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STRUCTURAL STUDIES ON ONCOFETAL CARBOHYDRATE ANTIGENS (CA 19-9, CA 50, AND CA 125) CARRIED BY *O*-LINKED SIALYLOLIGOSACCHARIDES ON HUMAN AMNIOTIC MUCINS*,†

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ABSTRACT

Mucins were extracted from human amniotic fluid in the presence of 45% vol. phenol and separated from the bulk of smaller-sized glycoproteins by exclusion on Sephacryl S400. The mucin-fraction FW, which still contained a minute proportion of mannose, strongly expressed oncofetal antigens recognized by monoclonal antibodies C 50, NS 19-9, OC 125, Leu M1, 49 H 8, and 115 C 2. The structures of the respective mucin-linked saccharides responsible for Ca 50-, Ca 19-9-, and Le^a-related antigenic activities were analyzed before or after reductive β-elimination from sialylglycoproteins, and purification of the derived alditols by gel-permeation chromatography on Bio-Gel P-4 or high performance liquid chromatography. Two ubiquitous (FW2, FW3) and three novel oligosaccharide alditols (FW5) were characterized by f.a.b.- and e.i.-m.s., combined with methylation analysis and chromium trioxide oxidation. The OC 125 epitope on mucin-carried *O*-glycans was destroyed during reductive cleavage of the saccharides, indicating a conformational involvement of the reducing terminal residue and its mode of conjugation to the protein. Exoglycosidase treatment of the mucin-bound antigen revealed that the epitope structure of OC 125 includes terminal β-D-galactosyl groups, and terminal sialyl groups that are almost inaccessible to *Vibrio cholerae* sialidase digestion.

INTRODUCTION

Since the early investigations of Morgan, Watkins, and Kabat[1,2], it is known that the pathological fluid from ovarian cysts, but also normal human body fluids like saliva or seminal plasma, are potent sources of blood group active mucins. The

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pioneering chemical work of these investigators resulting in the complete structural elucidation of the A, B, H, and Lewis blood group determinants[3-5] has provoked increasing interest in the structural and functional aspects of carbohydrates carried by mucins. This class of glycoconjugates further attracted attention since it became evident that mucins express a vast number of oncofetal and developmental antigens, which may represent incompletely synthesized blood group antigens or derivatives thereof originating from neosynthesis[6-9]. It has been demonstrated also that serum mucins, shed by tumor cells, represent the relevant antigens in the diagnosis of gastrointestinal cancer[10] by use of hybridoma-derived antibodies to a sialylated Lewis[a] blood-group epitope[11]. Surprisingly, this and other tumor-associated carbohydrate antigens could also be detected in normal body fluids as structural components of mucins[12-14].

Mucins from human amniotic fluid, which had been previously characterized by Lambotte and Uhlenbruck[5] to lack blood group activities, were shown by immunochemical or chemical analyses to express tumor-associated antigens Ca 125 (refs. 13, 16), sialyl-Le[a] (ref. 13), and sialyl-X (Ref. 17). Whereas the structures of those antigens carried by glycosphingolipids have well been established[11,18,19], information on the respective mucin-linked structures is rare[12,14,17]. We present herein chemical evidence for the expression of sialyl-Le[a] antigen on nona- or decasaccharides derived from human amniotic mucin, as well as preliminary information on the structure of Ca 125 antigen carried by amniotic mucins.

EXPERIMENTAL

F.a.b.–e.i. mass spectrometry. — F.a.b.–mass spectrometry was performed on a VG analytical ZAB-HF reversed-geometry mass spectrometer. Spectra of permethylated oligosaccharide alditols were recorded in the positive-ion mode[20]. After the target had been coated with sodium acetate, the sample (~5 μg of carbohydrate derivative dissolved in 1 μL of methanol) was added to the thioglycerol matrix. E.i.–mass spectra of methylated alditols were recorded at 70 eV with a CH5 MAT (Finnigan) spectrometer using the parameters described previously[12].

Analytical procedures. — Monosaccharides were analyzed by g.l.c. as trimethylsilyl derivatives of the corresponding methyl glycosides[21]. Methylation analysis of the purified sialyloligosaccharide alditols was performed according to Stellner *et al.*[22] with minor modifications. The partially methylated alditol acetates were analyzed by g.l.c.–m.s. using a fused-silica capillary column (17 m), wall-coated with OV-1, which was heated from 130 to 280° (10°/min). Chromium trioxide oxidation of bis(sialyl)oligosaccharide alditols (fraction FW-DS) was performed according to Fukuda *et al.*[23].

Preparative methods. — Preparation of mucins from amniotic fluid and other human body fluids including phenol extraction and chromatography on Sephacryl S400 has been described in detail by Hanisch *et al.*[13,14]. Reductive β-elimination of alkali-labile bound carbohydrates from sialylglycoproteins[12] and chromatographic

purification by ion-exchange chromatography on DEAE–Sephadex[14] and by h.p.l.c. on aminopropyl silicate were performed as described earlier[14]. The acidic oligosaccharides were fractionated according to their size by gel-permeation chromatography on a 1.5 × 150-cm column of Bio-Gel P-4 (200–400 mesh) in 50mM pyridinium acetate, pH 5.0. Two water-jacketed columns heated at 55° were used in series, the first in an upward, and the second in a downward flow which were maintained at 0.5 mL/min by a high-pressure pump. The eluate was monitored continuously by refractive-index detection or colorimetrically with the phenol–H_2SO_4 method on 2-mL fractions.

Immunoassays. — The quantitative measurement of Ca 19-9 and Ca 125 antigens was performed as a solid-phase radioimmunoassay, based on the "forward sandwich" principle (Centocor, Malvern, PA, U.S.A.), and analysis of Ca 50 activity with a commercial kit from Behringwerke AG (Marburg, FRG). Inhibition studies in the same systems (Ca 19-9, Ca 125) were carried out by incubation of the antibody-coated beads with antigen [1 mg mucin/mL of 20mM phosphate buffer–0.15M NaCl, pH 7.2, containing 5% (w/v) of bovine serum albumin], in the presence of carbohydrate fractions (1 mg) or purified alditols (0.1 mg) at 37°.

