Structural studies on sialylated oligosaccharides of bonnet monkey (*Macaca radiata*) luteal phase cervical mucus glycoprotein.

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(Received 15th October, 1996, revised 31st May, 1997)

Summary: Mucin glycoproteins were purified and fractionated from cervical epithelial secretion in luteal phase of the bonnet monkey *(Macaca radiata)*. Polyclonal antibody against the purified luteal phase glycoprotein cross reacted with crude luteal phase mucus as well as weakly with ovulatroy phase mucus and purified midcycle glycoproteins, thus suggesting some common epitopes in the ovulatory and luteal phase glycoproteins. Alkaline borohydride cleavage of a purified glycoprotein resulted in a mixture of acidic and neutral oligosaccharide alditols. Utilizing high performance chromatography, six oligosaccharide fractions (b-1 to b-6) have been purified from the sialylated oligosaccharide fraction b. Based on the results of enzymic and chemical studies, following structures are proposed for these oligosaccharides that bear similarities with those of the midcycle oligosaccharide structures.

Introduction

The variable biophysical behaviour of the cervical mucus during the ovulatory cycle is of great importance in reproductive physiology. The mechanism of sperm penetration through the cervix and cervical mucus depends on the intrinsic motility of the sperm and receptivity of the mucus [1,2]. Cervical mucus is a complex mixture produced continuously by endocervical cells. Minor quantities of endometrial, tubal and follicular fluids may also contribute to the cervical mucus [3]. The mucus exhibits a number of rheological properties, such as viscosity, spinnbarkeit, flow elasticity, and stickiness those are regulated by the ovarian function. The physical and chemical changes in the cervical mucus during the ovulatory cycle influences penetration as well as survival of the sperm. The cyclic changes in the physical properties of the mucus are accompanied by variations in carbohydrate composition [4,5]. Cervical mucus consists of high molecular weight, heavily glycosylated proteins, enzymes, metallic ions and low molecular weight proteins. Glycoproteins are the major constituents of the mucus. The results of physical studies on cervical glycoproteins suggest the presence of linear flexible chains that yield subunits following reduction of disulphide bonds [6,7]. Carbohydrate chains, which account for 50-75% of the molecular mass of the purified cervical glycoproteins in different ovulatory phases, are present in a heterogeneous population of *O*-linked neutral, acidic and sulphated oligosaccharides. The oligosaccharide chains on the protein core are distributed assymetrically resulting in protease susceptible and protease resistant domains of the glycoprotein [8,9]. The changes in biophysical properties of the mucus are accompanied by alteration in chemical structure, in particular the chemical structure of

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oligosaccharides is as yet to be clearly defined. In our earlier studies [10,11] it has been shown that the periovulatory and premenstrual glycoproteins show diversity in the attachment of NeuAc to sugar residues. It was also proposed [11] that this diversity in NeuAc linkage may provide rigidity as well as resistance against proteases to mucus glycoproteins, thereby enabling mucins to perform different functions during the ovulatory cycle.

In order to understand the relationship between the glycoprotein component of the mucus and function of this secretion during the cycle, it is necessary to characterize the glycoproteins from different phases of the ovulatory cycle responsible for the physiological role and the morphological outlay of the mucus. Purification and fractionation of periovulatory cervical mucus by gel-exclusion chromatography afforded high molecular weight glycoproteins. The major glycoprotein is a carbohydrate rich mucin type glycoprotein [12,13]. An antibody against this glycoprotein in immunodiffusion and in immunoelectrophoresis showed sharp and diffused precipitin lines [12]. Under the microscope, the antigen-antibody complex exhibited a morphology different from that of fibrillar channels of the periovulatory mucus [12]. The alteration induced in the morphological outlay by the antibody clearly suggests that the mucus channels are lined with glycoproteins and, thus, suggests the physiologically significant role played by the mucus in sperm migration and penetration [13].

Purification and fractionation of the glycoprotein of the bonnet monkey luteal phase mucus, raising antibody against a purified glycoprotein, liberation of oligosaccharides and subsequent purification and characterization of the acidic oligosaccharides is the subject of this report. The Sepharose 2B purified glycoprotein, Fraction 1, that was homogeneous in ion-exchange chromatography on DEAE-cellulose has been investigated in this study.

This study was pursued on *Macaca radiata* as this monkey produces copious amounts of cervical mucus and because of its phylogenetic closeness to the human.

Results Discussions

Purification of glycoproteins.

