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Primary Structure of the Glycans from Human Lactotransferrin

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The polypeptide chain of human lactotransferrin possesses two glycosylation sites to which glycans are linked through an *N*-(β-aspartyl)-*N*-acetylglucosaminylamine bond and which are structurally heterogenous. After chymotryptic or pronase digestions, glycopeptides with five different glycan structures could be isolated. For three of them, the structure has been determined by the application of methanolysis, methylation analysis, hydrazinolysis/nitrous deamination, enzymatic cleavage and ^{1}H-NMR spectroscopy at 360 MHz: Glycopeptides A and B: NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)[NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)]Man-(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc(β1-)Asn; Glycopeptide C: NeuAc(α2-6)(Gal(β1-4)GlcNAc(β1-2)Man-(α1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-2)Man(α1-6)}Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc(β1-)Asn. Glycopeptide D: NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)[Gal(β1-4)GlcNAc(β1-2)Man(α1-6)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc(β1-)Asn.

Two other glycopeptides were obtained in very low amount and possess more complex structures.

In 1960, Montreuil et al. [1] demonstrated the glycoprotein nature of human lactotransferrin (also called lactoferrin [2]). The lactotransferrins which are found in the milk of most mammalians constitute, with the serotransferrins present in serum of all vertebrates and with the ovotransferrins characterized in the egg white of birds, a homogenous family of proteins: the transferrins. These glycoproteins possess structural homologies with regard to their molecular weight [3–5], their single-chain nature [6,7], their amino-acid sequences [8–10] and the presence of two iron-binding sites [11].

The determination of the structure of the glycans of human serotransferrin [12,13], rabbit serotransferrin [14], hen ovotransferrin [15,16] and bovine lactotransferrin (Chéron, A., personal communication) shows that important differences exist between the number and the structure of the glycans present in these four kinds of transferrins.

In the case of human lactotransferrin, in 1966, the presence of two glycans linked by a 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine linkage was described as well as one *O*-glycosidically-linked glycan [17]. The presence of two *N*-glycosidically-linked glycans was confirmed in 1973 and for one of them the structure was proposed [18].

Microheterogeneity of the carbohydrate moieties was later demonstrated and partial results concerning different glycan structures were given in general reviews [19–21]. In the present paper we describe the complete primary structure of three types of glycans as determined by methanolysis, methylation analysis, hydrazinolysis/nitrous deamination, mass spectrometry, enzymatic cleavage and ^{1}H-NMR spectroscopy at 360 MHz. The two other glycans which were obtained in low amounts possess more complex structures.

Abbreviations. Fuc, L-fucose; Gal, D-galactose; Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; NeuAc, *N*-acetylneuraminic acid; Asn, asparagine; NMR, nuclear magnetic resonance.

Enzymes. Trypsin (EC 3.4.21.4); neuraminidase (EC 3.2.1.18); α-L-fucosidase (EC 3.2.1.51); endo-*N*-acetyl-β-D-glucosaminidase (EC 3.2.1.96).

MATERIALS AND METHODS

Materials

SP-Sephadex C-50 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), Biogel P-30 from Bio-Rad Laboratories (Richmond, CA, USA), anhydrous hydrazine from Pierce Chemical Co (Rockford, IL, USA), [^{14}C]acetic anhydride from C.E.A. (Gif-sur-Yvette, France), α-L-fucosidase from beef kidney from Boehringer, Mannheim (Meylan, France), twice-crystallized trypsin from Sigma Chemical Co. (St Louis, MO, USA) and pronase from Calbiochem AG (Lucerne, Switzerland). Other chemicals were of analytical grade.

Preparation of the Lactotransferrin Samples

Samples of human milk obtained at different stages of lactation were collected from a local milk bank, pooled and frozen at −20 °C until use. The thawed milk was defatted by centrifugation at 10000 × *g* for 30 min and the caseins were precipitated by adjusting the pH to 4.6 with dilute HCl. After elimination of caseins by centrifugation, the whey was submitted to chromatography on an SP-Sephadex C-50 column (3 × 50 cm) stabilized in 0.22 M sodium acetate buffer according to Chéron et al. [22]. However, the human lactotransferrin eluted with 0.50 M sodium acetate buffer pH 7 was contaminated by lysozyme; therefore its final purification was achieved by rechromatography on an SP-Sephadex C-50 column (3 × 50 cm) by elution with 0.40 M sodium acetate buffer pH 7.