Quantitative analysis of Leu M1 (Becton–Dickinson, Mountain View, U.S.A.), FH 3, FH 6 (kindly provided by Dr. S. Hakomori, Fred Hutchinson Cancer Research Center, Seattle, Washington, U.S.A.), 115 C 2 (kindly provided by Dr. J. Hilkens, Nederlands Kankerinstituut, Amsterdam, Netherlands), and 49 H 8 (kindly provided by Dr. B. M. Longenecker, University of Alberta, Edmonton, Alberta, Canada) binding activities was performed as enzyme immunoassay on Immulon plates (Dynatech, Plochingen, F.R.G.) according to Voller *et al.*[24].

Exoglycosidase digestion. — Mucins from human amniotic fluid (5 mg/mL) were incubated with one of the following exoglycosidases at 37°: Jack bean β-D-galactosidase (0.1 unit) in 0.1M citrate–phosphate buffer (pH 3.5); *E. coli* β-D-galactosidase (1 unit) in 0.02M phosphate buffer (pH 7.2)–0.15M NaCl; *Vibrio cholerae* or *Clostridium perfringens* sialidase (0.1 unit) in 0.1M acetate buffer (pH 5.5)–0.15M NaCl–9mM $CaCl_2$; bovine kidney α-L-fucosidase (0.05 unit) in 0.05M acetate buffer (pH 5.0); and bovine kidney *N*-acetyl-β-D-glucosaminidase (0.05 unit) in 0.1M citrate–phosphate buffer (pH 6.0).

Chemical treatments of mucus glycoproteins. — Partial periodate oxidation of the mucin-bound carbohydrate (5 mg PE-FW) was performed in 0.1M acetate buffer (2 mL), pH 4.5, containing 10mM $NaIO_4$ for 1 h at 25°. The reaction was stopped by addition of 1,2-ethanediol (50 μL).

For β-elimination of the *O*-glycosyl-linked carbohydrate chains by mild alkali treatment, the mucus preparation was incubated with (a) 1:1 (v/v) 0.17M KOH–dimethyl sulfoxide, or (b) 0.5M NH_3 for 2 h at 40°. The reaction was terminated by titration to pH 5 with (a) 0.5M HCl or (b) by freezing and lyophilization.

N-Acetylneuraminic acid was selectively hydrolyzed from the mucin-bound carbohydrates by incubation for 0.5 h at 100° with dilute acetic acid (pH 3.5). The chemically modified mucus preparations were dialyzed for 18 h at 4° against

phosphate-buffered 0.9% NaCl solution, and analyzed radiometrically for Ca 125 activity.

O-Deacetylation of mucin-bound *N*-acetylneuraminic acid was achieved by incubation of mucin (1 mg/mL) for 18 h at 4° with 4% (v/v) triethylamine in 50% (v/v) aqueous methanol.

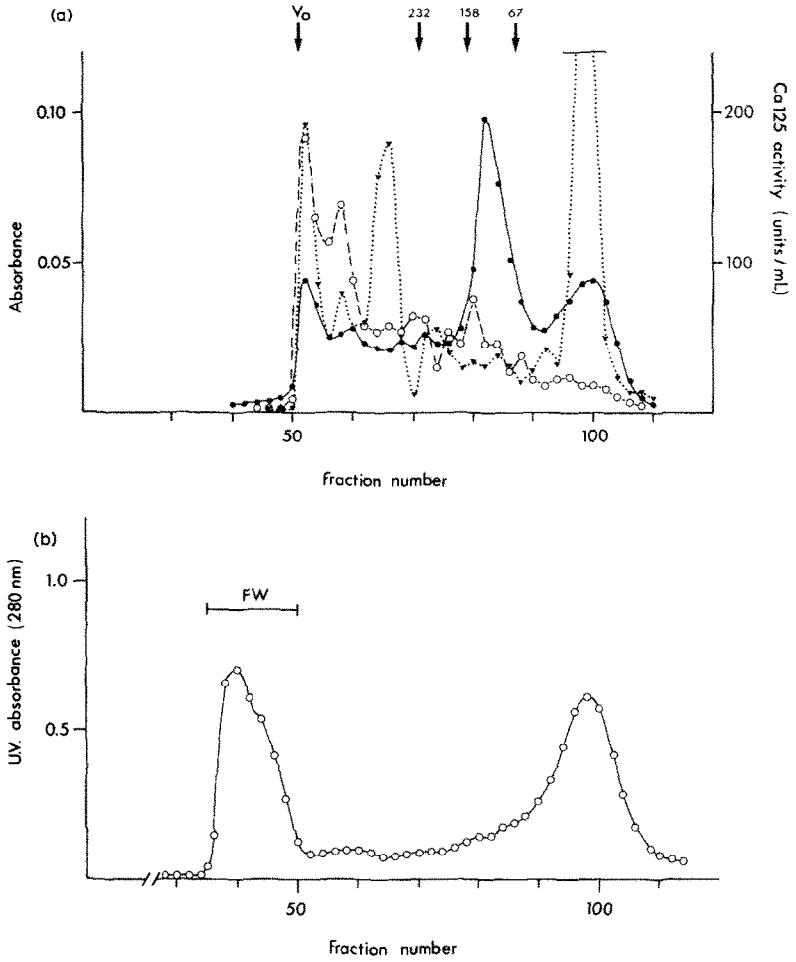


Fig. 1. Gel-permeation chromatography of sialylglycoproteins (Fraction PE-FW): (a) The sialylglycoproteins (25 mg) were chromatographed on a 2.5 × 100-cm column of Sephacryl S300 in 0.1M ammonium acetate, pH 4.35. (b) The sialylglycoproteins (160 mg) were chromatographed on a 2.5 × 100-cm column of Sephacryl S400 in 4M guanidium chloride, mM sodium EDTA, and mM sodium phosphate, pH 7.0. The columns were run at a flow rate of 24 mL/h, fractions (4 mL) were collected and analyzed: (a) by protein determination according to Lowry (—●—), by the phenol-H_2SO_4 method (···▼···), or by radioimmunoassay of Ca 125 activity (0.2-mL samples in 20mM phosphate and 0.15M NaCl corresponding to 1 mL of the eluate) (--○--), or (b) spectrophotometrically.

