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Primary Structure of the *N*-Glycosidically Linked Sialoglycans of Secretory Immunoglobulins A from Human Milk

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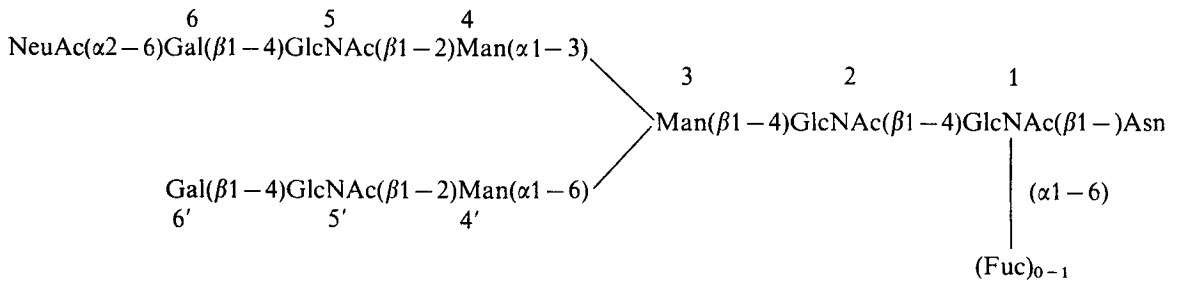
The alkali-stable sialoglycopeptides of secretory immunoglobulins A from human milk have been separated from the alkali-labile glycopeptides by gel filtration and from the asialoglycopeptides by ion-exchange chromatography. The structures of five of them have been determined on the basis of the results obtained by methylation analysis, mass spectrometry and 360 MHz ^{1}H-NMR spectroscopy. For glycopeptide B, the following structure has been found:



The other glycopeptides can be considered as extensions of this structure. The following extensions to Gal-6′ are proposed: NeuAc(α2–6) (glycopeptide A), Gal(β1–3) (glycopeptide D) and Fuc(α1–6) (glycopeptide E). Furthermore, in glycopeptide C a fucose residue in (α1–3) linkage to GlcNAc-5′ could be traced.

The secretory immunoglobulins of type A (sIgA) from human milk are glycoproteins [1] which are carbohydrate-rich (12%, w/w) and present an amazing diversity in the structures of their glycans. In fact, sIgA possess both *O*-glycosidically and *N*-glycosidically linked glycans [2,3]. The first ones are located, as in human serum IgA [4,5], in the hinge region of the α chains and we have described in previous papers the structures of some of them [3,6]. The *N*-glycosidically linked glycans are present on the α chains as in serum IgA [7] and on the junction [8] and secretory [9] pieces.

In order to understand the important roles probably played by the glycan moieties in the conformation and behaviour towards proteolytic enzymes of sIgA and in their interaction with receptors, we have investigated further the primary structure of the sIgA glycans, by studying the alkali-stable *N*-glycosidically linked sIgA glycopeptides. These latter fell into two categories, acidic and neutral, according to the presence or absence of sialic acid residues. In the present paper we describe the primary structure of the sialoglycans taking part of the acidic glycopeptide fraction. The neutral fraction of asialoglycans presents a large structural heterogeneity and will be described in a forthcoming paper.

Abbreviations. Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetylglucosamine; Man, D-mannose; NeuAc, *N*-acetylneuraminic acid; Asn, asparagine; sIgA, secretory immunoglobulins A; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Bio-Gel P-30 (200–400 mesh) and Dowex 1X2 (200–400 mesh) were from Bio-Rad Laboratories (Richmond, CA, USA). Sephadex LH-20 and Sephadex G-50 (fine) were obtained from Pharmacia Fine Chemicals A.B. (Uppsala, Sweden). Pronase was obtained from Calbiochem (Lucerne, Switzerland). Anhydrous hydrazine was from Pierce Chemical Company (Rockford, IL, USA).