Iron was completely removed from the lactotransferrin under the conditions described by Mazurier and Spik [23].

The apolactotransferrin was desialylated by *Clostridium perfringens* neuraminidase [24], reduced and alkylated according to Crestfield et al. [25].

Preparation and Analysis of the Glycopeptides

Glycopeptides were prepared either by pronase digestion of the apolactotransferrin as described previously for human serotransferrin [26] or by trypsin digestion [27] on the reduced and alkylated sialo- or asialoapolactotransferrin.

The proteolysates were purified by filtration on Biogel P-30 columns (6 × 100 cm). Elution was carried out with water and the glycopeptide fractions were characterized by a phenol/sulfuric acid reagent [28]. Free-flow electrophoresis was performed in an Elphor-Vap II apparatus under the following conditions: 0.5 M acetic acid, pH 2.5, 1700 V, 24 h. Preparative electrophoresis was realized in 1 M acetic acid, pH 2.4, 7 V/cm for 16 h.

The sugar composition of glycopeptides was determined by classical colorimetric methods [29] and by gas-liquid chromatography after treatment of the glycopeptides with 0.5 M HCl/methanol for 24 h at 80 °C and pertrifluoroacetylation [30]. Amino acid analysis was performed on a Multichrom Beckman auto-analyzer according to the method of Spackman et al. [31] as modified by Charet et al. [32].

Permethylation of the Glycopeptides

Glycopeptides were methylated according to Hakomori [33]. Identification of methylated derivatives of neutral monosaccharides and hexosamines was performed after hydrolysis (4 M HCl, 4 h at 100 °C), peracetylation (pyridine/acetic anhydride (1:1, 100 °C; 30 min), methanolysis (0.5 M HCl/methanol; 4 h; 80 °C) and a new peracetylation as described before [34].

The peracetylated methyl derivatives were separated by gas-liquid chromatography (glass column 0.3 × 300 cm; Carbowax 6000 on Chromosorb W-HMDS, 60–80 mesh; N_2; flow rate 30 ml/min; temp. 110–220 °C; 2 °C/min).

To improve the separation of the monomethyl derivatives of hexosamines, the methylated hexosamines were separated from the methylated neutral monosaccharides by paper electrophoresis [35] and subsequently analyzed by gas-liquid chromatography (glass capillary column, 0.4 mm × 60 m, of Carbowax 20 M; N_2; temp. 130–225 °C; 2 °C/min).

Hydrazinolysis/Nitrous Deamination

Hydrazinolysis/nitrous deamination was carried out according to Bayard et al. [36]. The oligosaccharides containing 2,5-anhydro-mannose were reduced by sodium borodeuteride and methylated. The methylated derivatives were separated by gas-liquid chromatography on a column of SE-30 or on a capillary column of OV-101 and detected by total ionization current and fragment mass spectrometry as described by Strecker et al. [37].

Glycosidase Digestions

Desialylation was performed with the neuraminidase from *Cl. perfringens* [24]. Fucosidase digestion was realized with 50 µl of an enzyme solution (4 U/ml) in citrate/phosphate buffer pH 4.6 for 24 h and repeated for 24 h with another 50 µl fucosidase solution. The amount of free fucose was determined by the method of Finch et al. [38]. The asialoglycopeptides obtained by pronase digestion were radioactively labeled on the asparagine residues by *N*-acetylation with [^{14}C]acetic anhydride and subjected to digestion with the endo-*N*-acetyl-β-D-glucosaminidase B isolated from a Basidiomyces (*Sporotricum dimorphosporum*) according to Bouquelet et al. [39].

360-MHz ^{1}H-NMR Spectroscopy

For ^{1}H-NMR spectroscopic analysis the neutralized glycopeptides were repeatedly exchanged in ^2H_2O (100% ^{2}H, Aldrich, Milwaukee, USA) at room temperature with intermediate lyophilization. The 360-MHz ^{1}H-NMR spectra were recorded on a Brucker HX-360 spectrometer, operating in the Fourier-transform mode at a probe temperature of 25 °C. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate, indirectly to acetone in ^2H_2O: δ = 2.225 ppm with an accuracy of 0.003 ppm.