RESULTS

Preparation of amniotic mucin. — From 800 mL of cell-free amniotic fluid, 0.5 g of sialylglycoproteins (fraction PE-FW) were recovered after treatment of the liquid with hot 90% (v/v) phenol. The mucins in this fraction were separated from smaller-sized, mannose-containing glycoproteins by gel permeation chromatography on Sephacryl S400 or Sephacryl S300 (Fig. 1). The high-molecular-weight material (fraction FW) excluded from Sephacryl S400 comprised ~23% (w/w) of the total sialylglycoproteins contained in fraction PE-FW. According to monosaccharide analysis, the mucins in fraction FW still contained minute proportions of mannose (4.5%, w/w, of total carbohydrates constituting on their part 72.4%, w/w, of mucin) which is assumed to be not a regular constituent of this class of glycoconjugates (Table I). Concomitant with the purification process, the activities of a series of oncofetal antigens were determined by use of monoclonal antibodies in radio- or enzyme-immunoassays (Fig. 1). Increase of specific activities by a factor of ~3 measured for fraction FW after separation of non-mucin-type glycoproteins (specific Ca 19-9 activity of Fraction FW, 23 500 units/mg) suggested that the respective carbohydrate antigens occur on mucin-carried *O*-glycans[13]. Qualitative results (Table II) indicated that amniotic mucins express tumor-associated carbohydrate antigens T, X, sialyl-Le^a (Ca 19-9), Ca 50, Ca 125, and a Le^a related structure (Le^a-X). A qualitative comparison with the activities measured for mucins from other normal and pathological body fluids (seminal plasma and tumor ascites fluid) revealed the marked "embryonic character" of the amniotic material.

Isolation and purification of bis(sialylated) saccharide alditols. — Acidic carbohydrates isolated by base–borohydride treatment of sialylglycoproteins (Fraction PE-FW) were separated from neutral carbohydrates (0.01M pyridinium acetate, Fraction FW-N), and fractionated into monosialyl- (0.1M pyridinium acetate, Fraction FW-MS) and bis(sialyl)oligosaccharide alditols (0.5M pyridinium

TABLE I

MONOSACCHARIDE ANALYSIS OF MUCIN FRACTION FW[a]

Sugar	*Weight (μg)*	*% w/w*	*Molar proportion relative to GalNAc*
Fuc	166.7	23.0	3.6
Man	32.8	4.5	0.6
Gal	234.1	32.4	4.6
GalNAc	61.3	8.5	1.0
GlcNAc	158.1	21.8	2.5
NeuAc	70.7	9.8	0.8
Total	723.7	100	

[a]Sugars derived from 1 mg of sample were analyzed as their methyl glycosides, after trimethylsilylation, by g.l.c. chromatography on a fused-silica capillary column (30 m) coated with RSL 300 (Alltech, Unterhaching, F.R.G.).

TABLE II

ONCOFETAL ANTIGENS ON SECRETED MUCINS FROM HUMAN BODY FLUIDS

Monoclonal antibody	*Oncofetal antigen*	*Structure*	*Mucins derived from*		
			Seminal plasma	*Amniotic fluid*	*Tumor ascites fluid*
49 H 8[a]	T	**1**	+	+	+
OC 125[b]	Ca 125	[c]	−	+	+
C 50[b]	Ca 50	**2**	+	+	+
NS 19-9[b]	Ca 19-9 (sialyl Le[a])	**3**	+	+	+
Leu M1[a]	X-antigen	**4**	+	+	(+)
F H 3[a]	X-antigen	**5**	−	−	−
F H 6[a]	Sialyl dimeric X-antigen	**6**	−	−	−
115 C 2[a]	Le[a]-X-antigen	**7**	−	+	−

[a]Binding of monoclonal antibodies was analyzed by quantitative enzyme immunoassay on Immulon plates precoated with the mucin preparation (0.03–5 μg). Assays were considered positively when 1 μg of mucin yielded an absorbance of at least 0.1 extinction unit. [b]Activity of mucin preparations (1–100 μg) was analyzed in commercial radioimmunoassays. Assays were considered to be positive when the specific activity exceeded 10 standard units per 0.1 mg. Mucins were prepared from pooled human body fluids as described in the Experimental section. Tumor ascites fluid was obtained from patients suffering from gastric adeuocarcinoma. [c]Structure unknown.

acetate, Fraction FW-DS) by stepwise elution from a DEAE–Sephadex A-25 column. The yield from 200 mg of sialylglycoproteins was 57 mg of Fraction FW-N, 17 mg of Fraction FW-MS, and 40 mg of Fraction FW-DS. Fraction FW-DS was further chromatographed on Bio-Gel P-4 in the low pressure or on aminopropyl silicate in the high pressure mode, or both (Fig. 2). As judged from the elution profile of the carbohydrates from the Bio-Gel P-4 chromatography, the fraction of bis(sialyl)oligosaccharide alditols comprises six major subfractions, separated on the basis of size exclusion in a range from 6–30 hexose units. Subfractions 1–3 contained significant proportions of mannose and, as may be inferred from f.a.b.–mass spectrometry, are composed preponderantly of complex-type *N*-glycans, which probably have been reductively cleaved during base–borohydride treatment. The smaller-sized components in Fractions 4–6 lacked mannose, but contained significant proportions of 2-acetamido-2-deoxygalactitol, which is a constituent of mucin-type *O*-glycans.

A final purification of oligosaccharide alditols contained in subfractions 3–6 was achieved by high performance liquid chromatography. The correspondence of Fractions FW2–FW6 and subfractions from the Bio-Gel P-4 chromatography is illustrated in Fig. 2. The major oligosaccharide alditols of Fractions FW2, FW3, and FW5 were methylated and subjected to structural analysis.