Preparation of the Sialoglycopeptides

sIgA were isolated from a pool of human milk and the glycans *N*-glycosidically linked to the glycopeptide chains were prepared according to the procedure described previously [3]. The tryptic-pepsic fraction called GP-II which had a molecular weight of 10000 was submitted to further pronase digestion [10]. The mixture of glycopeptides was separated from the peptides by gel filtration on a column containing Bio-Gel P-30 (4 × 130 cm) and the carbohydrate fractions visualized by the phenol/sulfuric acid reagent [11] were further submitted to ion-exchange chromatography on a column containing Dowex 1X2 (200–400 mesh, acetate form) stabilized in 2 mM pyridine/acetic acid buffer pH 5 [12]. Elution was performed using a discontinuous gradient of ionic strength at a flow rate of 25 ml/h. The neutral fraction (GP-N) was eluted by the initial buffer and the acidic fraction

(GP-A) with a 500 mM pyridine/acetic acid buffer pH 5. All the fractions were desalted on a column containing Sephadex G-50 (2.2 × 45 cm) equilibrated in water, at a flow rate of 18.5 ml/h. The sialoglycopeptides (GP-A) were fractionated by preparative paper electrophoresis at pH 2.4 (acetic acid 1 M), for 12 h at 10 V/cm.

Determination of the Structure of the Sialoglycopeptides

The molar carbohydrate composition of the glycopeptides was determined by gas-liquid chromatography after methanolysis and trifluoroacetylation [13]. Exhaustive methylation was carried out according to Hakomori [14] and the methylglycosides were separated by gas-liquid chromatography before (for neutral monosaccharides) and after (for neutral monosaccharides and hexosamines) peracetylation according to Fournet et al. [15]. The identification of the methyl derivatives of glucosamine residues was carried out after hydrolysis (4 M HCl, 4 h, 100 °C) of the permethylated glycopeptides and after peracetylation, reduction with $NaBD_4$, purification on Dowex 50X8 (25–50 mesh, H^+) followed by peracetylation [16]. The sialoglycopeptide GP-A3 was submitted to hydrazinolysis–nitrous acid deamination [17] and the liberated oligosaccharides were methylated and analyzed by gas-liquid chromatography–mass spectrometry [18]. For ^{1}H-NMR spectroscopic analysis the neutralized glycopeptides were repeatedly exchanged in D_2O (100% D, Aldrich, Milwaukee, USA) at room temperature with intermediate lyophilization. The 360-MHz ^{1}H-NMR spectra were recorded on a Bruker HX-360 spectrometer, operating in the Fourier transform mode at a probe temperature of 25 °C. Chemical shifts are given relative to sodium-4,4-dimethyl-4-siliapentane-1-sulphonate (indirectly to acetone in D_2O: δ = 2.225 ppm) with an accuracy of 0.003 ppm.

RESULTS

Preparation of the Sialoglycopeptides

From the trypsin-pepsin hydrolysate of 1 g of sIgA, two fractions were isolated by gel filtration on Bio-Gel P-30 [3]: GP-I (43 mg) and GP-II (240 mg). The structures of four *O*-glycosidically linked glycans present in the GP-I fraction were previously determined [3]. Fraction GP-II, containing the *N*-glycosidically linked glycopeptides, was separated by ion-exchange chromatography into a neutral fraction GP-N (17.5 mg) which was extremely heterogeneous. The mixture of sialylated glycopeptides GP-A (61 mg) was separated into three fractions by preparative paper electrophoresis. The proportions of these fractions were 8% for glycopeptide GP-A1; 42% for GP-A2 and 50% for GP-A3.

Primary Structure of the Sialoglycopeptides

The amino acid composition of the three sialoglycopeptides (GP-A1 to GP-A3) shows the presence in all of the glycopeptides of one aspartic acid residue for four *N*-acetylglucosamine residues. Threonine, serine, glutamic acid, glycine, alanine and valine residues were characterized in trace amounts. The three glycopeptides were essentially differentiated by their *N*-acetylneuraminic acid, fucose and galactose content (Table 1). The primary structure of the different glycans was established by determination of the carbohydrate composition, methylation analysis, mass spectrometry and 360-MHz ^{1}H-NMR spectroscopy.

