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STRUCTURE OF A GLYCOLIPID REACTING WITH MONOCLONAL IgM IN NEUROPATHY AND WITH HNK-1

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An acidic glycolipid antigen that reacts with monoclonal IgM in patients with demyelinating neuropathy and with the mouse monoclonal antibody, HNK-1, was purified from human peripheral nerves. This lipid sharing antigenic determinants with the myelin-associated glycoprotein was shown to be an unusual glucuronic acid-containing sulfated glycosphingolipid with five sugars, but without sialic acid. Mild acid methanolysis converted the GlcUA to its methyl ester, removed the acidic sulfate group and abolished the antigenicity. Results from chemical, enzymatic, infrared, and mass spectral analysis suggested the following structure with a sulfate in a position that remains to be determined: GlcUA β1→3Gal β1→4GlcNAcβ1 → 3Galβ1→4Glcβ1→1 ceramide.

Peripheral neuropathies are often associated with patients having monoclonal IgM due to plasma cell abnormalities (1,2). Experiments in various laboratories have demonstrated that monoclonal IgM in some of the patients binds to the myelin associated glycoprotein (MAG) (3-6). More recently Ilyas et al. (6) have reported that IgM of these patients also binds to an acidic glycolipid present in the ganglioside fraction of the human peripheral nervous system. A mouse IgM monoclonal antibody, HNK-1 (Leu-7) raised to a membrane antigen from human T cell line HSB-2 recognizes a surface antigen on a subset of human lymphocytes including natural killer cells (7). HNK-1 also reacts with MAG (8-10) and with the same acidic glycolipid antigen (11). We have isolated and purified this acidic glycolipid antigen from human sciatic nerves and cauda equina. A partial structure of this glycolipid antigen and the nature of the antigenic determinant are presented in this report.

MATERIALS AND METHODS

Isolation of the glycolipid antigen: Human peripheral nerves or cauda equina (about 10 g) were extracted overnight with 20 volumes of chloroform/-methanol/water (1:1:0.15). The extract was then adjusted to a final solvent volume of chloroform/methanol/water (2:1:1.5) to form two separate phases. The upper phasewas collected and the lower phase was washed once with 0.5

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volume of methanol:water (1:1). The lipids in the combined upper phases were recovered by reversed-phase Bond-Elute method (12), treated with mild alkali to remove traces of acidic phospholipids, desalted (13) and chromatographed on a DEAE-Sephadex column (13,14). The bound acidic lipids were sequentially eluted with different concentration of ammonium acetate in methanol (15). The antigenic glycolipid along with the tetrasialogangliosides was eluted with 0.5 M ammonium acetate in methanol. This fraction was desalted (12) and the tetrasialogangliosides were digested with *V. cholera* sialidase (14) to form GM1. The antigenic compound was stable to this treatment. The GM1 formed was removed by another DEAE-Sephadex column with 0.02 M ammonium acetate whereas the antigenic glycolipid was eluted with 0.5M ammonium acetate in methanol. After removing the salt the purity of the antigen was checked by TLC procedure in several solvent systems (16).

TLC-Overlay technique: Binding of the patients' IgM paraprotein and HNK-1 with the glycolipid antigens was done using TLC overlay technique as described previously (6,11).

RESULTS AND DISCUSSION

The ganglioside fractions obtained from the DEAE-Sephadex column were analyzed for antigenic activity by TLC overlay technique. The antigenic activity was eluted mainly with 0.5M ammonium acetate in methanol along with the tetrasialoganglioside (Fig. 1a,b). The major antigenic compound in this