Partial chemical and enzymic characterization of Ca 125 antigen of amniotic mucin. — As previously shown for the antigen detected in human milk[13], the Ca

β-D-Gal*p*-(1→3)-D-GalNAc
1

α-NeuAc*p*-(2→3)-β-D-Gal*p*-(1→3)-D-GlcNAc
2

α-NeuAc*p*-(2→3)-β-D-Gal*p*-(1→3)-D-GlcNAc
4
↑
1
α-L-Fuc*p*
3

β-D-Gal*p*-(1→4)-D-GlcNAc
3
↑
1
α-L-Fuc*p*
4, 5

α-NeuAc*p*-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc
3 3
↑ ↑
1 1
α-L-Fuc*p* α-L-Fuc*p*
6

β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc
4 3
↑ ↑
1 1
α-L-Fuc*p* α-L-Fuc*p*
7

125 antigen of amniotic mucin may be located on a sialyloligosaccharide *O*-glycosyl-linked to protein. Several chemical evidences support this classification, *i.e.*, partial destruction of the carbohydrate chain by mild periodate oxidation, β-elimination of the carbohydrate chains by mild alkali treatment under conditions that are known not to affect the structural integrity of the protein, (Table III). More specific information on the structure of the antigen was obtained by enzymic digestion with various exoglycosidases (Table III): (a) the nonreducing, unsubstituted β-D-galactosyl group was accessible to digestion by β-D-galactosidases from jack bean and *E. coli* and is part of the epitope structure of OC 125; (b) an *N*-acetyl-α-neuraminosyl group is linked to an internal sugar residue of the carbohydrate chain, as only *Clostridium perfringens* sialidase significantly decreased the antigen activity, whereas the *Vibrio cholerae* enzyme [which is known to attack predominantly terminal (2→3)-linked *N*-acetyl-α-neuraminosyl groups] had only little effect (Table III); and (c) α-L-fucosyl and 2-acetamido-2-deoxy-β-D-glucosyl residues, if present at all, were not accessible to enzymic cleavage by the respective exoglycosidases from bovine kidney, or may not be involved in the epitope structure of the antibody. Inhibition studies with the mucin-derived oligosaccharides (FW-N, FW-MS, FW-DS) showed that reductive cleavage eliminates Ca 125 antigenic activity, which points to a possible involvement of carbohydrate and protein in the maintainance of the conformation of the antigenic determinant. Loss of Ca 125 activity during

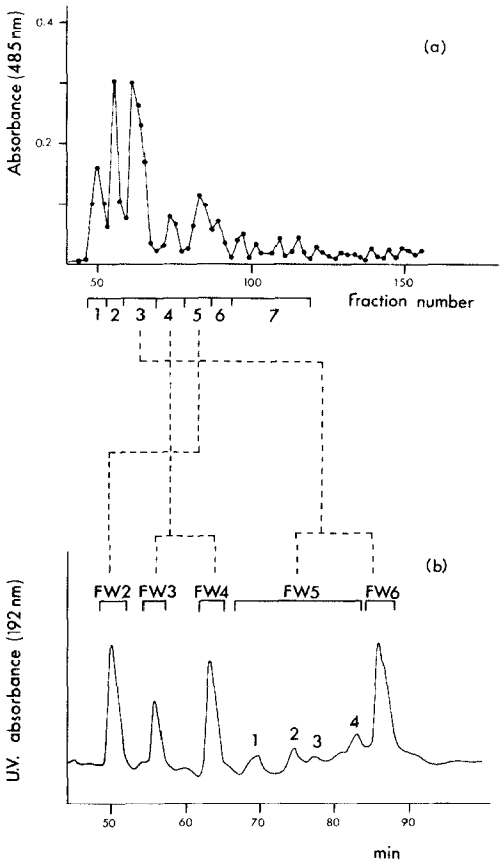


Fig. 2. Fractionation of acidic oligosaccharide alditols (Fraction FW-DS): (a) On Bio-Gel P-4: at low pressure; for chromatographic conditions, see Experimental section. (b) On aminopropyl silicate, at high pressure; gradient elution was performed with CH_3CN–15mM KH_2PO_4 (pH 4.7), starting from 30% (v/v) of aqueous phase up to 80% (duration 100 min).

alkali–borohydride treatment, by *O*-deacetylation of the sialic acid residue could be excluded (see Table III).

Structural analysis of the major bis(sialyl)oligosaccharide alditols. — L.c.-fraction FW2. The monomer composition of the oligosaccharide alditol FW2 could be deduced from the monosaccharide analysis and the pseudomolecular ions in the f.a.b.–mass spectrum (Table IV). Homogeneity of the sample was indicated by the absence of alternative major signals in the region of pseudo-molecular ions, although some degradation during the workup and derivatization procedures may have occurred, as evidenced by the ion (M − NeuAc) at *m/z* 873 (Fig. 3). The sequence of residues was obtained from the fragmentation patterns in the f.a.b.- and e.i.-mass spectra. In both, the nonreducing, terminal sequence was represented by intense primary ions at *m/z* 376 and 580 indicating a terminal monosaccharide group (NeuAc) and a disaccharide unit (NeuAc–Hex), respectively. The major ion

TABLE III

CHEMICAL AND ENZYMIC CHARACTERIZATION OF CA 125 ANTIGEN FROM AMNIOTIC MUCIN

Chemical treatment	*Conditions*	*Time of incubation (h)*	*Recovery of Ca 125 activity (%)*
Mild alkali	0.5M NH_3, 40°	2	52
Mild alkali	0.17M KOH/Me_2SO, 40°	2	0
Mild periodate oxidation	0.01M $NaIO_4$, 25°	1	0
O-Deacetylation	4% (v/v) Triethylamine, 4°	20	90
EXOGLYCOSIDASE TREATMENT			
α-D-Sialidase *(Vibrio cholerae)*		3	90
α-D-Sialidase *(Clostridium perfringens)*		3	6
α-L-Fucosidase (bovine kidney)		3	99
β-D-Galactosidase (Jack bean)		3	58
β-D-Galactosidase *(E. Coli)*		3	46
N-Acetyl-β-D-glucosaminidase (bovine kidney)		3	100

from the (potential) reducing-end was observed at *m/z* 637, which indicated a substitution of the terminal alditol residue by a sialyl group. The substitution pattern of the 2-acetamido-2-deoxygalactitol residue became evident from a series of intense alditol fragments registered in the e.i.-mass spectrum at *m/z* 739, 783, and 406. These data and the results of the methylation analysis (Table IV) suggested substitution of the terminal alditol residue at O-3 and O-6, establishing the structure of the FW2 oligosaccharide alditol as **8**.