The luteal phase glycoproteins were eluted from the Bio-Gel P-200 column in the void volume. There were overlapping components those were eluted in the void volume (Fig.1) and contained carbohydrates and proteins (Table 1). Two major heterogeneous glycoprotein components in addition to five proteins were obtained. The carbohydrate and amino acid composition of the two glycoproteins were similar (Table 1). The two glycoprotein fractions in agarose gel electrophoresis showed the presence of periodate-Schiff and Coomassie-blue staining materials, although these components had the minimal entry into the gel. The Bio-Gel P-200 purified glycoprotein, Fraction 1, afforded two components in Sepharose 2B chromatography (Fig.2). The two fractions, Fraction 1 (60%) and Fraction 2 (40%) contained sugars and amino acids (Table 1). The major glycoprotein fraction, Fraction 1, in DEAE-cellulose chromatography provided a single glycoprotein (Table 1). Fraction 1 glycoprotein in polyacrylamide-agarose electrophoresis barely entered in gel, although it reacted positively with Coomassie-blue and periodate-Schiff stains. Antibody against Fraction 1 glycoprotein in immunodiffusion reacted with crude luteal phase mucus, Bio-Gel P-200 and Sepharose 2B purified glycoprotein fractions. The antibody also weakly reacted with midcycle and follicular phase mucuses, and Bio-Gel P-200 and Sepharose 2B purified glycoproteins from the periovulatory phase.

Preparation of sialylated oligosaccharide alditols.

Fraction 1 glycoprotein (54 mg) from the Sepharose 2B column was subjected to alkaline borohydride treatment [14], yielding a mixture of oligosaccharide alditols. A decrease of serine and threonine and a corresponding increase of alanine and the appearance of α-aminobutyric acid were observed, concomitant with partial conversion of GalNAc residues to GalNAc-ol. The acidic oligosaccharides, obtained from the column of AG1-X2, were separated on a Bio-Gel P-4 column into two main components, Fraction a and Fraction b in addition to overlapping fractions. The

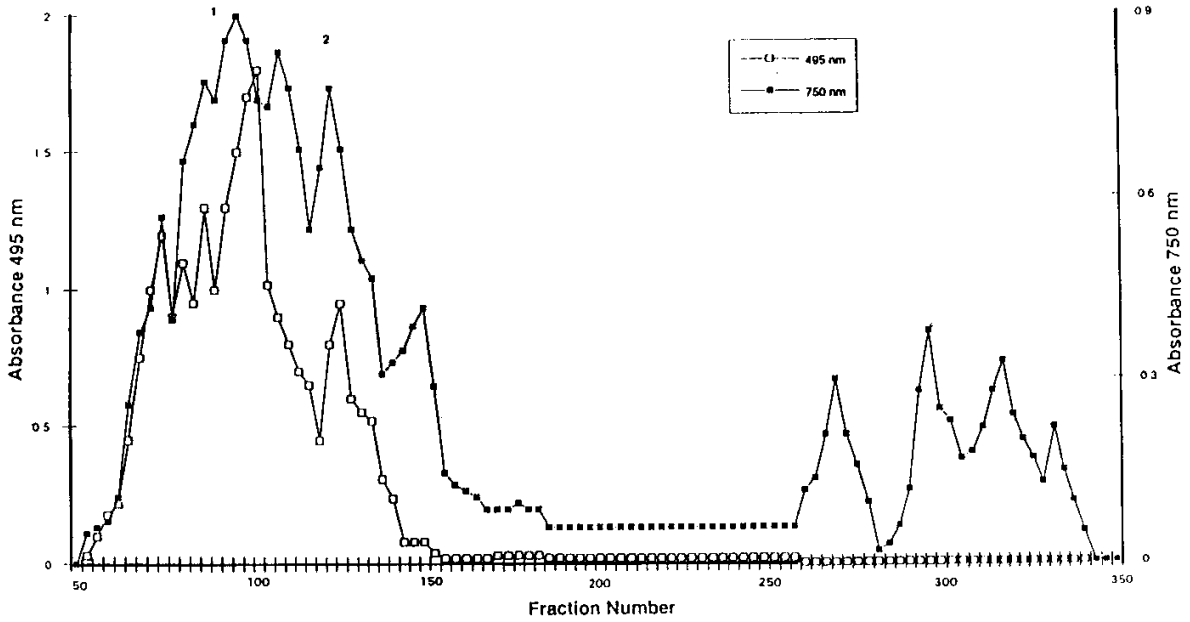

Fig. 1: Purification of crude luteal phase cervical mucus in Bio Gel P-200. The column was washed with 50 mM sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide. Fractions of 2 ml were collected and every third fraction was examined for the presence of carbohydrates (495 nm) and proteins (750 nm).

Table 1 Carbohydrate and amino acid composition of glycoproteins purified on Bio-Gel P-200, fractionated on Sepharose 2B and DEAE-cellulose.

