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MASS SPECTROMETRY OF A HUMAN TUMOR GLYCOLIPID ANTIGEN BEING DEFINED BY MOUSE MONOCLONAL ANTIBODY NS-19-9

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SUMMARY. With an antibody-to-chromatogram binding assay to follow the preparation a glycolipid was isolated from human pancreatic carcinoma using a mouse monoclonal antibody of apparent specificity for gastrointestinal tumors. Direct probe mass spectrometry of three derivatives established the sugar sequence as NeuAc-hexose-(fucose)N-acetylhexosamine--hexose-hexose and the ceramide to be composed mainly of phytosphingosine and 16-24 carbon 2-hydroxy fatty acids. NMR spectroscopy of two of the derivatives made likely the presence of the sequence Galβ1→3GlcNAc(4←1α Fuc)β1→, which is the blood group Lewis a determinant. This is in agreement with recent results from degradation studies.

Using the hybridoma technique devised by Köhler and Milstein (1) several laboratories are immunizing mice with human tumor cells with the objective to obtain monoclonal antibodies useful for tumor diagnosis and therapy. Of a large number of antibodies prepared in this way only a few have been defined as concerns the structure of their target antigens. These are all carbohydrates, mostly glycolipids of blood group type (2-10) and only one of them appears to be a novel structure (9,10). The two antibodies obtained against this antigen were from immunizing with human colorectal adenocarcinoma cell line SW 1116 and they showed an apparent tumor specificity (11). Binding to tumor cells was inhibited by serum from most patients with gastrointestinal cancer but not by the serum from normal individuals or most patients with other malignancies (12). The structure of the antigenic glycolipid isolated from SW 1116 cells, where

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it is a main acid glycolipid, was recently proposed from degradation studies to be a hexaglycosylceramide with a sialylated Lewis a antigen with the saccharide structure NeuAcα2→3Galβ1→3GlcNAc(4←1αFuc)β1→3Galβ1→4Glc (ref. 10).

Using the same monoclonal reagent to follow the preparation the present investigation isolated the glycolipid in much lower yield from solid tumor and characterized it structurally by mass spectrometry and NMR spectroscopy.

EXPERIMENTAL

One case of pancreas adenocarcinoma (primary tumor and liver metastases) and seven cases of colon adenocarcinoma (mainly liver metastases) were investigated. The detailed data to follow came from the pancreas case. Lyophilized tumor tissue (325 g) was extracted with a Soxhlet apparatus in two steps (13) and the lipids were subjected to mild alkaline degradation (14) and partition (chloroform-methanol-water 8:4:3, by vol.). The upper phase was evaporated and dialyzed against tap water and the lower phase was filtered through an overloaded silicic acid column to remove cholesterol and methyl esters of fatty acids (eluted with pure chloroform; main weight of total lipid). The upper and lower phase glycolipids were separately put on a DEAE-cellulose column to obtain acid glycolipids (14). After dialysis the acid fractions weighed 1 g (upper) and 0.4 g, respectively. The combined samples were separated into 12 fractions on Iatrobeads (80-60, Iatron Chemical Co., Tokyo) using a stepwise elution with increasing amounts of methanol in chloroform. Fractions containing the actual glycolipid as shown by the NS-19-9 antibody (11) and a chromatogram binding assay (6) were combined (intogether 199 mg). A continuous gradient elution with chloroform-methanol-water from Iatrobeads into 90 fractions gave 84 mg in 14 fractions showing reactivity with the antibody. A monosialoganglioside fraction (43 mg) was obtained by elution with 0.02 M NH_4Ac in CH_3OH from DEAE-Sepharose (15). A continuous gradient elution with chloroform-methanol - 3.5 M NH_3 in water (from 70:30:5 to 50:40:10) from Iatrobeads gave 19 active fractions (23 mg) which were further separated on Iatrobeads with a continuous gradient of chloroform-methanol-water (from 65:25:4 to 60:35:8). Two active fractions were obtained (6.6 mg and 3.5 mg) which were apparently homogeneous on thin-layer chromatography (Si60 nanoplates, Merck) using 4 different solvents. However, as shown by mass spectrometry the two fractions still contained at least 4 different glycolipid oligosaccharides.