Table 1. *Carbohydrate composition of the glycopeptide fraction GP-A and of the glycopeptide sub-fractions GP-A1 to GP-A3 isolated from human milk secretory IgA*
The molar ratios were calculated on the basis of three mannose residues

Nature of the monosaccharides	Molar ratio in			
	mixture of sialoglycopeptides GP-A	GP-A1	GP-A2	GP-A3
Fuc	1.46	1.08	1.25	1.33
Gal	2.22	2.05	2.09	2.50
Man	3	3	3	3
GlcNAc	4.09	3.92	4.04	3.49
NeuAc	0.78	1.94	0.88	1.39

Table 2. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated glycopeptides isolated from human milk secretory IgA*
The molar ratios were calculated on the basis of two residues of (3,4,6)-Me_3Man

Monosaccharide methyl ethers	Molar ratio in		
	GP-A1	GP-A2	GP-A3
(2,3,4)Me_3Fuc	1.00	1.2	0.81
(2,3,4,6)Me_4Gal	0	0.9	1.00
(2,3,4)Me_3Gal	2.02	1.2	0.98
(2,4,6)Me_3Gal	0	0	0.49
(3,4,6)Me_3Man	2	2	2
(2,4)Me_2Man	0.98	1.19	1.00
(3,6)Me_2GlcNAc(Me)	2.90	2.65	3.20
(6)Me_1GlcNAc(Me)	0	0.27	0.20
(3)Me_1GlcNAc(Me)	0.40	0.28	0.30

Structure of the Glycopeptide Fraction GP-A1

The carbohydrate composition of glycopeptide fraction GP-A1 is presented in Table 1. The results suggest the sample to be homogeneous. The identification of the different methylated derivatives (Table 2) and particularly the identification of the methylated derivatives of mannose led to the conclusion that a biantennary structure of the *N*-acetyllactosamine type is present. The 360-MHz ^{1}H-NMR spectral data of glycopeptide fraction GP-A1 point to a bi(α2–6)-sialo biantennary *N*-acetyllactosamine type of structure. This can be deduced from the chemical shifts of the H-1 of Man-4 (δ = 5.133 ppm) and Man-4′ (δ = 4.940 ppm) in combination with those of the H-2 of Man-3 (δ = 4.259 ppm), Man-4 (δ = 4.196 ppm) and Man-4′ (δ = 4.116 ppm). Independent evidence for the (α2–6) type of linkage between *N*-acetylneuraminic acid and galactose stems from the chemical shifts of the structural reporter group signals of the *N*-acetylneuraminic acid residues (δH-3ax = 1.725 ppm, δH-3eq = 2.672 ppm and δNAc = 2.030 ppm) and from the chemical shift of the H-1 of the galactose residues (δH-1 = 4.447 ppm) [19]. The (α1–6) type of linkage of fucose to GlcNAc-1 is characterized by the positions of the structural reporter group signals of fucose (δH-1 = 4.88 ppm, δH-5 = 4.12 ppm and δCH_3 = 1.20 ppm) and by the chemical shift of the *N*-acetyl signal of GlcNAc-2 (δNAc = 2.096 ppm) [19]. This result is in accordance with the characterization of 3-*O*-methyl derivative of *N*-acetylglucosamine. The low