	Bio-Gel P-200 Fractions				Sepharose 2B Fractions				DEAE-cellulose	
	1		2		1		2			
	%	M/R*	%	M/R*	%	M/R*	%	M/R*	%	M/R*
Carbohydrates										
Fuc	7.0	1.06	6.8	1.04	5.7	0.83	6.2	1.03	6.3	0.82
Gal	16.0	2.19	14.9	2.08	15.8	2.1	15.1	2.29	21.0	2.48
GlcNAc	9.0	1.00	8.8	1.00	9.3	1.00	8.1	1.00	10.4	1.00
GalNAc	14.0	1.55	13.6	1.54	13.2	1.42	10.9	1.34	15.6	1.50
NeuAc	9.0	0.71	9.4	0.76	10.3	0.79	7.9	0.60	11.3	0.78
Total	55.0		50.0		54.3		48.2		64.6	
Sulphate					0.4		0.5			
Amino acids[§]										
Asp	5.1		4.8		5.3		5.2		5.6	
Thr	18.2		17.1		19.6	-10.4	18.1		19.4	
Ser	12.1		12.9		11.9	-4.1	12.3		11.7	
Glu	7.1		6.9		6.8		7.1		5.8	
Pro	5.6		7.1		6.4		6.9		6.8	
Gly	9.0		8.5		8.2		9.2		7.8	
Ala	10.2		11.1		10.2	+4.1	10.3		9.6	
Aba						+9.6				
Cys/2	1.8		2.4		1.6		1.9		1.4	
Val	6.8		5.9		6.4		5.7		6.0	
Ileu	5.8		5.1		5.6		4.9		5.8	
Leu	5.8		4.9		5.9		5.4		6.7	
Tyr	2.1		1.9		2.2		1.8		2.3	
Phe	2.3		1.8		2.1		1.9		2.6	
Lys	2.6		3.1		2.9		3.4		3.4	
His	2.2		2.8		2.1		2.7		2.4	
Arg	3.3		3.7		2.8		3.2		2.7	

* M/R (molar ratio) relative to GlcNAc.
§ Residues per 100 residues.
¶ Fraction 1 after treatment with alkali.

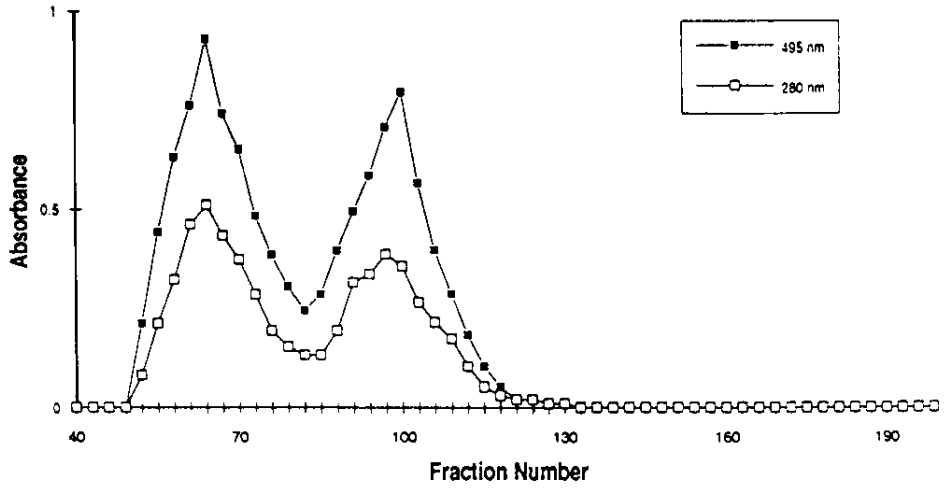

Fig. 2: Gel chromatography of the Bio-Gel P-200 purified Fraction 1 glycoprotein on Sepharose 2B. The glycoprotein were eluted from the column with 50 mM sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide. Fractions of 1.5 ml were collected and every third fraction was examined for the presence of carbohydrates (495 nm) and proteins (290 nm).

Table 2:Molar composition of oligosaccharides obtained by alkaline-borohydride treatment of glycoprotein fraction F-1 and fractionation.