Of the 6.6 mg fraction 2 mg were permethylated (16) and fractionated on 2 g of Iatrobeads using a continuous gradient of 0 to 10 vol.% methanol in chloroform giving 50 fractions. After rechromatography on 10 g Iatrobeads five out of 100 fractions were apparently pure glycolipid (0.4 mg) as defined by direct probe mass spectrometry (17) on a new ZAB-HF mass spectrometer (VG Analytical) using electron ionization and the in-beam technique. Part of the permethylated sample was reduced with $LiAlH_4$ and silylated (17) and these two derivatives were also subjected to mass spectrometry. Permethylated and permethylated-reduced derivatives were in addition analyzed by NMR spectroscopy (18).

RESULTS AND DISCUSSION

The glycolipid antigen obtained pure by a final fractionation as a permethylated derivative was structurally characterized by mass spectrometry and NMR spectroscopy. By direct inlet mass spectrometry of three derivatives the sequence and lipophilic components were safely assigned, at present possible for glycolipids with up to 12 sugars in size (19).

Fig. 1 shows the spectrum of the derivative obtained after permethylation, $LiAlH_4$-reduction (reduction of amide to amine at 3 sites and

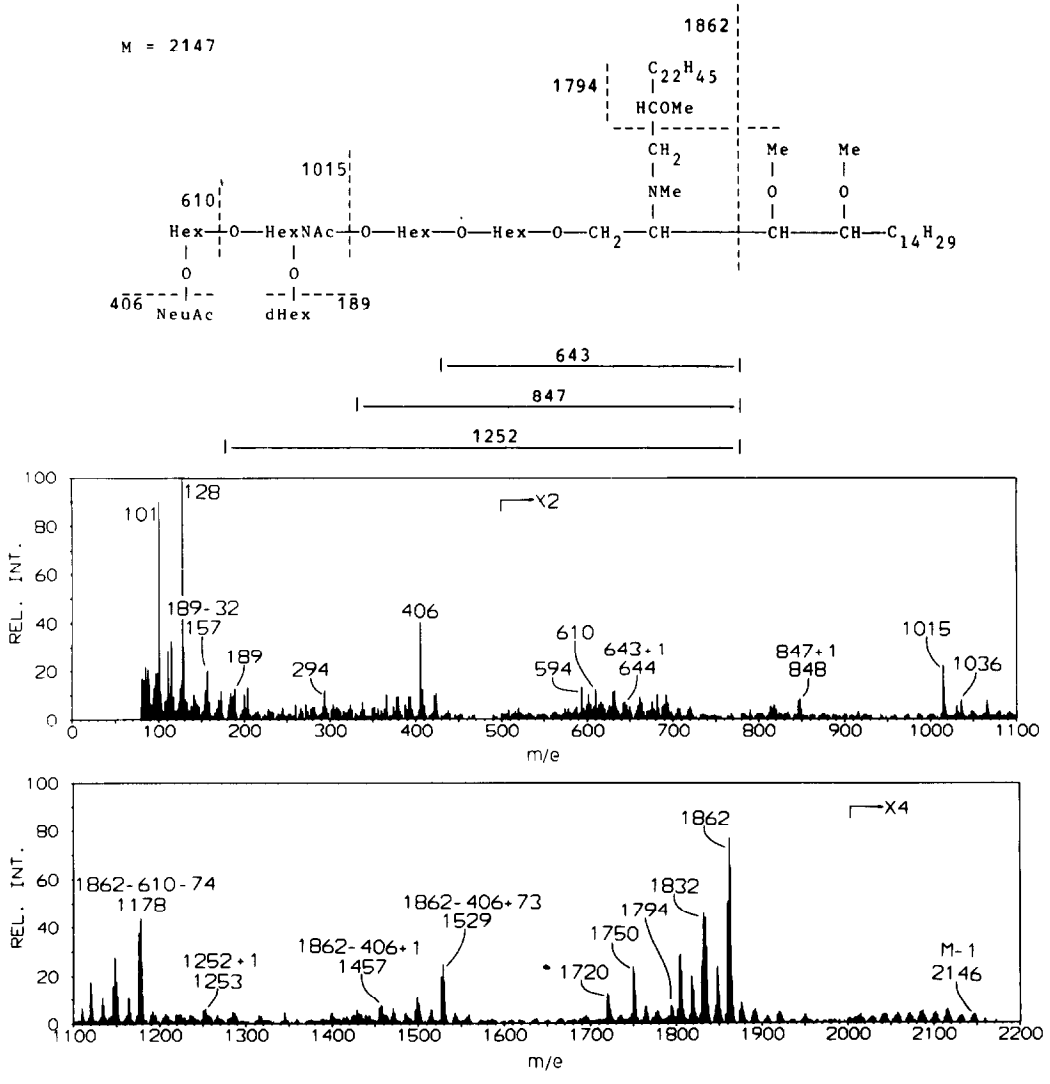

Fig. 1. Mass spectrum and simplified formula of the permethylated, $LiAlH_4$-reduced and silylated derivative (10 µg) of an acid glycolipid isolated from human pancreas tumor. The conditions of analysis were: acceleration voltage 6 kV, filament current 200 µA, electron energy 62 eV, and ion source temperature 270 °C. NeuAc means N-acetylneuraminic acid, Hex hexose, dHex deoxyhexose, HexNAc N-acetylhexosamine.

