1→4 [GlcNAc1→4, GlcNAc1→2]	GlcNAc1→4		L		
$(Gal1\rightarrow 4)_2$ [GlcNAc1→2, GlcNAc1→4, GlcNAc1→2]	GlcNAc1→4			L	

analytical data on desialylated oligosaccharides GlcN-3A and GlcN-3N, the charge heterogeneity is most likely to be due to the difference in the content of the terminal sialic acid.

As described above, a hybrid-type oligosaccharide is demonstrated for Asn-159 which is located within the disulfide loop of the last domain of the Fc region of IgD. This is the first case in which mode of the sugar linkage in a hybrid-type oligosaccharide is established for immunoglobulins. The unique feature not only the kind of oligosaccharide but also its location in the Fc region, may have some correlations to possible biological significance of IgD.

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