Glycan A

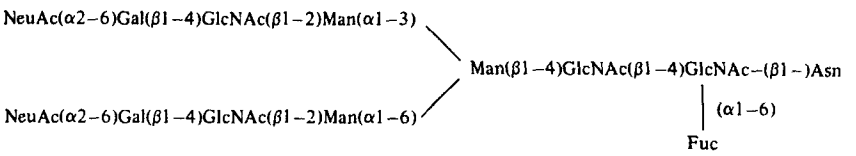
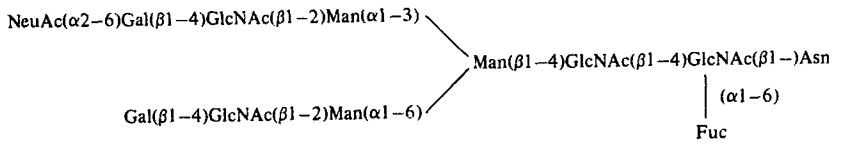


Fig. 1. *Structure of the disialylated glycan A from human milk sIgA*

Glycans B and F



Glycans C and G

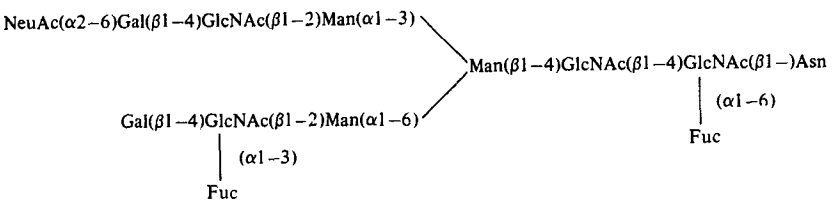


Fig. 2. *Structure of the glycans B and C constituting the glycopeptide fraction GP-A2*

amount of this component (Table 1) is due to the fact that, in the conditions of hydrolysis we used, the linkage between GlcNAc-1 and the asparagine residue has not been completely hydrolysed [16].

Structure of the Glycopeptide Fraction GP-A2

The glycopeptide fraction GP-A2 is a mixture of two glycopeptides (glycopeptides B and C) of the *N*-acetyllactosamine type. Both glycans possess one *N*-acetylneuraminic acid residue but they differ in their fucose content. This conclusion was reached on the basis of the following results. (a) The carbohydrate composition shows that the fucose residues were not present in an integer number of residues (Table 1); (b) methylation analysis demonstrates the presence of mono 3-*O*-methyl and 6-*O*-methyl derivatives of *N*-acetylglucosamine (Table 2); (c) the 360-MHz ^{1}H-NMR spectrum of the glycopeptide fraction GP-A2 shows that it consists of a mixture of closely related biantennary glycans of the *N*-acetyllactosamine type. The kind of branching is evident from the chemical shift values of the mannose H-2 signals (δH-2 Man-3 = 4.258 ppm, δH-2 Man-4 = 4.195 ppm and δH-2 Man-4′ = 4.105 ppm) [19]. In all glycopeptides present in this mixture the upper branch contains *N*-acetylneuraminic acid (α2–6)-linked to Gal-6. This conclusion is based on the chemical shift values of H-3ax and H-3eq of *N*-acetylneuraminic acid (δ = 1.725 ppm and 2.667 ppm, respectively), in combination with that of H-1 of Man-4 (δ = 5.133 ppm). The presence of fucose (α1–6)-linked to GlcNAc-1 is proved by the positions of the structural reporter group signals of fucose: δH-1 = 4.88 ppm, δH-5 = 4.12 ppm and δCH_3 = 1.21 ppm. For the *N*-acetyl signal of GlcNAc-2 two singlets are observed at δ = 2.080 ppm and δ = 2.094 ppm with a relative intensity ratio 1:1. For the H-1 of GlcNAc-2 two doublets are found at δ = 4.626 ppm and δ = 4.699 ppm, in a similar intensity ratio as observed for the *N*-acetyl signals of this residue. The set of signals at 2.080 ppm and 4.626 ppm belongs to a glycopeptide devoid of fucose (α1–6)-linked to GlcNAc-1 whereas the other set, at δ = 2.094 ppm and δ = 4.699 ppm, belongs to a glycopeptide bearing Fuc in an (α1–6) linkage to GlcNAc-1 [19]. The occurrence of a second fucose residue (α1–3)-linked to a peripheral *N*-acetylglucosamine residue is obvious from the set of chemical shifts of the fucose structural reporter group protons: δH-1 = 5.12 ppm and δCH_3 = 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 = 4.909 ppm [20]. The occurrence of two signals for the *N*-acetyl protons of GlcNAc-5′ at δ = 2.048 ppm and δ = 2.042 ppm in an intensity ratio of 1:1 indicates that the (α1–3)-linked fucose is only partly present [20, 21]. The structure of the glycans B and C present in the glycopeptide fraction GP-A2 are given in Fig. 2.