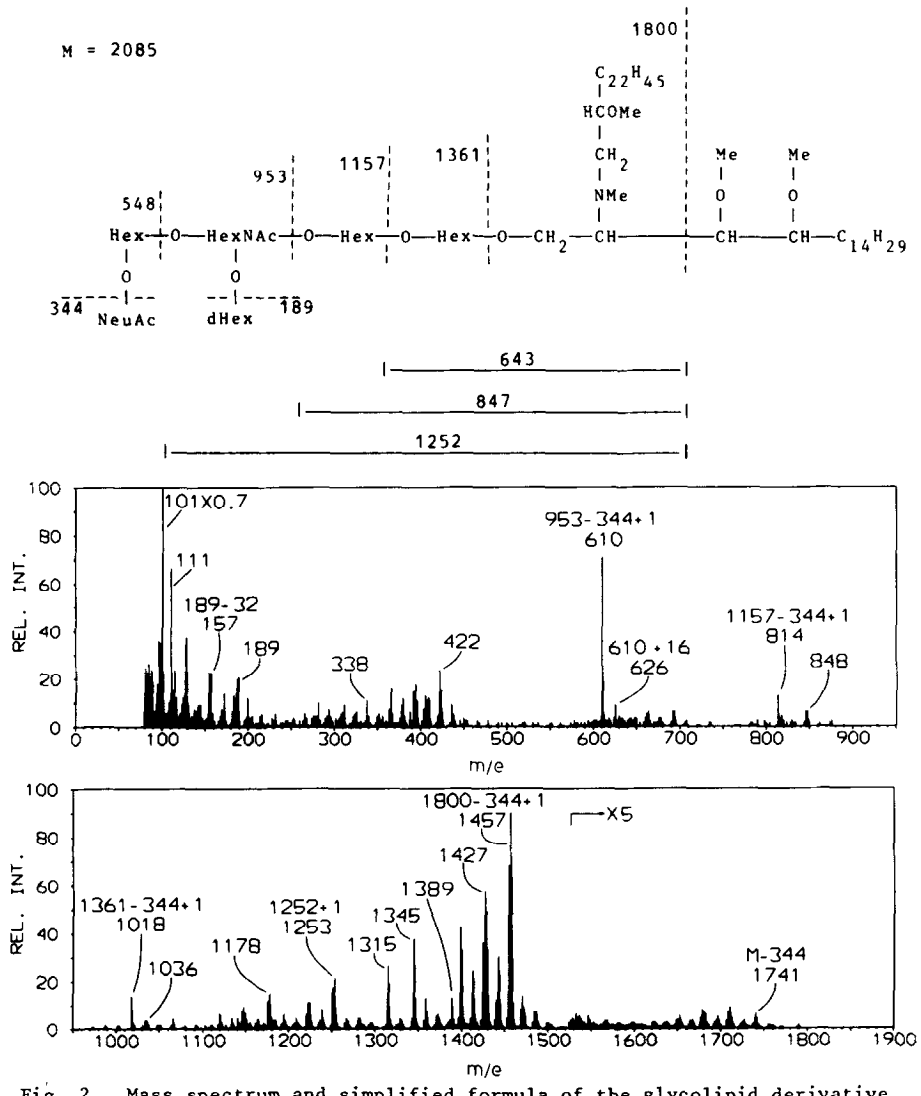

Fig. 2. Mass spectrum and simplified formula of the glycolipid derivative of Fig. 1 without silylation (10 μg). The conditions of analysis were: acceleration voltage 6 kV, filament current 200 μA, electron energy 64 eV, and ion source temperature 265 °C.

reduction of methyl ester to alcohol of the sialic acid) and trimethylsilylation (of the alcohol). A series of abundant ions diagnostic for the oligosaccharide and the fatty acid (17,19) is shown by m/e 1720-1862, due to a mixture of 16-24-carbon non-hydroxy acids (at 1720-1832) and hydroxy acids (at 1750-1862) and the six-membered oligosaccharide shown by the top formula. Terminal sialic acid derivative is shown by m/e 406 and terminal fucose by 189 and 157, trisaccharide by 610 and tetrasaccharide by 1015. Also, there are sequence ions containing the fatty acid

and an increasing number of sugars from the ceramide end, at 644, 848 and 1253 for the heaviest fatty acid (see below top formula). The intense series of peaks at m/e 1036-1178 is due to a loss of the two terminal sugars and part of the HexNAc and has been found only for type 1 chain glycolipids (13,20,21), that is Galβ1→3GlcNAc, and not for the type 2 chain, Galβ1→4GlcNAc. The peak at m/e 1794 (and at 1389 of Fig. 2) is evidence for the 2-hydroxy substituent of some of the fatty acids and that phytosphingosine is part of the long-chain bases. This interpretation is supported by data from the permethylated and reduced derivative (Fig. 2), lacking the trimethylsilyl group compared to the derivative of Fig. 1. Strong peaks are due to loss of the reduced sialic acid from several fragments (17), e.g. m/e 610, 814, 1018, 1315-1457. Finally, the only permethylated derivative (Fig. 3) gave sequence ions for terminal sugars at m/e 157, 189, 344 and 376, for two sugars at 548 and 580, four sugars at 624 and 999 and five sugars at 828 and 1203 (not shown). The rather strong peak at 402 is evidence for Galβ1→3GlcNAc(←Fuc) (20,21). A type 2 chain isomer instead produces m/e 432 (22). About half

