

2. MATERIALS AND METHODS

2.1. Preparative methods

Amniotic fluid from individual donors (0.5–1.0 l) was obtained from the University Clinic of Gynecology and Obstetrics, Cologne, FRG. Insoluble material was centrifuged off or removed by filtration prior to extraction of the clear, yellow liquid with an equal volume of 90% aqueous phenol at 65°C for 15 min. Further steps of mucin preparation, including gel filtration on Sephacryl S400 in phosphate-buffered guanidinium chloride (4 M), have been described [10]. Reductive β -elimination of bound carbohydrates and their chromatographic purification comprising ion-exchange chromatography on DEAE-Sephadex and high-performance liquid chromatography (HPLC) on aminopropyl silicate were performed as described [8]. Sialyl saccharides of fraction FW4 (0.1 mg) were solubilized in 0.1 ml acetate (50 mM), CaCl_2 (9 mM), NaCl (0.15 M), pH 5.5, containing 0.1 unit of *Vibrio cholerae* or *Clostridium perfringens* sialidase. After incubation for 1 h at 37°C the reaction mixture was diluted with water (9/1) and applied onto a column of DEAE-Sephadex A25 (0.5 \times 3 cm). Desialylated saccharides of the run through were purified on HPLC and methylated.

2.2. Analytical methods

Monosaccharides were analyzed by gas-liquid chromatography (GLC) as trimethylsilyl derivatives of the corresponding methyl glycosides [11]. The oligosaccharide alditols (approx. 0.1 mg) were methylated, hydrolyzed, reduced and acetylated according to Stellner et al. [12] with minor modifications and the resulting mixture of partially methylated alditol acetates was analyzed by GLC/mass spectrometry [8]. Prior to direct probe mass spectrometry the methylated saccharide alditol in fraction FW4 was purified from contaminating material on high-performance thin-layer chromatography (HPTLC) using chloroform/benzene/ethanol (2:2:1, v/v) for development of the plates. Fast atom bombardment-mass spectrometry (FAB-MS) was performed on a VG analytical ZAB-HF reversed-geometry mass spectrometer. FAB spectra of underivatized sialyl saccharides were recorded in the negative [13], those of the permethylated derivatives in the positive ion mode

[14]. Conditions for direct-probe electron impact-mass spectrometry (EI-MS) have been described [9].

3. RESULTS

Acidic carbohydrates isolated by reductive β -elimination from mucins of human amniotic fluid (250 mg) were separated from neutral carbohydrates on DEAE-Sephadex A25 (fraction I eluting with 0.1 M pyridine-acetate, 17 mg; fraction II eluting with 0.5 M pyridine-acetate, 40 mg) and fractionated by HPLC on aminopropyl silicate columns. Fraction FW 4 (major peak at 63 min), representing approx. 6–7% of the acidic carbohydrates in fraction II, was methylated and further purified on HPTLC. The major component of fraction FW 4 was finally subjected to structural analyses in FAB- and EI-MS.

According to the pseudo-molecular ion $M + \text{Na}^+$ in FAB-MS at m/z 1879 (fig. 1a) and the known mass increments of deoxyhexose, hexose or *N*-acetylhexosamine residues, in conjunction with results of a monosaccharide analysis the saccharide alditol is composed of *N*-acetylneuramic acid (NeuAc) [2], galactose [2], *N*-acetylglucosamine [1], L-fucose [1] and *N*-acetylgalactosaminitol (GalNAc-ol) [1]. The combined FAB- and EI-derived fragmentation pathways for the saccharide alditols were used to substantiate the carbohydrate sequence and linkage pattern of terminal residues. In FAB and EI-MS a terminal di- and a tetrasaccharide unit gave rise to formation of ions at m/z 580 and m/z 999, respectively, which may indicate the structures of two branches NeuAc-Hex and NeuAc-Hex-[dHex-]HexNAc, where Hex denotes hexose and dHex represents deoxyhexose.

From the intense secondary ion at m/z 793 in FAB-MS which originates from the primary fragment at m/z 999 through elimination of a deoxyhexose residue, it may be concluded that deoxyhexose is linked to C3 of the subterminal *N*-acetylhexosamine in the NeuAc-Hex-[dHex-]HexNAc branch. This conclusion is based on well-documented evidence [15,16].