RESULTS

Carbohydrate Composition of Lactotransferrin Samples

The lactotransferrin isolated from human whey by two ion-exchange chromatographies on an SP-Sephadex C-50 column was pure by serological examination. The molar carbohydrate compositions of ten different preparations of

Table 1. *Characteristics of the seven tryptic glycopeptide fractions isolated from sialylated, reduced and alkylated lactotransferrin*
The molar compositions were calculated on the basis of three mannose residues per glycan

Monosaccharide	Proportion in						
	A	B	C	D	E	F	G
	mol/mol						
NeuAc	2.0	1.8	1.1	0.9	0.8	0.5	0.3
Fuc	1.1	1.0	1.8	1.0	1.5	2.2	4.2
Gal	2.2	2.2	2.3	2.1	2.3	2.9	4.3
GlcNAc	4.1	4.2	4.2	4.0	3.7	4.7	5.5
Man	3	3	3	3	3	3	3
Percentage of total	11	10	40	23	8	5	3
Type of peptide chain	I	II	I	II	II	I + II	I + II

Table 2. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated glycopeptides from human lactotransferrin*

Glycopeptide	Methyl ethers							
	(2,3,4)Me_3-Fuc	(2,3,4,6)Me_4-Gal	(2,3,4)Me_3-Gal	(3,4,6)Me_3-Man	(2,4)Me_2-Man	(3,6)Me_2-GlcNAcMe	(3)Me_1-GlcNAcMe	(6)Me_1-GlcNAcMe
	mol/mol							
A	0.40	0	1.80	1.75	1.00	2.60	0.92	0
B	0.45	0	1.75	1.80	1.00	2.65	0.70	0
C	1.35	1.10	0.90	2.05	1.00	1.75	0.70	0.82
D	0.60	1.20	0.95	1.80	1.00	2.70	0.60	0
E	0.95	1.35	0.80	1.85	1.00	2.10	0.65	0.40

iron-free human lactotransferrin prepared from a pool of human milk were determined by gas-liquid chromatography. The results indicate a high dispersion since the molar carbohydrate ratio calculated on the basis of a molecular weight of 80000 and of six mannose residues were as follows: Gal, 4.87 ± 0.60; Fuc, 2.86 ± 0.64; GlcNAc, 8.96 ± 0.62 and NeuAc, 2.60 ± 0.30. These results constitute a first indication of the polydispersity of the glycan structures.

Isolation and Characteristics of the Glycopeptides

The glycopeptides obtained from a trypsin digest of reduced and alkylated sialo-apolactotransferrin were separated by free flow and paper electrophoresis. Seven fractions (A–G) were obtained, and their carbohydrate compositions are reported in Table 1. The variations in the number of NeuAc (0 to 2), of Fuc (1 to 4) and of GlcNAc (4 to 6) suggest that the glycans possess different types of structure.

The amino acid composition of the fractions A–G indicates that glycopeptides A and C which contained four proline residues possess the characteristics of peptide chain type I, the glycopeptides B, D and E which did not contain proline residues possess the features of the peptide chain type II. The amino acid sequences of both peptide chains were previously described [10, 40]. The more complex glycopeptides F and G represent a mixture of glycopeptides possessing the two types of peptide chain.

Methylation of the Glycopeptides

The molar ratios of the monosaccharide methyl ethers of the permethylated glycopeptides A–E given in Table 2 indicate that all the glycans possess a biantennary structure of *N*-acetyllactosamine type. A good separation of the methyl derivatives of glucosamine enables the identification of the 3,6-di-*O*-methyl derivative of *N*-acetylglucosamine in all glycopeptides, of the 3-*O*-methyl derivative of *N*-acetylglucosamine in glycopeptides A, B and D and of 3-*O*-methyl and 6-*O*-methyl derivatives of *N*-acetylglucosamine in glycopeptides C and E. After defucosylation, no modification was noticed in the presence of the methyl derivatives of the neutral monosaccharides while the mono-methyl derivatives of *N*-acetylglucosamine were replaced by the 3,6-di-*O*-methyl derivative of *N*-acetylglucosamine, indicating that the fucose residues were only linked in 6 or 3 position to the *N*-acetylglucosamine residues. In all asialoglycopeptides the 2,3,4-tri-*O*-methyl derivative of galactose was replaced by the permethylated derivative indicating that *N*-acetylneuraminic acid residues were always linked to galactose in the 6 position.