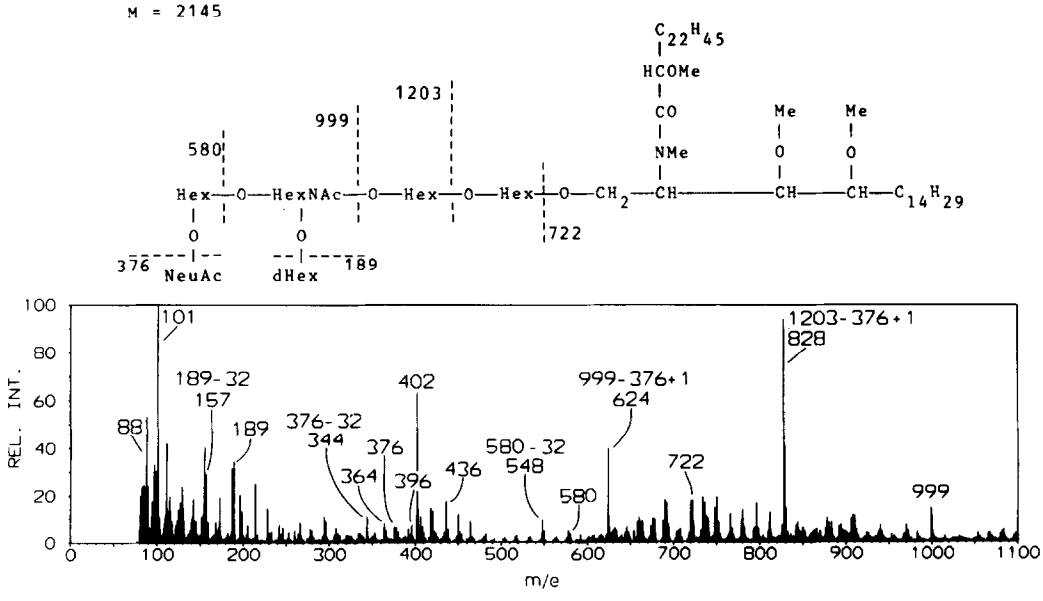

Fig. 3. Mass spectrum and simplified formula of the permethylated derivative (10 μg) of the pancreas tumor glycolipid. The conditions of analysis were: acceleration voltage 8 kV, filament current 200 μA, electron energy 44 eV, and ion source temperature 330 °C.