A minor component comigrating with the heptasaccharide alditol gives rise to $M + \text{Na}^+$: 2154; its composition corresponds to NeuAc₂-Hex₃-HexNAc₂-HexNAc-OH. Because the contaminating octasaccharide does not contain a deoxy-

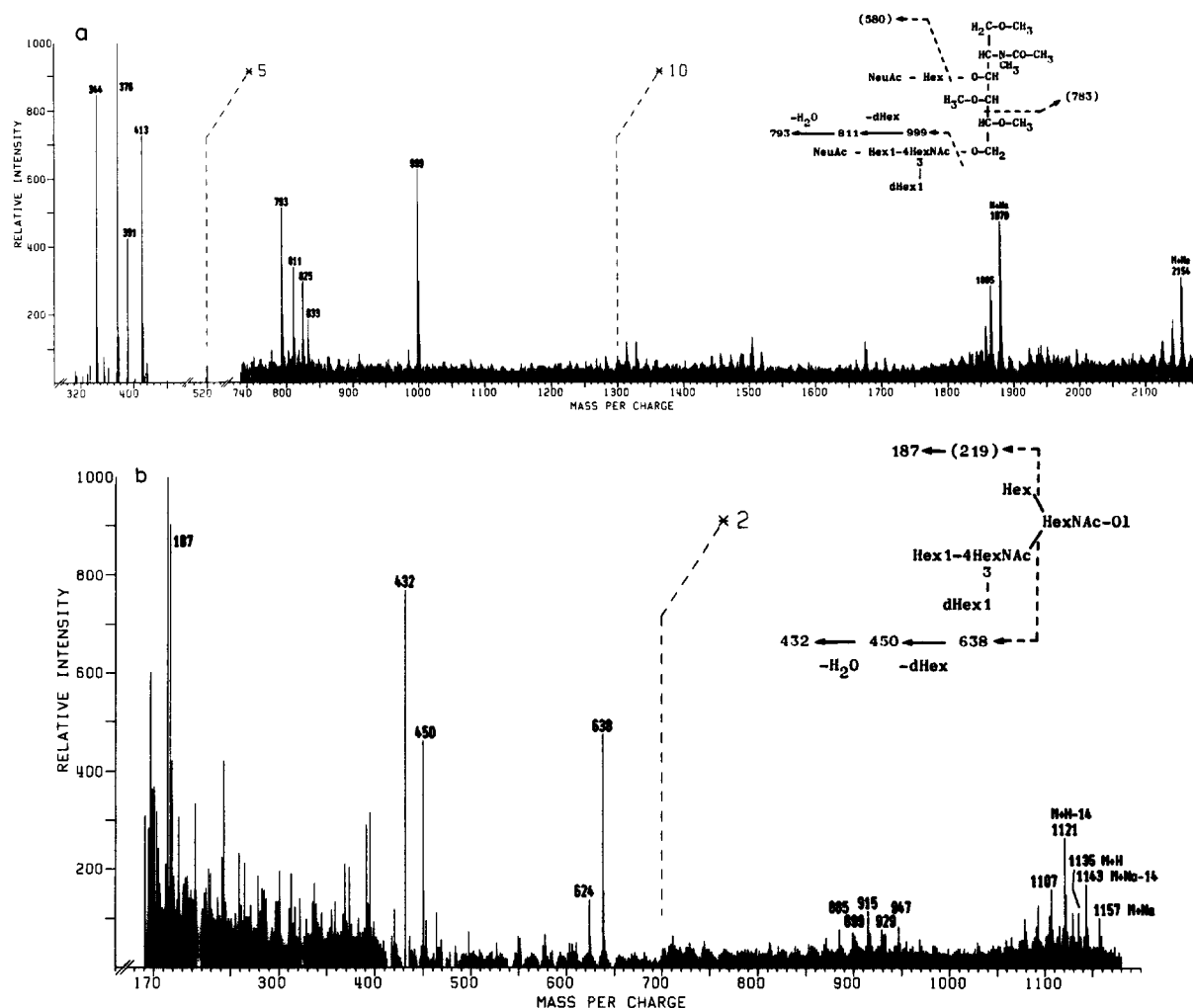


Fig.1. FAB mass spectrum of permethylated FW4 component registered in the positive ion mode. Fragment ions observed only in the EI-MS are shown in parentheses (a); FAB mass spectrum of methylated FW4 component after treatment with *V. cholerae* sialidase and fractionation on DEAE-Sephadex A25 (b).