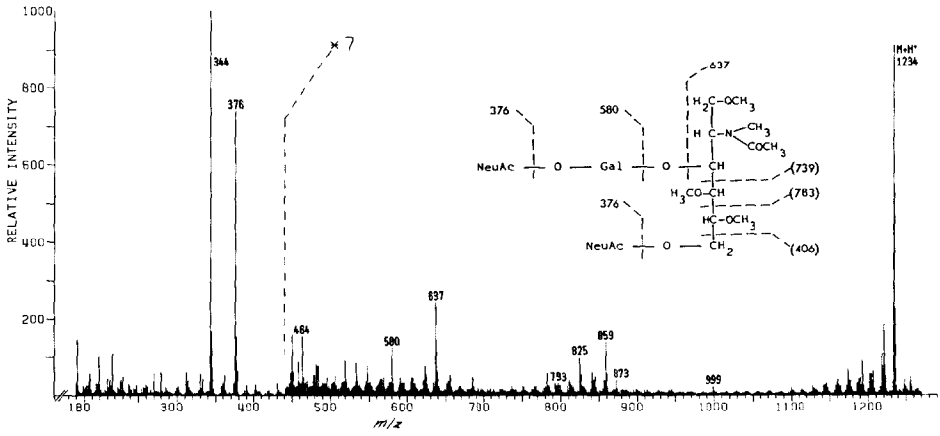


Fig. 3. F.a.b.-mass spectrum of permethylated compound FW2 recorded in the positive-ion mode (in the absence of added sodium ions). Major sequence ions are indicated in the fragmentation scheme. Fragments observed only in e.i.-m.s. are given in parentheses.

TABLE IV

METHYLATION ANALYSIS OF OLIGOSACCHARIDE ALDITOLS IN FRACTIONS FW2 AND FW3[a]

O-*Methyl derivatives*	*Oligosaccharide alditol*	
	FW2	*FW3*
2,3,4,6-Me_4-Galol		
2,4,6-Me_3Galol	+	+
1,4,5,6-Me_4-GalNAc(NMe)ol		
1,4,5-Me_3-GalNAc(NMe)ol	+	+
3,6-Me_2-GlcNAc(NMe)ol	(+)	+
4,6-Me_2GlcNAc(NMe)ol		
6-Me-GlcNAc(NMe)ol	(+)	(+)
3-Me-GlcNAc(NMe)ol	(+)	(+)

[a]Derivatives detected in minor quantities [<10% of 1,4,5-Me_3-GalNAc(NMe)ol registered by total ion detection] are indicated in parentheses.

NeuAc*p*-(2→3)-D-Gal*p*
1
↓
3
NeuAc*p*(2→6)-D-GalNAcol

8

L.c.-fraction FW3. The f.a.b.-mass spectrum obtained for Fraction FW3 revealed no indication for a significant heterogeneity on inspection of the high-mass range. The only signals in that range which fit the masses of pseudomolecular ions ($M + H^+$) and ($M + Na^+$) were observed at *m/z* 1683 and 1705, respectively (Fig. 4), representing a hexasaccharide of the calculated composition 2 NeuAc, 2 Hex, 1 HexNAc, and 1 HexNAcol (Table V). In the lower mass range, a series of primary

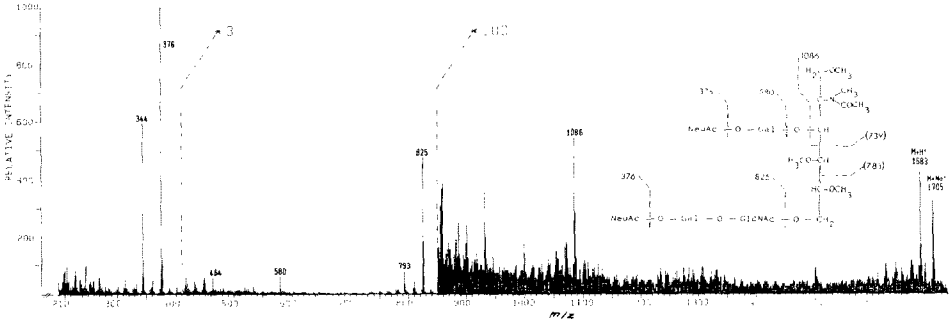


Fig. 4. F.a.b.-mass spectrum of permethylated compound FW3 recorded in the positive-ion mode (in the absence of added sodium ions). Major sequence ions are indicated in the fragmentation scheme. Fragments observed only in e.i.-m.s. are given in parentheses.

TABLE V

DATA FROM POSITIVE ION F.A.B.- AND E.I.-M.S., AND SUGAR COMPOSITION OF OLIGOSACCHARIDE ALDITOLS IN FRACTIONS FW2, FW3, AND FW5

L.c. fractions	*Major sequence ions in f.a.b.- or e.i.-m.s.*[a] (m/z) *from the*		*Pseudomolecular ion* $M + H^+$ *and* $(M + Na^+)$[a] (m/z)	*Monosaccharide composition*[b]				
	Nonreducing end group	*Reducing end group*		*Fuc*	*Gal*	*GalNAcol*	*GlcNAc*	*NeuAc*
FW2	376, 580	637 (739, 783)	1234 (1256)		1	1		2
FW3	376, 580, 825	1096 (739, 783)	1683 (1705)		2	1	1	2
FW5-1 (minor)	376, 580, 825, 1274		2132 (2154)		3	1	2	2
FW5-1 (major)	376, 580, 999, 1448	1723	2306 (2328	1	3	1	2	2
FW5-2	376, 580, 999, 1622		2480 (2502)	2	3	1	2	2

[a]In parentheses. [b]Based on pseudomolecular ions in f.a.b.-m.s. and on chemical analysis.

ions at *m/z* 376, 580, and 825 may be attributed to fragments from the nonreducing terminal sequence which indicate a disaccharide (NeuAc→Hex) and a trisaccharide unit (NeuAc→Hex→HexNAc). The e.i.-mass spectrum (not shown) exhibited a similar fragmentation pattern but, moreover, yielded information on the branching of the terminal HexNAcol residue by lacking the primary fragment ion at *m/z* 276, which would indicate a monosubstitution, and by showing a series of alditol fragments at *m/z* 739 and 783 (Table V), which point to the presence of a disaccharide unit NeuAc→Hex linked to O-3 of the alditol. In conjunction with the results of methylation analysis, which gave 2-acetamido-1,4,5-tri-*O*-acetyl-3,6-di-*O*-methylglucitol as the major GlcNAc derivative (Table IV), the relative preponderance of the secondary ion at *m/z* 182 over that at *m/z* 228 reflects the predominant existence of a Type 2 chain in the sequence NeuAc→Hex→HexNAc. From the combined data of f.a.b.- and e.i.-m.s. in connection with g.l.c.–m.s. analysis of partially methylated alditol acetates and monosaccharide analysis, structure **9** was postulated for FW3 saccharide alditol.