Oligosaccharide		Molar ratio* of monosaccharides					
Bio-Gel P-4	hplc of Fraction b	NeuAc	Fuc	Gal	GlcNAc	GalNAc	GalNAc-ol
a		0.58	0.59	2.14	0.79	0.56	1.0
b		0.68	0.61	1.40	0.82	0.30	1.00
	b-1	1.50			0.60		1.00
	b-2	0.70		1.00	0.90		1.00
	b-3	0.70		0.90	0.80		1.00
	b-4	0.80	0.70	0.90	0.80		1.00
	b-5	0.70	0.80	0.90	0.80		1.00
	b-6	0.70	0.60	1.70	0.80		1.00

* Molar ratio relative to GalNAc-ol.

carbohydrate composition of the oligosaccharide fractions is given in Table 2. The oligosaccharide Fraction b was separated by hplc into six fractions (b-1 to b-6, Fig.3). The molar ratios of the oligosaccharide b-1 to b-6 sugars are given in Table 2. Because of the limited amount of purified oligosaccharides available for structural analysis, characterization was performed on labelled oligosaccharides.

Oligosaccharide b-1

Oligosaccharide b-1, a major oligosaccharide component, was homogeneous in pc and has the composition NeuAc-GlcNAc-GalNAc-ol (1.5:0.6:1; relative to GalNAc-ol). This oligosaccharide in pc moved slower than NeuAcα2-6GalNAc-ol and Neuα(2-3)Galβ(1-3)GalNAc-ol. Treatment of the oligosaccharide with neuraminidase resulted in cleavage of NeuAc and release of GalNAc-ol and a component similar to the standard disaccharide, NeuAcα(2-6)GalNAc-ol in pc. Hydrolysis of the neuraminidase treated material obtained from pc showed the presence of GlcNAc and GalNAc-ol. Digestion of b-1 with *N*-acetylglucosaminidase removed GlcNAc. Periodate oxidation-borohydride reduction, acid hydrolysis and re-*N*-acetylation of b-1 showed therein the presence of SerNAc-ol and ThrNAc-ol. Methylation results (Table 3) combined with the above data suggested that oligosaccharide Fraction b-1 was a mixture of a

Table 3: O-Methyl derivatives obtained by acid hydrolysis of methylated oligosaccharides before and after enzyme treatment.

Oligosaccharide O-Methyl derivative.	Fuc	Gal					GlcNAc			GalNAc-ol		
	2,3,4,	2,3,4,6,	2,4,6,	3,4,6,	2,3,6,	3,4,6,	3,4,	4,6,	3,6,	6,	3,4,	4.
b-1							+				+	+
b-2		+						+				+
b-2[a]		+						+			+	
b-3			+			+						+
b-3[a]			+			+					+	
b-4	+		+					+				+
b-4[a]	+	+						+				+
b-4[b]						+					+	
b-5	+	+								+		+
b-5[a]	+	+								+	+	
b-6	+		+	+				+				+
b-6[c]			+					+			+	
b-6[d]		+						+			+	

[a]Nueraminidase treated.
[b]Sequentially neuraminidase, α-fucosidase and β-galactosidase treated.
[c]α-L-Fucosidase and β-D-galactosidase treated.
[d]Neuraminidase treatment of α-L-fucosidase and β-galactosidase treated oligosaccharide.

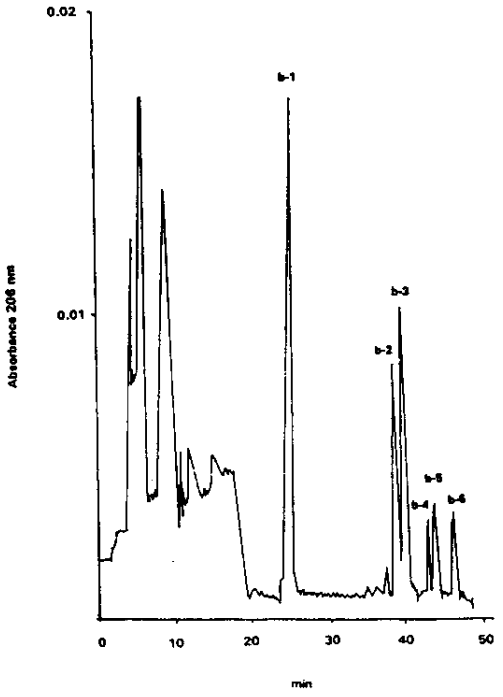

Fig. 3: Hplc separation of acidic oligosaccharides of Fraction b oligosaccharide on a column of Lichsorob-NH_2 with a linear gradient of 4:1 to 1:1(v/v) acetonitrile-water containing 2.5 mM ammonium hydrogen carbonate for 60 min at a flow rate of 1.2 ml/min.

disaccharide, and a trisaccharide with the structures b-1-a and b-1-b shown in Fig.4.