Structure of the Glycopeptide Fraction GP-A3

The results obtained by methanolysis (Table 1) and by methylation analysis (Table 2) of the glycopeptide fraction GP-A3 indicate that the monosaccharides and the methyl derivatives of these monosaccharides were not present in a ratio of integer numbers. Consequently, fraction GP-A3 is a mixture of glycopeptides. In fact, the glycopeptide fraction GP-A3 differs from the two others by an excess of galactose. After methylation, hydrolysis, reduction with sodium boro-

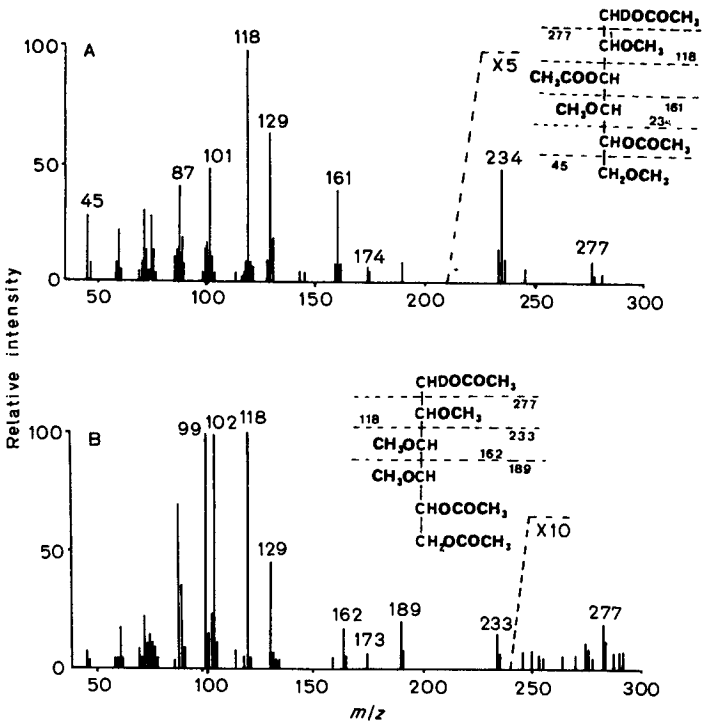


Fig. 3. *Mass spectra of the two methylated, reduced and acetylated derivatives of the galactose residues present in the glycopeptide fraction GP-A3.* (A) 2,4,6-Tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol; (B) 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetylgalactitol

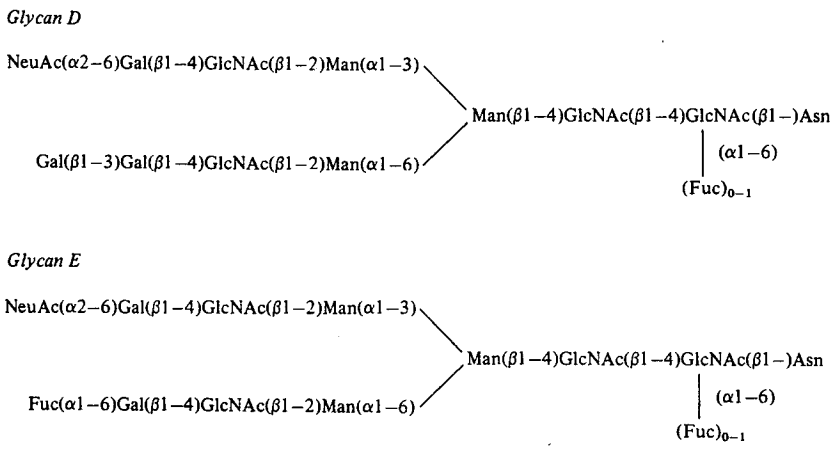


Fig. 4. *Structure of the glycans D and E composing the glycopeptide fraction GP-A3*