Table 3. *^{1}H-NMR chemical shifts of anomeric protons, mannose H-2, sialic acid H-3 and* N*-acetyl group protons for the four glycopeptides from human lactotransferrin*
For numbering of monosaccharide residues and complete structures see Table 4

Reporter group	Residue	Shift in glycopeptide fraction			
		A	B	C	D
		ppm			
H-1	1	5.074	5.072	5.074	5.067
	2	4.685	4.688	4.686	4.684
	3	≈ 4.77	≈ 4.77	4.769	4.771
	4	5.135	5.135	5.133	5.133
	4′	4.941	4.942	4.910	4.925
	5	4.607	4.609	4.605	4.606
	5′	4.607	4.609	≈ 4.58	4.579
	6	4.448	4.447	4.444	4.445
	6′	4.448	4.447	4.444	4.474
	a	4.872	4.882	4.876	4.871
	b	–	–	5.123	–
H-2	3	4.258	4.259	4.252	4.254
	4	4.197	4.198	4.191	4.191
	4′	4.112	4.115	4.103	4.103
H-3ax	NeuAc (α2→6)	1.722	1.722	1.717	1.717
H-3eq	NeuAc (α2→6)	2.670	2.674	2.669	2.667
H-5	a	≈ 4.12	4.128	≈ 4.12	≈ 4.12
	b	–	–	≈ 4.8	–
H-6	a	1.206	1.209	1.209	1.205
	b	–	–	1.173	–
NAc	1	2.016	2.019	2.016	2.017
	2	2.097	2.097	2.094	2.092
	5	2.070	2.070	2.068	2.068
	5′	2.070	2.070	2.042	2.047
	NeuAc	2.031	2.032	2.030	2.030

Hydrazinolysis of the Glycopeptides

The permethylated oligosaccharides obtained from glycopeptides A–D after hydrazinolysis/nitrous deamination and sodium borodeuteride reduction were separated by gas-liquid chromatography and identified by mass spectrometry. In the four glycopeptides A–D, the following derivatives were found: Man(1-3)[Man(1-6)]Man(1-4)–2-anhydro[1-^{2}H]man-

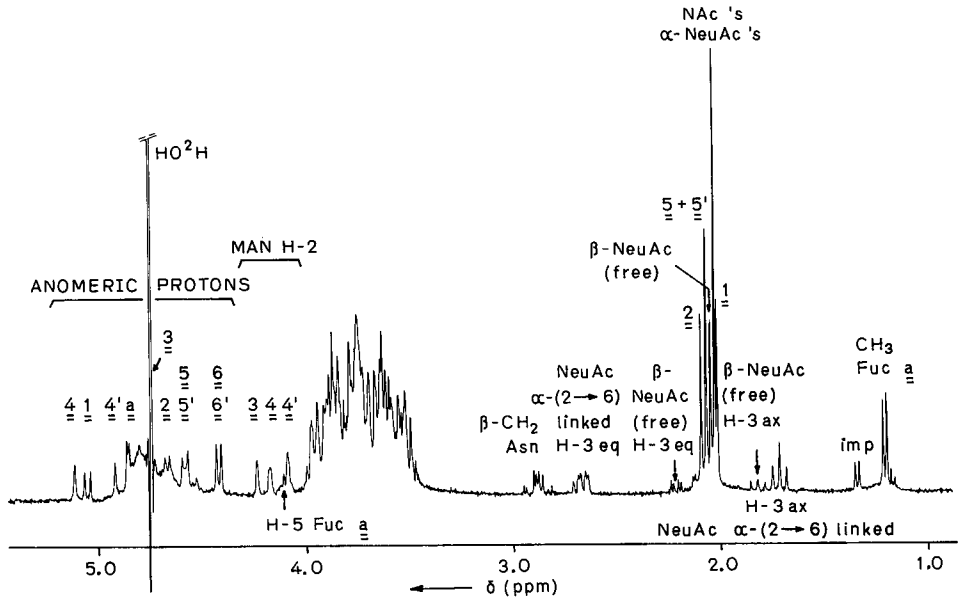

Fig. 1. *360-MHz ^{1}H-NMR spectrum of glycopeptides A or B isolated from human lactotransferrin in ^2H_2O at 25 °C*