hexose residue, the ion m/z 825 which represents a NeuAc-Hex-HexNac-sequence can be attributed to this component. The ion at m/z 811 probably does not result from undermethylation of the carbohydrate fragment NeuAc-Hex-HexNac represented at m/z 825. Its abundant appearance in common with a secondary ion at m/z 793 more likely supports the assumption that both ions are derived from the fragment at m/z 999 through successive elimination of (dHex-H₂O) or (dHex), respectively.

The branching pattern of the heptasaccharide

alditol becomes evident from the complete absence of m/z 276 in both EI- and FAB-MS, indicating a doubly substituted hexosaminitol residue. In addition, a fragment at m/z 783 in EI-MS (not shown) suggests that the *N*-acetylneuraminic acid-hexose unit is linked to C3 of the *N*-acetylgalactosaminitol residue. Methylation analysis corroborated the presence of 3,6-di-*O*-substituted *N*-acetylgalactosaminitol in the FW4 component. Moreover, partially methylated alditol acetates were detected and identified in capillary GLC-MS which point to the presence of 3,4-di-*O*-substituted *N*-acetylglucos-

amine and 3-*O*-substituted galactose. After treatment of fraction FW4 with *V. cholerae* sialidase the asialo derivative of the major component could be separated from the minor (enzyme-resistant) octasaccharide on DEAE-Sephadex A25. FAB-MS of the permethylated compound yielded $M + Na^+$: 1157 (fig.1b). As expected, the trisaccharide ion $Hex(1 \rightarrow 4)[dHex(1 \rightarrow 3)]HexNAc$ represented by m/z 638 gave rise to intense daughter ions at m/z 450 and 432, again indicating a 1 \rightarrow 3 linkage of the fucose residue to the *N*-acetylglucosamine.

The anomeric configuration of the glycosidic bonds was established on the basis of exoglycosidase digestion and CrO_3 oxidation. On digestion with sialidases from *V. cholerae* or *C. perfringens* the disialylated saccharide alditol is quantitatively converted into the corresponding asialo derivative as judged from the HPLC profile after treatment and FAB-MS analysis of the reaction products (fig.1b). Both *N*-acetylneuraminic acid residues are presumed to be α -glycosidically linked to the subterminal sugar. L-Fucose was not accessible to digestion by the α -L-fucosidase from bovine kidney. The α -anomeric configuration of fucose became evident on CrO_3 oxidation of the acetylated sugar since 90% of the deoxyhexose was resistant to the oxidative treatment. On the other hand, galactose and *N*-acetylglucosamine were destroyed quantitatively under the same conditions, suggesting that these residues have the β -configuration.

4. DISCUSSION

Although most of the monoclonal antibody defined, tumor-associated carbohydrate antigens belong to the class of glycolipids [17], evidence has been obtained for mucins representing the relevant antigens in the diagnosis of cancer in patients' sera [18]. However, the immunochemical detection of these aberrant carbohydrate epitopes on mucins of normal body fluids and their structural characterization in this and in previous contributions [8,9] pose two questions. (i) Are there any structural differences between the mucin oligosaccharides of normal body fluids and their counterparts in cancer sera, which are not readily detectable by means of monoclonal antibodies with glycolipid-epitopes? (ii) Are the same postulated

variants of known glycosyltransferases involved in the biosynthesis of aberrant carbohydrate structures both on mucins shed by the tumor cell and those secreted into normal body fluids? Therefore, work is in progress aimed at the production of monoclonal antibodies with mucin epitopes, which recognize the sialyl-X and sialyl-Le^a antigenic determinants. Moreover, the ready availability of the source may enable us to isolate partially a rarely expressed α -3-L-fucosyltransferase from amniotic fluid, which is involved in sialyl-X biosynthesis.

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