Oligosaccharide b-2

Oligosaccharide b-2 was a tetrasaccharide with the molar composition NeuAc:Gal:GlcNAc:GalNAc-ol (0.7:1:0.9:1, relative to GalNAc-ol). Digestion of the oligosaccharide with neuraminidase removed NeuAc. Methylation analysis of sialo and asialo-oligosaccharides (Table 3) suggested the presence of 3,6-disubstituted GalNAc-ol and sialic acid linked to C-6 of GalNAc-ol in the oligosaccharide. In addition, the presence of a terminal galactose residue and a 3-substituted GlcNAc residue was observed. Digestion of b-2 with α-galactosidase had no effect on the oligosaccharide, and treatment with β-galactosidase removed the galactose residue. Periodate oxidation-borohydride reduction resulted in destruction of Gal and NeuAc residues, conversion of GalNAc-ol to ThrNAc-ol and GlcNAc was recovered unchanged. Based on these results the b-2 tetrasaccharide was assigned the structure b-2 shown in Fig.4.

Oligosaccharide b-3.

Oligosaccharide b-3 had the molar composition NeuAc:Gal:GlcNAc:GalNAc-ol (0.7:0.9:0.8:1., relative to GalNAc-ol). Treatment of the oligosaccharide with neuraminidase removed NeuAc. Methylation analysis of the methylated sugars of sialo and asialo-oligosaccharides showed derivatives in these oligosaccharides (Table 3) that suggested the presence of terminal GlcNAc, 3-linked Gal, 3,6-linked GalNAc-ol and NeuAc linked to C-6 of GalNAc-ol. Digestion of b-3 with

Abbreviations

Abbreviations used are Fuc, fucose; Gal, galactose; NeuAc, N-acetylneuraminic acid; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; glc, gas liquid chromatography; hplc, high performance liquid chromatography; pc, paper chromatography.

b-1-a, NeuAcα(2-6)GalNAc-ol.,
b-1-b, NeuAcα(2-6)[GlcNAcβ(1-3)]GalNAc-ol.,
b-2, Galβ(1-3)GlcNAcα/β(1-3)[NeuAcα(2-6)]GalNAc-ol.,
b-3, GlcNAcβ(1-3)Galα/β(1-3)[NeuAcα(2-6)]GalNAc-ol.,
b-4, Fucα(1-3)GlcNAcβ(1-6)[NeuAcα(2->3)Galβ(1-3)]GalNAc-ol.,
b-5, Galβ(1-3/4)[Fucα(1-3/4)]GlcNAcα/β(1-3)[NeuAcα(2-6)]GalNAc-ol.,
b-6, NeuAcα(2-3)Galβ(1-4)GlcNAcβ(1-6)[Fucα(1-2)Galβ(1-3)]GalNAc-ol.,

Fig. 4: Proposed structures for oligosaccharides b-1 to b-6.

β-*N*-acetylglucosaminidase removed GlcNAc residue. Periodate oxidation-borohydride reduction of b-3 eliminated NeuAc and GlcNAc, GalNAc-ol was converted to ThrNAc-ol and Gal was not affected. These results suggested that b-3 was a tetrasaccharide with the structure shown in Fig.4.

Oligosaccharide b-4.

Oligosaccharide b-4 had the molar sugar composition NeuAc:Fuc:Gal:GlcNAc:GalNAc-ol (0.8:0.7:0.9:0.8:1, relative to GalNAc-ol). Treatment of b-4 with neuraminidase removed sialic acid. Analysis of methylated sialo and asialo oligosaccharides showed the presence of terminal Fuc, 3-linked Gal, 3-linked GlcNAc, 3,6-substituted GalNAc-ol (Table 3), and NeuAc linked to C-3 of Gal. Sequential digestion of the oligosaccharide with neuraminidase, α-fucosidase and β-galactosidase removed residues of NeuAc, Fuc and Gal. Methylation analysis of enzyme-treated oligosaccharide suggested therein the presence (Table 3) of terminal GlcNAc and 6-linked GalNAc-ol. Periodate oxidation-borohydride-reduction of b-4 removed Fuc and NeuA, converted GalNAc-ol to ThrNAc-ol, Gal and GlcNAc were recovered unchanged. These data revealed that b-4 was a pentasaccharide with the structure shown in Fig.4.

Oligosaccharide b-5.

Oligosaccharide b-5 was homogeneous in pc and had the molar composition NeuAc:Fuc:Gal:GlcNAc:GalNAc-ol (0.7:0.8:0.9:0.8:1, relative to GalNAc-ol). Periodate oxidation-borohydride-reduction eliminated NeuAc, Fuc and Gal, GalNAc-ol was converted to ThrNAc-ol and GlcNAc was not affected. Methylation of the oligosaccharide showed the presence of terminal Fuc, NeuAc and Gal, 3,4-linked GlcNAc and 3,6-linked GalNAc-ol (Table 3). Treatment of b-5 with neuraminidase removed NeuAc, and subsequent methylation of the asialo-oligosaccharide showed that NeuAc was linked to C-6 of GalNAc-ol. Treatment of the asialo-oligosaccharide with β-galactosidase removed a galactose residue. These results provided evidence that oligosaccharide b-5 was a pentasaccharide with the structure given in Fig.4.