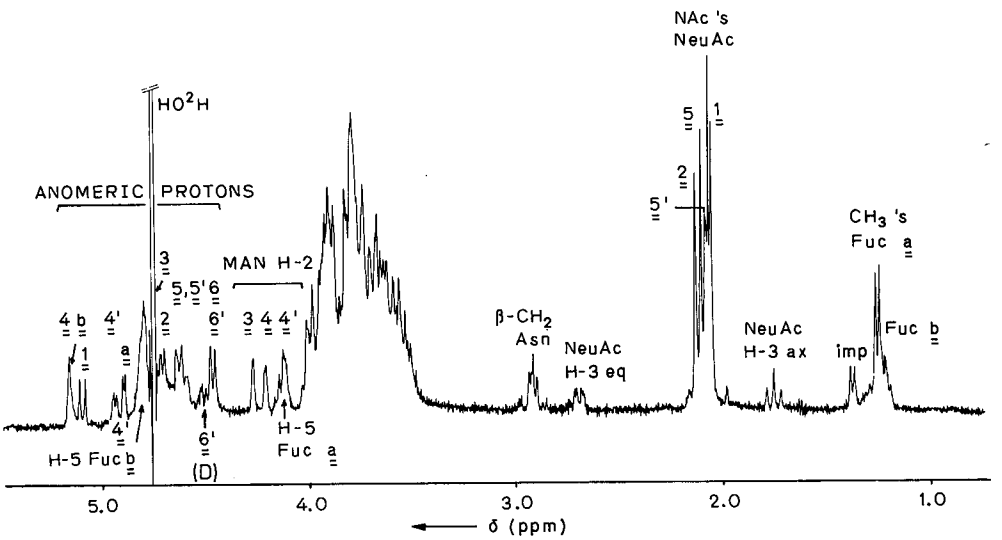

Fig. 2. *360-MHz ^{1}H-NMR spectrum of glycopeptides C and D isolated from human lactotransferrin in ^2H_2O at 25 °C*

nitol–2-keto-3-deoxynonulosic acid-(2-6)Gal(1-4)–2,5-anhydro[1-^{2}H]mannitol and the disaccharide Fuc(1-6)–2,5-anhydro[1-^{2}H]mannitol. The presence in the hydrazinolysis/nitrous deamination products from glycopeptide C of the nonulosic acid–(2-6)Gal(1-4)–2,5-anhydro[1-^{2}H]mannitol and Gal(1-4)[Fuc(1-3)]–2,5-anhydro[1-^{2}H]mannitol derivatives in a ratio of 1:1 indicated that the *N*-acetylneuraminic acid and fucose are bound to two different *N*-acetyllactosamine units. In addition to these derivatives, the compound Gal(1-4)-[Fuc(1-6)]–2,5-anhydro[1-^{2}H]mannitol was characterized in the glycopeptide C and the compound Gal(1-4)–2,5-anhydro-[1-^{2}H]mannitol in the glycopeptide D. The mass spectra of these different derivatives were described previously [37].

*Hydrolysis by Endo-*N*-acetyl-β-D-glucosaminidase B*

The presence of Fuc(α1-6)GlcNAc(β1-4)Asn in the four glycopeptides was confirmed by the identification of Fuc-(α1-6)GlcNAc(β1-4)-*N*-[^{14}C]acetylasparagine after hydrolysis of the four asialoglycopeptides by endo-*N*-acetyl-β-D-glucosaminidase B from a Basidiomyces and separation by thin-layer chromatography as described previously [39].

360-MHz ^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were recorded of the glycopeptides A–D. The ^{1}H-NMR data are compiled in Table 3 and spectra of these glycopeptides are presented in Fig. 1 and 2.

The structural reporter group signals of the Man residues confirm that the four glycopeptides all possess the biantennary structure [41,42]. The presence of fucose (α1-6)-linked to GlcNAc-1 in all glycopeptides is evident from the structural reporter group signals of Fuc (δ for H-1 = 4.88 ppm, for H-5 = 4.12 ppm and for H-6 = 1.21 ppm) and of GlcNAc-2 (δ for H-1 = 4.69 ppm and for NAc = 2.097 ppm) [43,44]. The chemical shift value of H-1 of Man-4 shows that in the

Table 4. *Structures of the four glycopeptides isolated from human lactotransferrin*

Glycopeptides	Structure
A and B	NeuAc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→3) NeuAc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→6) Man(β1→4)GlcNAc(β1→4)GlcNAcβ1→Asn |(α1,6) Fuc a
C	NeuAc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→3) Gal(β1→4)GlcNAc(β1→2)Man(α1→6) |(α1,3) Fuc b Man(β1→4)GlcNAc(β1→4)GlcNAcβ1→Asn |(α1,6) Fuc a
D	NeuAc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→3) Gal(β1→4)GlcNAc(β1→2)Man(α1→6) Man(β1→4)GlcNAc(β1→4)GlcNAcβ1→Asn |(α1,6) Fuc a

four glycopeptides the upper branch contains NeuAc (α2-6)-linked to Gal-6 [14].