NeuAc*p*-(2→3)-D-Gal*p*-(1→4)-D-Glc*p*NAc-(1→6)-D-GalNAcol
3
↑
NeuAc*p*-(2→3)-D-Gal*p*

9

L.c.-fraction FW5. As may already be expected from the l.c. profile, Fraction FW5 exhibited a complex picture in f.a.b.–m.s. (Fig. 5). The observed pseudomolecular ions of major prominence suggested that part of the fraction represents a mixture of closely related octa- to deca-saccharide alditols derived from mucin-linked *O*-glycan chains, each deriving from another through successive additions of deoxyhexose residues [$(M + Na^+)$ at *m/z* 2154, 2328, and 2502]. Another series of signals in the pseudomolecular range, but of lower intensities, probably originated from a complex-type *N*-glycan devoid of deoxyhexose residues and appearing as three molecular compounds [$(M + Na^+)$ at 2562, 2603, and 2807]. The smaller-mol. wt. species, may represent biosynthetic precursors or *in vivo* degradation products (loss of terminal HexNAc or Hex groups) of the larger-mol. wt. molecule, calculated to be composed of 2 NeuAc, 5 Hex, 3 HexNAc, and 1 HexNAcol. The presence of minor amounts of mannose in Fraction FW5 may be regarded as the principal support of partial *N*-glycan cleavage.

A subfractionation of oligosaccharides from Fraction FW5 revealed that the presumed *N*-glycan-derived alditols were contained in Peaks FW5-3 and FW5-4 (Fig. 2), and the presumed *O*-glycan-derived compounds in Subfractions FW5-1 and FW5-2. F.a.b.–mass spectra obtained for these subfractions allowed the unambiguous assignment of the major fragment signals to the respective compounds in FW5-1 and FW5-2 (Table IV). The minor saccharide alditol of Fraction FW5 represented by the pseudomolecular ion at *m/z* 2154 and shown previously to

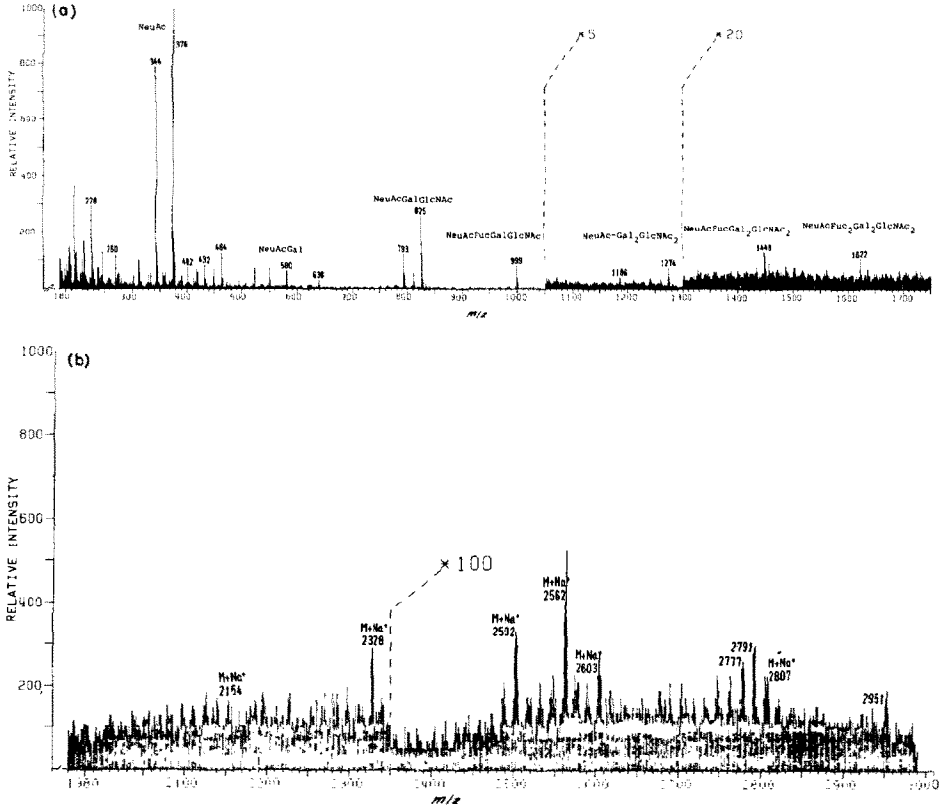
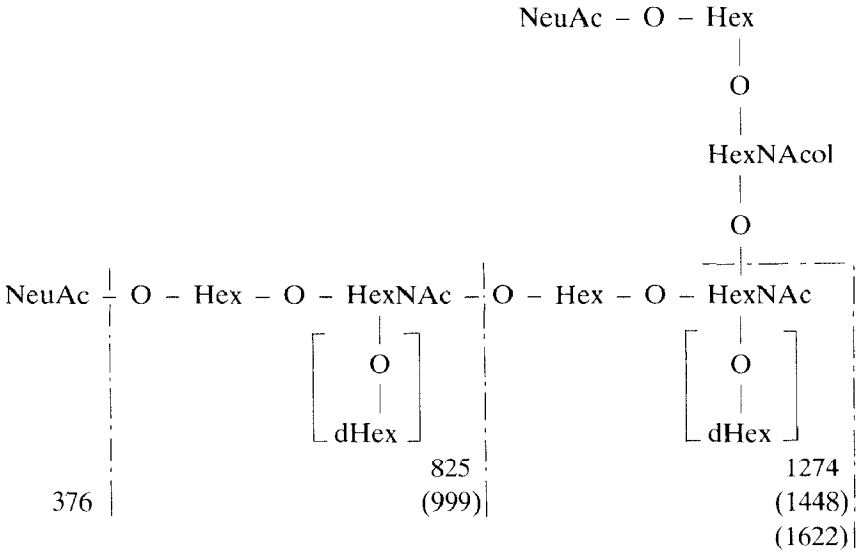


Fig. 5. F.a.b.-mass spectrum of permethylated oligosaccharide alditols in fraction FW5 recorded in the positive-ion mode. Structural elements represented by major sequence ions are shown in the spectrum: (a) The range up to *m/z* 2000 was recorded in the absence of sodium ion, (b) the spectrum of ions above *m/z* 2000 in the presence of added sodium ions.

cochromatograph mainly with Fraction FW4 (ref. 17) was accordingly characterized by the sequence ions at *m/z* 825 and 1274. Similarly the major compounds in Subfractions FW5-1 and FW5-2 [having (M + Na^+) at *m/z* 2328 and 2502, respectively], were assigned to the sequence ions listed in Table V. From these data, the structure models for three related saccharide alditols may be derived from the fragmentation diagram illustrated in Scheme 1.