Oligosaccharide b-6.

Oligosaccharide b-6 had the molar composition NeuAc:Fuc:Gal:GlcNAc:GalNAc-ol (0.7:0.6:1.7:0.8:1, relative to GalNAc-ol). Periodate oxidation-borohydride-reduction degraded NeuAc, Fuc and a Gal residue, GalNAc-ol was converted to ThrNAc-ol, and a Gal and GlcNAc residues were resistant to oxidation. Methylation of b-6 showed the presence of terminal NeuAc and Fuc, 2-linked and 3-linked Gal, 4-linked GlcNAc and 3,6-linked GalNAc-ol. Treatment of the oligosaccharide with α-fucosidase and then with β-galactosidase removed fucose and Gal residues. Methylation of the residual oligosaccharide showed the presence of terminal NeuAc, 3-linked Gal, 4-linked GlcNAc and 6-linked GalNAc-ol. Treatment of the α-fucosidase

and β-galactosidase-treated oligosaccharide with neuraminidase removed NeuAc, and subsequent methylation of the residual oligosaccharide showed therein the presence of terminal Gal, 4-linked GlcNAc and 6-linked GalNAc-ol. Treatment of the asialo oligosaccharide with β-galactosidase removed a galactose residue. These results suggested the oligosaccharide b-6 to be a hexasaccharide with the structure shown in Fig.4.

In the present study the mucus was collected from several monkeys in the luteal phase of the menstrual cycle. The isolation of the secreted glycoproteins and their separation from other polymeric materials, namely enzymes and serum proteins, was readily accomplished by gel filtration on Bio-gel P-200. The glycoprotein component in the mucus was accompanied by several proteins as shown in the gel chromatography (Fig.1). The composition analysis of the proteins (data not shown) indicated variable amounts of cystine residues in these proteins, suggesting possibilities of inter-or intra-molecular disulphide linkages. The purified glycoprotein fractions had similar carbohydrate and amino acid composition. The main glycoprotein fraction in Sepharose 2B chromatography afforded two glycoproteins with similar carbohydrate and amino acid composition. The carbohydrate moiety of the two glycoproteins differred in Fuc, GalNAc, NeuAc and sulphate contents. Amongst the amino acids there was a noticeable similarity in the hydroxy amino acids. Fraction-2 glycoprotein exhibited a slight increase in cystine and basic amino acids. The main glycoprotein fraction, Fraction-1, was homogeneous in DEAE-cellulose chromatography. In gel electrophoresis it had the minimal entry, like the periovulatory phase glycoprotein, and showed the absence of contaminating proteins. The antibody against the main luteal phase glycoprotein, Sepharose Fraction-1, reacted with luteal phase mucus, Bio-Gel P-200 purified glycoproteins, Sepharose 2B glycoprotein fractions as well as DEAE-cellulose purified glycoprotein. In addition, this antibody also weakly reacted with ovulatory phase mucus, Bio-Gel P-200 and Sepharose 2B purified glycoproteins from the ovulatory phase, suggesting some common epitopes in glycoproteins of different phases of the ovulatory cycle, interalia the presence of molecules with similar structures during the cycle.

Despite the fact that there are morphological and rheological differences between the ovulatory phase and the luteal phase mucuses, the carbohydrate and amino acid composition of the glycoproteins from both mucuses were similar. There are minor variations in the amounts of carbohydrates as well as of amino acids. These minor variations in carbohydrates during the cycle have been observed in human mucus as well [4]. It has been proposed that functions, viscoelasticity and morphology of the mucus are reflected on the carbohydrate composition and /_ or in their structure, and that this relationship is unique for each individual [15]. Furthermore, it has been proposed [15] that mucin concentration and not composition changes in response to hormonal alteration during the menstrual cycle. The current findings on the purified and fractionated glycoprotein components are in line with these observations. There are number of proteins in the mucus in addition to glycoproteins in each phase of the menstrual cycle, and their functional role in conjunction with the glycoproteins has yet to be investigated. Variations in the protein contents, such as cystine and basic amino acids, may contribute significantly to the functions of the glycoproteins.