deuteride and peracetylation the glycopeptide fraction GP-A3 was analyzed by gas-liquid chromatography and mass spectrometry. The presence of the 2,3,4-*O*-trimethyl and 2,4,6-*O*-trimethyl derivatives of galactose (Fig. 3) indicates that these residues are substituted at C-6 or C-3, respectively. The hydrazinolysis-nitrous deamination data confirmed that the glycopeptide fraction GP-A3 was a mixture of glycopeptides with different structures since the following oligosaccharides were identified: Man(1–3)[Man(1–6)]Man(1–4)-2,5-anhydro[1-^{2}H]mannitol; 3-oxo-3-deoxynonulosic acid (2–6)-Gal(1–4)-2,5-anhydro[1-^{2}H]mannitol; Gal(1–3)Gal(1–4)-2,5-anhydro[1-^{2}H]mannitol; Fuc(1–6)Gal(1–4)-2,5-anhydro[1-^{2}H]mannitol; Gal(1–4)[Fuc(1–3)]2,5-anhydro[1-^{2}H]-mannitol; Gal(1–4)-2,5-anhydro[1-^{2}H]mannitol and Fuc-(1–6)2,5-anhydro[1-^{2}H]mannitol. The separation, the proportion and the mass spectra of these different oligosaccharides were previously reported [18]. Their characterization allowed the identification of four structures: those of glycans B and C (Fig. 2) and of glycans D and E (Fig. 4) having unusual linkages such as Gal(1–3)Gal-R and Fuc(1–6)-Gal-R.

The 360-MHz ^{1}H-NMr spectrum of glycopeptide fraction GP-A3 revealed that this fraction contains glycans with the biantennary structure. *N*-Acetylneuraminic acid is exclusively (α2–6)-linked to Gal-6. The *N*-acetyl signals, observed for GlcNAc-2 at $\delta = 2.078$ ppm and $\delta = 2.093$ ppm in an intensity ratio of 1:1, indicate that fucose (α1–6)-linked to GlcNAc-1 is present for about 50% [19]. Evidence exists for a second fucose linkage type viz. (α1–3) to GlcNAc-5′. The structural reporter group signals of this fucose residue are found at $\delta = 5.121$ ppm (H-1) and $\delta = 1.168$ ppm (CH_3).

The Fuc(α1–6)Gal moiety, which was found by mass spectrometry to occur in a low amount (5–10%) could not be identified by 360-MHz ^{1}H-NMR spectroscopy. The

chemical shift data of the structural reporter signals of fucose in such a type of linkage are not yet available. It cannot be excluded that they coincide with structural reporter group signals of fucose in other linkage types. The more so since in glycopeptide GP-A3 it is expected to be present in a small amount.