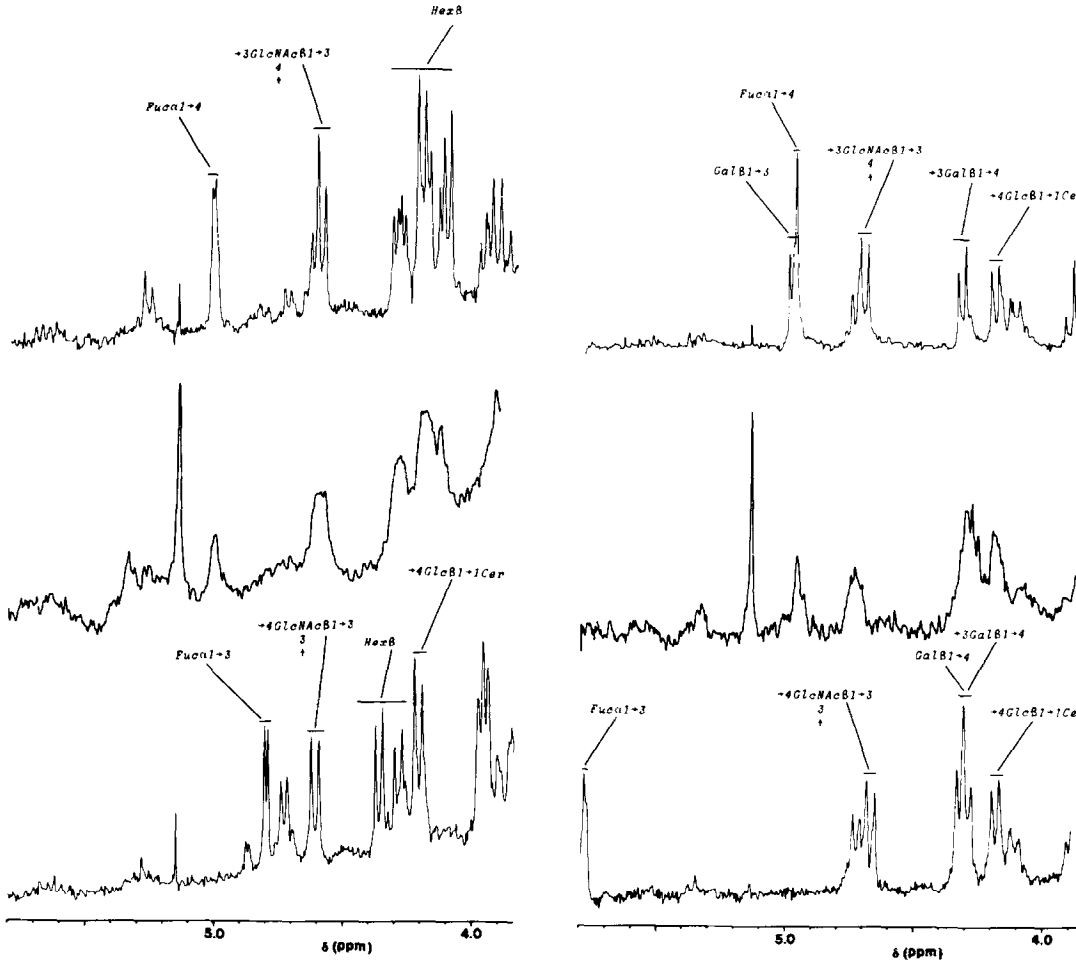

Fig. 4. Partial NMR spectra of permethylated (left) and $LiAlH_4$-reduced permethylated (right) derivative of the pancreas tumor glycolipid in comparison with published references of type 1 (top; Lewis a glycolipid) and type 2 (bottom; Lewis x glycolipid) chain non-acid glycolipids (18). 6000 pulses (left) and 9000 pulses were recorded from about 400 µg in 0.45 ml C^2HCl_3. The great change in chemical shift upon reduction is diagnostic for sugars in 3-position of GlcNAc (Galβ1→3 of top reference and Fucα1→3 of bottom reference, ref. 18). The absence of Fucα1→3 of the sample is clear. The peak at 5.15 ppm is due to an impurity of the solvent.

of the long-chain base was phytosphingosine as shown by several data, e.g. the relation of m/e 364 (sphingosine) to 396 (phytosphingosine) of Fig. 3 (23).

There is no alternative interpretation to the saccharide sequence concluded from direct inlet mass spectrometry of the three derivatives (Figs. 1-3). One such sequence is known from the literature, a fuco-ganglioside isolated from human kidney with the structure NeuAc2→3Gal1→

4GlcNAc(3←1Fuc)1→3Gal1→4Glc1→1Cer (24). NMR spectroscopy of two of the derivatives in comparison with known references (Fig. 4) excluded Fucα1→3GlcNAc and instead proposed the presence of the isomeric Fucα1→4GlcNAc produced by the fucosyltransferase coded by the Lewis gene (25). Our data, obtained without use of conventional degradation methods, therefore together strongly support the recent conclusion from degradation studies of a sialylated Lewis a glycolipid (10). The lipophilic components with a dominance of hydroxy fatty acids and phytosphingosine suggest an origin in epithelial cells (26).