Alkaline-borohydride treatment of the glycoprotein, Sepharose Fraction 1, gave a mixture of oligosaccharides that was fractionated by gel filtration. An oligosaccharide fraction, Fraction b, obtained from Bio-Gel P-4, was furhter separated by hplc into six oligosaccharide fractions. Seven oligosaccharides, disaccharides to hexasaccharides, were characterized. The oligosaccharides had the following core structures :

i) Galβ(1-3)GalNAc-ol.

ii) GlcNAcβ(1-3)GalNAc-ol

iii) Galβ(1-4)GlcNAcβ(1-6)GalNAc-ol.

Elongation of the carbohydrate chains takes place on these core residues, resulting in the sturctures characterized as shown in Fig.4. The structures of sialylated oligosaccharide chains have been identified from human midcycle cervical glycoproteins [16], and these bear similarities to the core structure of the bonnet monkey midcycle

oligosaccharides [17,18]. The sialylated oligosaccharides from bonnet monkey midcycle cervical glycoproteins have been characterized [18]. The sialylated oligosaccharides from the luteal phase glycoprotein share core structures as well as some structural features with bonnet monkey and human midcycle glycoproteins. There are structural variations in the oligosaccharides of the luteal phase and midcycle glycoproteins. The core structure ii has neither been observed in the midcycle bonnet monkey glycoprotein oligosaccharide chains nor in human midcycle glycoproteins. Similarly, Fuc residues in the sialylated oligosaccharide chains of midcycle bonnet monkey mucus are mainly reported to be linked 1->2 to Gal [17],, whereas the luteal phase oligosaccharide chains have Fuc linked 1->2 to Gal as well as 1->3 to GlcNAc residues. The Fuc residues in sialylated oligosaccharides from human midcycle glycoprotein are solely linked 1->3/4 to GlcNAc [16]. The fucosyltransferases responsible for the transfer of fucose residues to position C-2 of Gal and C-3 of GlcNAc from the human cervical epithelium have been characterized [19]. The NeuAc residues in the luteal phase oligosaccharides have α2-> 3 linkage to Gal and α2->6 linkage to GalNAc-ol, a feature common with midcycle oligosaccharide chains [17,18] of the bonnet monkey as well as those of human [16].

Diverse core structures and variety of oligosaccharide chain lengths in mucin glycoproteins are known [20,21], and the heterogeneity in the oligosaccharide structure and chain length in the cervical mucus glycoproteins could be even wider because of changing physicochemical behaviour during the menstrual cycle.

Experimental

Materials

Bio-Gel P-2, Bio-Gel P-4, Bio-Gel P-200, AG50W-X8, AG1-X2 ion-exchange resins were purchased from Bio-Rad Laboratories, Sepharose 2B from Pharmacia Fine Chemicals, and exoglycosidases were purchased from Sigma Chemical (α-L-fucosidase from beef epididymis and emulsin; α-D-galactosidase from *Aspergillus niger*; β-N-acetylglucosaminidase from jack-bean) and Boehringer-Mannheim (neuraminidase from Vibrio cholerae and β-D-galactosidase from *E. coli*).

Collection of cervical mucus.

The cervical mucus of the bonnet monkey was collected by aspiration with a suction pump during the cycle. The bonnet monkey menstrual cycle was regular and similar to human [22,23]. The secretion was promptly frozen and was retained in the frozen state until use.

Analytical methods.

The hexose content from the column eluates was estimated by the phenol-sulphuric acid method [24], the protein content by measuring the absorbance at 280 nm or by using the procedure of Lowry *et al.* [25], and sialic acid by the thiobarbituric acid method [26], after mild acid hydrolysis with 50 mM sulphuric acid or by glc [27]. Pc was performed on Whatman No.1 paper in ethyl acetate:pyridine:acetic acid:water (5:5:1:3). All solutions were dialyzed in hydrogen carbonate-treated cellulose tubing at 4 °C.

Standard oligosaccharides.

The oligosaccharides NeuAcα(2-3)Galβ(1-3)GalNAc-ol and NeuAcα(2-3)Galβ(1-3)[NeuAcα(2-6)]GalNAc-ol were prepared from fetuin as previously described [28]. The oligosaccharides NeuAcα(2-6)GalNAc-ol, Galβ(1-3)[NeuAcα(2-6)]GalNAc-ol and Fucα(1-2)Galβ(1-3)[NeuAcα(2-6)]GalNAc-ol were prepared from bonnet monkey cervical mucus [17].

Isolation and purification of luteal phase mucus glycoproteins.