The terminal sequence NeuAc→Hex→HexNAc, common to all three saccharide alditols, is assumed to be a Type 1-based carbohydrate chain, as judged from the relative abundance of secondary ions at *m/z* 228 (derived from the primary ion at *m/z* 825) and at *m/z* 402 (derived from the corresponding ion of the fucosylated compound at *m/z* 999) in f.a.b.- and e.i.-mass spectra. Furthermore, evidence has been obtained in inhibition studies with fraction FW5 that the nonreducing terminal sequences of these oligosaccharides fit the epitope structure of NS 19-9 antibody[11]. In accordance with this observation, the amniotic mucin did not bind F



Scheme 1.

H 6 (Table II), which recognizes a repetitive Type 2 chain (sialylated dimeric X antigen)[25], but strongly 115 C 2 antibody (Table II), which reacts with a difucosylated backbone hybrid chain having a Type 1 and Type 2 structure (Le^a-X antigen[26]). The presence of this structure on the amniotic mucin was confirmed by the inhibition of 115 C 2-binding by mucin-derived alditols from Fraction FW-DS (Fig. 6); 50% of 115 C 2-antibody binding was inhibited by less than 0.1 nmol of desialylated FW5-2 oligosaccharide alditol, which is comparable to the inhibitory activity of lacto-*N*-difucohexaose II (ref. 26). The results suggest structures **10** and **11** for the oligosaccharides of Fraction FW5-1 and FW5-2, respectively.

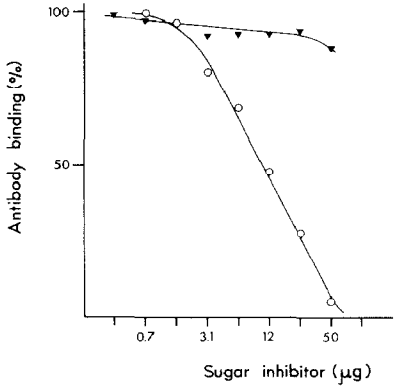
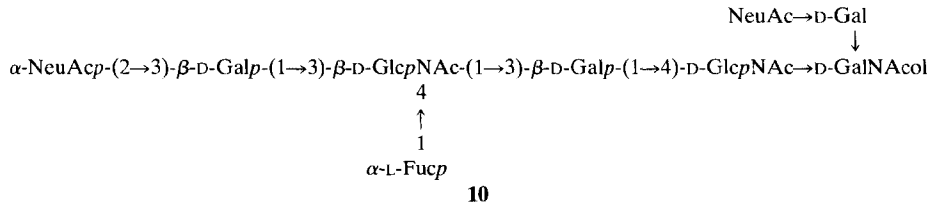


Fig. 6. Binding inhibition of monoclonal antibody 115 C 2 by mild acid-treated carbohydrate fractions. Serial twofold dilutions of 50 μg of sugar inhibitor per well were used after desialylation of the mucin-derived oligosaccharide alditols by mild acid treatment (pH 3.5, 100°, 15 min): (—▼—) sialylated carbohydrates from mucins of human seminal plasma; (—○—) bis(sialyl)ated carbohydrates from mucins of human amniotic fluid.

Anomeric configuration of glycosidic bonds. — Unambiguous determination of the anomeric configuration of the terminal *N*-acetylneuraminic acid groups was based on exoglycosidase digestion with sialidases from *Vibrio cholerae* and *Clostridium perfringens*. Both enzymes were able to convert the bis(sialyl)ated oligosaccharide alditols of Fractions FW2–FW6 into the corresponding asialo derivatives as judged by l.c. analysis. Accordingly, *N*-acetylneuraminic acid groups were presumed to be linked α-glycosidically to the subterminal sugar residues. The α-L-anomeric configuration of fucose and α-D of mannose, the latter being detected in



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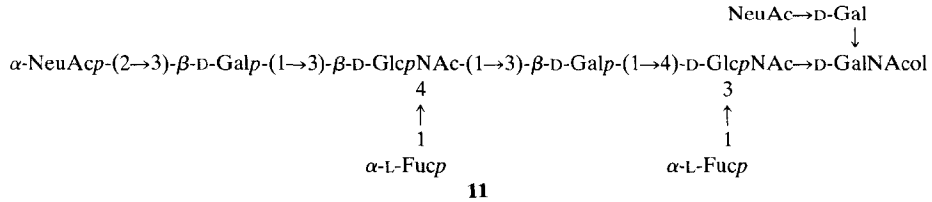

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significant proportions in Fraction FW-DS, were established by CrO_3 oxidation of the acetylated oligosaccharide alditols. Comparison of treated and untreated samples by g.l.c. analysis revealed that both sugar components were resistant to oxidation. On the other hand, galactose and 2-acetamido-2-deoxyglucose were oxidized quantitatively under the same conditions, suggesting a β-D configuration for these sugar residues.