Concerning the Gal(1→3)Gal moiety, which was traced by mass spectrometry [18]. The ^{1}H-NMR spectrum is not conclusive. Previously, we observed that the attachment of a galactose residue in (β1→3) linkage to another galactose residue gives rise to a downfield shift of the H-4 signal of the 3-substituted galactose residue from $\delta = 3.92$ ppm to $\delta = 4.19$ ppm [22]. If such a structural element occurs in glycopeptide GP-A3 in a relatively low amount it cannot be excluded that the H-4 signal of the penultimate galactose residue is hidden under the H-2 signal of Man-4. Regarding the anomeric proton signals of the involved galactose residues, it is interesting to compare those with the signals of Gal(β1→3)Gal(β1→4)Xyl(β1→0)Ser, a reference compound possessing the Gal(β1→3)Gal moiety. In this reference compound the H-1 signal of the terminal galactose is found at $\delta = 4.610$ ppm (H. van Halbeek, unpublished results). The H-1 signal of the terminal galactose in glycopeptide GP-A3 may coincide at $\delta = 4.61$ ppm with the H-1 signal of GlcNAc-2 for that portion of the glycopeptide fraction that does not bear a fucose in (α1→6) linkage to GlcNAc-1. However, an H-1 signal for the penultimate galactose at $\delta = 4.52$ ppm is missing, suggesting that if the Gal(1→3)Gal moiety is present, the amount is less than 5%.

DISCUSSION

The *O*-glycosidically and *N*-glycosidically conjugated glycans of sIgA have been separated by gel filtration in two fractions, the main one being constituted of the *N*-glycosidically linked glycans. The preponderance of these latter could be explained by the fact that the $sIgA_2$ population, devoided of the *O*-glycosylated hinge region, is present in human milk in an amount three times higher than that of $sIgA_1$ which possess the hinge region [23].

The sialylated *N*-glycosidically linked glycans represent the major and less heterogeneous fraction, in contrast to the neutral glycopeptides. The sub-fractionation of the acidic fraction has led to the isolation of five oligosaccharides the structure of which are described in the present paper. All have in common a biantennary basic structure of the *N*-acetyllactosamine type and possess an *N*-acetylneuraminic acid residue (α2→6)-linked to the terminal galactose of the 'Man(α1→3) branch'. Most of the compounds possess a fucose residue (α1→6)-linked to the asparagine-conjugated *N*-acetylglucosamine. The heterogeneity of the structures is essentially due to the presence of additional Gal(β1→3) or Fuc(α1→6) linked to the terminal galactose residue, or of Fuc(α1→3) linked to the *N*-acetylglucosamine taking part of the *N*-acetyllactosamine residue present in the 'Man(α1→6) branch'.

The sequence Fuc(α1→6)Gal(β1→4)-R represents a novel type of oligosaccharide structure, while Gal(β1→3)Gal-(β1→4)-R has been previously demonstrated in glycopeptides from calf thymocyte plasma membranes [24], in angiotensin-converting enzyme [25] and in bovine glycophorin [26]. The location of these structures on the different polypeptide chains of sIgA has not yet been established. However, it may be noted that the structure of the oligosaccharides from the J-chain of Waldenström macroglobulin [8] shows strong homologies with those from sIgA here described. The major difference, however, is the absence of fucose in the former. On the other hand, the structure of a glycan from the free secretory piece isolated from human milk has been proposed [9]. However, we have not characterized this structure in the acidic glycopeptide fraction we isolated.

The *N*-glycosidically linked glycans from human milk sIgA, together with those from human serum immunoglobulins such as IgM [27], IgE [28], IgA [4,5] and IgG [29], from bovine serum immunoglobulins [30,31] and from bovine colostral IgG_1 [32] show a relative unity of structure. They are biantennary and of the *N*-acetyllactosamine type with a fucose residue linked to the *N*-acetylglucosamine of the protein attachment point. However they differ by the presence of particular oligosaccharide sequences like: Gal(β1→4)[Fuc-(α1→3)]GlcNAc, Fuc(α1→6)Gal and Gal(β1→3)Gal(β1→4)-GlcNAc. Moreover, in human serum IgA_1 [5] and IgG [29], an additional intersecting *N*-acetylglucosamine residue linked to the Man(β1→4) has been demonstrated. This residue is absent in the acidic glycopeptides from human milk sIgA, but has been recently identified in practically all the neutral glycopeptides of the *N*-acetyllactosamine type (A. Pierce, unpublished results).

The biological significance, if any, of the structural specificity of human milk sIgA glycans remains to be established.

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