The crude luteal phase mucus was partially solubilized in 50 mM sodium phosphate, pH 7.0, containing 0.02% NaN_3. The cellular debris and other insoluble materials were removed by centrifugation (2500 rpm) at 4°C, the supernatant was dialyzed and the non diffusible material was lyophilized to give the crude mucus. This material (0.4 g) was dissolved in 50 mM sodium phosphate, pH 7.0, 120 ml, containing sodium azide (0.02%), by stirring overnight, at 4 °C. The solution was applied to a column (5x80 cm) of Bio-Gel P-200

(50-100 mesh). The column was washed with phosphate buffer (pH 7.0, 50 mM). The column eluate fractions containing carbohydrate and protein were pooled, the pH was adjusted to 5.5 with 4 M acetic acid, the solution was extensively dialyzed and lyophilized to give the partially purified mucus glycoproteins and proteins (Fig.1). The glycoprotein fractions were examined in agarose gel electrophoresis as previously described [9]. The partially purified glycoprotein (Fraction 1, 180 mg) in phosphate buffer (40 ml, pH 7.0) was applied to a column (2.5x70 cm) of Sepharose 2B. The column was washed with the phosphate buffer and fractions containing carbohydrates and proteins were combined and lyophilized. Two fractions, Fraction 1 and Fraction 2, were obtained (Fig.2). These fractions were examined in polyacrylamide-agarose gel electrophoresis as previously described [9]. The main fraction, Fraction 1 (100 mg), was further purified on a column (1.8x65 cm) of DEAE-cellulose. The column was washed with 100 mM NaCl followed by a gradient of 0.1 M to 1 M NaCl in 10 mM HCl. Antibodies to Fraction 1 glycoprotein were prepared as previously described [12].

Alkaline borohydride (borotritide) treatment.

The major glycoprotein fraction (Fraction 1, 60 mg) eluted from the Sepharose 2B column was treated with 2 M sodium borohydride in 50 mM sodium hydroxide (8 ml) for 18 h at 45 °C according to Iyer and Carlson [14]. The reaction mixture was adjusted to pH 5.4 with 4 M acetic acid. The [^{3}H]-labelled oligosaccharides were prepared by subjecting a portion of glycoprotein (7 mg) to β-elimination using NaB[^{3}H]$_4$ (5 mCi) under conditions described above. The two reaction mixtures were combined and desalted on a column (1.4x60 cm) of AG50W-X8 (H^+, 50-100 mesh). The oligosaccharide alditols were separated into neutral and acidic oligosaccharides on a column of AG1-X2 (OAc^- 200-400 mesh). The column was washed with water, 50 mM pyridine-acetic acid, pH 5.4, and then with 100 mM acetic acid. The acidic oligosaccharides were further fractionated on a column (1.8x90 cm) of Bio-Gel P-4 (200-400 mesh) into two main fractions, fraction "a" and fraction "b". Hplc of oligosaccharide fraction b [29], obtained from the Bio-Gel P-4 column, was performed on a Lichrosorb-NH_2 column (Fig.3). The oligosaccharides from the column were eluted with a gradient of acetonitrile-water (4:1 to 1:1) containing 2.5 mM ammonium hydrogen carbonate, for 60 min at a flow rate of 1.2 ml/min.

Treatment with glycosidases.

[^{3}H]-Labelled oligosaccharides were digested in 100 mM pyridine-acetic acid or 50 mM sodium citrate, pH 5.0, at 37°C for 24-72 h under a toluene atmosphere. Reactions were terminated by heating the enzyme containing solution in a boiling water-bath for 1 min, and the solutions were dried under vacuum. In the case of sodium citrate buffer, the solutions were desalted by gel filtration on Bio-Gel P-2.

Methylation analysis.

The oligosaccharide alcohols were methylated by the modified procedure of Hakomori [30]. The methylated oligosaccharide alditols were processed, and the methylated sugars were acetylated and identified by glc-ms as previously reported [18].

Periodate oxidation-borohydride reduction.

Oligosaccharide alcohols were oxidized in 50 mM sodium acetate (100 µl, pH 4.6) containing 50 mM sodium periodate. The reaction was performed for 6-10 h at 22 °C in the dark. Excess of periodate was consumed by the addition of ethylene glycol. Samples were treated with $NaBH_4$ at 22 °C for 30 min. The excess of borohydride was destroyed and the mixture was desalted simultaneously by the addition of AG50W-X8 (H^+) resin. The solution was filtered and the resin washed with methanol. The combined eluates were dried in vacuo. The residue was treated with 0.25 M H_2SO_4 for 2.5 h and deionized with AG1-X8 (OAc^-, 100-200 mesh) resin. The products were examined by glc in comparison with standards as reported earlier [18].

Acknowledgement

Nasir-ud-Din thanks the National Scientific Research Development Board, Pakistan, for partial financial support.

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