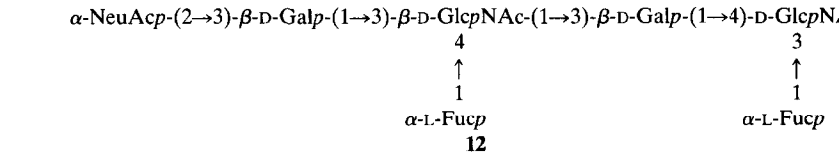
DISCUSSION

The structures of mucin-carried epitopes are difficult to elucidate owing to the heterogeneity of the multiple carbohydrate chains found on these glycoconjugates and to a possible elimination of the antigenicity by reductive cleavage of the oligosaccharides. This elimination hampers or totally forestalls the molecular assignment of antigenic specificities to the corresponding antibodies; thus, the present study of the Ca 125 antigen on amniotic mucin can give only partial insight into the structure of the antigen. The results suggest that the carbohydrate structure recognized by monoclonal antibody OC 125 includes sugar residues of the non-reducing end (β-D-Gal, α-NeuAc) and is dependent on its conjugation to the protein as judged from exoglycosidase digestion and from inhibition analysis with reductively cleaved sialyloligosaccharide alditols. The terminal sialic acid group,

which is involved in the epitope structure of the antibody, may be linked to an internal sugar residue where it is less accessible to enzymic cleavage by *Vibrio cholerae* sialidase. The classification of Ca 125 as a mucin-carried, *O*-glycan epitope is partially based on its alkali lability which, on the other hand, is not a reliable criterion for discrimination between *N*- and *O*-glycosyl linkages on glycoproteins. A second line of evidence was obtained from analyses of specific Ca 125 activities during the purification process[13].

It is generally accepted that *N*-glycosyl-linked carbohydrate chains are more stable to base-catalyzed elimination than are *O*-glycosyl-linked under the conditions used by Iyer and Carlson[27]. However, base–borohydride cleavage of alkali-labile *O*-glycans from hybrid glycoproteins always results in a considerable cleavage of *N*-glycan chains[28,29], which appear to be released, for the major part, as oligosaccharide alditols. Indeed, liberation of *N*-glycan chains hindered seriously the preparation of homogeneous oligosaccharides for structural work, but immunochemical analysis indicated that antigen activities Ca 19-9, Ca 50, Ca 125, T, Leu M1, and Le^a-X could be assigned to the mucin-linked *O*-glycans chains.

With the exception of antibodies OC 125 (ref. 7), 115 C 2 (ref. 26), and 49 H 8 (ref. 30), the remainder antibodies used in this study were characterized by glycosphingolipids as primary target antigens. The cross-reactivity of antibodies C 50 (ref. 31), NS 19-9 (ref. 11), and Leu M1 (ref. 32) with mucins, as described in the present contribution, may be due to restriction of the respective epitopes to a likewise small section located at the nonreducing terminal of the carbohydrate chains. With regard to antibodies F H 3 (ref. 33) and F H 6 (ref. 25) which recognize a more extended epitope, the different core structures of mucin-like *O*-glycan chains (as compared to glycolipids) may have some influence on the binding capacity and prevent cross-reaction even though the respective antigen structures might be present. Also a lack of expression of repetitive 3-fucosyllactosamine units (di- or tri-meric X antigen) and of the corresponding sialyl derivative (sialyl dimeric X antigen) on mucins from human body fluids has to be considered. Accordingly, these antigens would be restricted to polylactosaminoglycans[34], glycosphingolipids[35], and mucins in cancer sera[25]. Although direct chemical evidence from methylation analysis is lacking, the novel carbohydrate structures of *O*-glycan-derived oligosaccharide alditols from Fractions FW5-1 and FW5-2 were assumed to be hybrids of Type 1 and Type 2 chains on the basis of inhibition studies with monoclonal antibody 115 C 2. Similar sequences have been established for glycosphingolipids of human erythrocytes[36], and for mucins from ovarian cysts[37,38]. The common features of these carbohydrate structures are an internal Type 2 and a nonreducing Type 1 terminal sequence, which is a fucosylated Le^a-active trisaccharide unit. The FW5-2 oligosaccharide alditol exhibited a structure unique in that a second L-fucosyl group is attached to an internal 2-acetamido-2-deoxy-D-glucose residue of a hybrid backbone and it expresses a sialylated Le^a determinant **12**.



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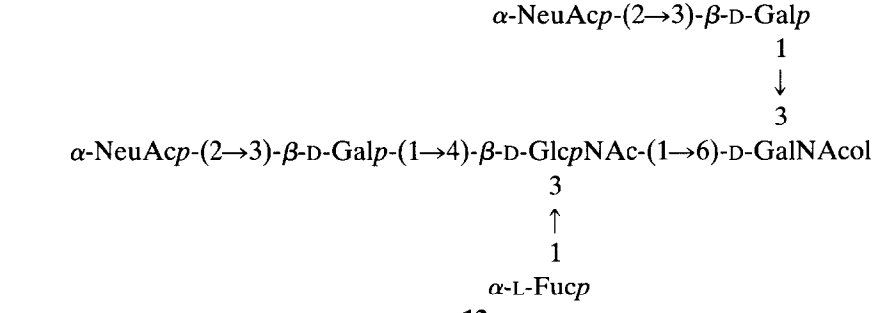
The desialylated derivative of this oligosaccharide chain on the amniotic mucin is assumed to be responsible for binding of monoclonal antibody 115 C 2, which recognizes a difucosyl hybrid having Type 1 and Type 2 backbone structures[26]. Ca 19-9 activity in Fraction FW5 was unambiguously attributed to the fucosylated compounds in Subfractions FW5-1 and FW5-2, whereas Ca 50 activity was likely due to a nonfucosylated compound, contained mainly in Fraction FW4 (ref. 17), which has the composition $NeuAc_2Hex_3HexNAc_2HexNAcol$.

Another oligosaccharide alditol analyzed in this study (FW3) had previously been reported to occur on ascites hepatoma cells[39,40] and ascites sublines from rat mammary adenocarcinoma[9], on human mammary proteoglycans[41], and on normal granulocytes[42]. It is presumed[43] to represent the biosynthetic precursor of a fucosylated oligosaccharide alditol (FW4) expressing the sialyl-X antigenic determinant[17] (**13**).



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The results from our structural work on mucin-derived *O*-glycans of normal human body fluids suggested that these oligosaccharide chains expressing various oncofetal antigens may represent target antigens for the production of diagnostically relevant antibodies, in particular since the activities of several tumor antigens (Ca 19-9, Ca 125, and Ca 50) are associated with the mucin fraction of patients' sera. As has been pointed out in an earlier investigation of Lewis and ABH substances in amniotic fluid, all these carbohydrate chains are probably of fetal origin[44]. Thus, the abundance of oncofetal carbohydrate antigens in this normal body fluid, which is expected to be a source of novel tumor markers, may be explained.

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