Our study has not shown for sure that this glycolipid is the receptor for the NS-19-9 antibody as our preparation before final purification as a permethylated derivative contained three other gangliosides of the following probable sequences, as concluded from mass spectrometry, all with a NeuAc at the non-reducing end: lactotetraosylceramide, hexosylglobotetraosylceramide and a hexaglycosylceramide with two hexosamines and four hexoses. However, the earlier study made this very likely as this glycolipid is a dominating acid glycolipid of SW 1116 cells (10).

From our preparation in many steps from solid pancreas tumor at most 2 mg of the actual glycolipid was estimated from 325 g dry tissue. In one case of colon adenocarcinoma (four out of seven cases analysed contained the antigen) 0.5 mg was estimated from 750 g dry tissue. These yields are in contrast to the preparation from the cell line SW 1116 from which 0.3 mg was obtained from 10 g wet cells using only two chromatographic steps (10). One explanation for this difference may be that the solid tumor contains only a small fraction of tumor cells reacting with NS-19-9 antibody (L. Olding and C. Svalander, unpublished observation) and that the cell line is based on such positive cells.

As shown in the present work a glycolipid antigen present in very low amounts in a solid tumor may be possible to prepare by use of a specific monoclonal antibody and a chromatogram binding assay to follow the

preparation. Direct inlet mass spectrometry of suitable derivatives may give an overview sequence information in the final stages of purification where still a mixture of glycolipids exists. A special problem of purification is a consequence of the enrichment of a chromatographic interval where several glycolipids with similar properties may come up, especially when using tissue with heterogeneous cell composition.

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