In the glycopeptides A and B the lower branch contains NeuAc (α2-6)-linked to Gal-6′. This conclusion is based on the chemical shift value of H-1 of Man-4′. Furthermore, the chemical shift values of H-1 of both Gal-6 and Gal-6′, the H-1 and the *N*-acetyl protons of GlcNAc-5 and GlcNAc-5′ confirm the proposed structure.

The occurrence of a second fucose residue (1-3)-linked to a peripheral GlcNAc residue in the fraction C is obvious from the set of chemical shifts of the fucose structural reporter group protons (δ for H-1 = 5.12 ppm, for H-5 $\approx$ 4.8 ppm and for H-6 $\approx$ 1.17 ppm). This fucose residue is located in the asialo lower branch as can be inferred from the chemical shift value of H-1 of Man-4′: δ = 4.910 ppm [45,46]. The accompanying signal at 4.925 ppm, which is ascribed to the H-1 of Man-4′ in an afuco-asialo lower branch, indicates that the (α1-3)-linked fucose residue is only partly ($\approx$ 50%) present. It should be noted that the attachment of fucose in (α1-3) linkage to GlcNAc-5′ causes upfield shifts for H-1 of Gal-6′ (4.474 to 4.444 ppm) and for the *N*-acetyl protons of GlcNAc-5′ (2.047 to 2.042 ppm). These effects are in accordance with those described for fucose (α1-3)-linked to peripheral GlcNAc residues [45,47].

Structures of the Glycopeptides A – E

The results obtained from methanolysis, methylation analysis, hydrazinolysis/nitrous deamination, enzymic hydrolysis and 360-MHz ^{1}H-NMR spectroscopy lead to structures for the glycopeptides A – D as given in Table 4. These results show that the heterogeneity of the glycans is essentially due to the number of Fuc and NeuAc residues. The glycopeptide E which contains 1.5 Fuc residues and gives after methylation 3-*O*-methyl and 6-*O*-methyl derivatives of *N*-acetylglucosamine, corresponds to a mixture of glycopeptides with structures identical to glycopeptides A and C. The preliminary results concerning the structure of glycopeptides F and G are in favor of the presence in these glycopeptides of one or two supplementary fucosyl-*N*-acetyllactosaminic units. Further analysis are needed in order to determine the positions of these units precisely on the two antennae of the biantennary glycans.

DISCUSSION

In the two monosialylated glycopeptides, the NeuAc is always located on the antenna corresponding to the (α1-3)-linked mannose of the pentasaccharide core. When the glycopeptide is monosialylated and difucosylated one of the fucose residues is located on the antenna corresponding to the (α1-6)-linked mannose. These results are in a good accordance with the observations made by Paulson et al. [48] about the mutual exclusion of the *N*-acetylneuraminyl-transferase and fucosyltransferase activities.

The peptide chain seems to have no influence on the activity of the fucosyltransferase and sialyltransferase since the glycans A and B which possess identical structure can be attached to either of the glycosylation sites of the molecule as can the glycans C (with two fucose residues) and D (with one fucose residue).

Two major differences exist between the glycans of human serotransferrin and lactotransferrin, since, first, the lactotransferrin glycans possess one or two supplementary fucose residues. Secondly, the position of the two glycans on the polypeptide chain of serotransferrin and lactotransferrin are different: while in the lactotransferrin, one of the glycans is situated in the N-terminal part and the second one in the C-terminal part [49], in the case of the serotransferrin both glycans are located in the C-terminal part of the molecule [9,10]. The differences which have been found in the structure of the glycans and in their location may constitute a factor

which will explain the specificity of their biological activities. In fact, it has been shown that the serotransferrins recognize specifically membrane receptors of the reticulocytes [50], that ovotransferrins recognize embryo bird cells [51] and that the lactotransferrins recognize the membrane receptors of human enterocytes [52]. The recognition of human lactotransferrin by the membrane receptors of the macrophages has been described by Van Snick et al. [53]. This recognition may probably be mediated by the fucose residues, since Shepherd et al. [54] have recently described the presence of fucose receptors on rat macrophages. In order to check this hypothesis and the role of the different glycans found in the lactotransferrin, the determination of biological activities of human lactotransferrin with partially modified glycans is now under investigation.

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