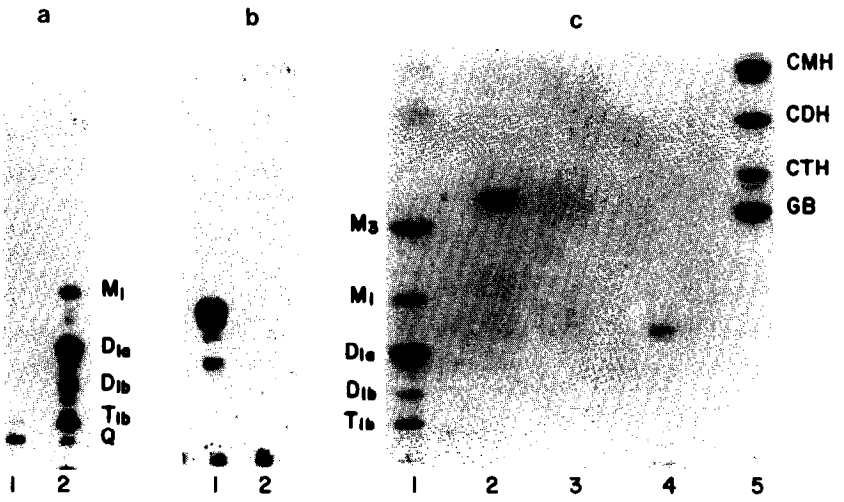


Fig. 1. (a) Lane 1, HPTLC of human peripheral nerve tetrasialoganglioside fraction obtained after DEAE-Sephadex column chromatography. Lane 2, rat brain gangliosides. The gangliosides were detected with resorcinol spray.

(b) An autoradiograph of the chromatogram in (a) after overlaying with a neuropathy patient's serum diluted 1:500, followed by radioiodinated goat anti-human IgM (6). The lane numbers correspond to the fractions in (a).

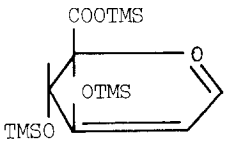
(c) HPTLC of purified antigenic glycolipid. Lane 1, standard gangliosides; lane 2, antigen after treatment with mild acid; lane 3, antigen after treatment with mild acid and then alkali; lane 4, native antigen; lane 5, neutral glycolipid standards.

fraction migrated between GM1 and GD1a standards. A second, less prominent, slower migrating antigen was also detected. It became apparent that both the antigenic compounds did not react with resorcinol spray (for sialic acid containing glycolipids) and thus are not gangliosides. The antigens, however, reacted with orcinol spray giving purple color indicating the presence of a reducing sugar.

The purified major antigenic compound did not bind to DEAE-Sephadex after mild acid methanolysis (0.05 M anhydrous methanolic HCl for 3.5 hour at room temperature). It migrated on a TLC plate close to globoside and paragloboside (Fig. 1c) and completely lost its antigenicity to IgM from the patients and HNK-1 (not shown). The acid treated antigen after treatment with mild alkali (0.2 M NaOH in aqueous methanol) was retained on a DEAE-Sephadex column but was eluted earlier with 0.02 M ammonium acetate in methanol (corresponding to the monosialoganglioside fraction). It migrated on a TLC plate slightly above the 'native' antigen (Fig. 1c) and its immunoreactivity with the human IgM was at least partially restored. The alkaline treatment, however, did not restore reactivity with HNK-1. Both the antigen and the acid treated antigen were susceptible to endo-β-galactosidase (17) producing glucosylceramide suggesting the GlcNAcβ1$\rightarrow$3Galβ1$\rightarrow$4Glcβ1$\rightarrow$1ceramide core structure. The native antigen was not susceptible to β-glucuronidase (Limpet), but the acid methanolized alkali treated product (Fig 1c, lane 3) was and yielded paragloboside. Fourier transform infrared spectra of the purified antigen showed absorption at 1225 cm^{-1} due to S=O stretching vibrations. This absorption was absent in the acid methanolyzed antigen indicating a loss of sulfate.

Mass spectrometric analysis of the acid treated antigen after pertrimethylsilylation gave m/z 407 and a strong m/z 317 (Fig. 2a) which showed that the acid hydrolyzed antigen contained a terminal persilylated methylester of glucuronic acid. Ions that show a loss of OCH_3 to produce m/z 376 from m/z 407 and a loss of CH_3COOH to produce m/z 257 from m/z 317 were also found. The mass spectrum had a weak m/z 451 indicating a lack of terminal hexose in the molecule (18). Ion at m/z 311 indicated the presence of C_{18}-sphingenine in the molecule.

The acid and alkali treated antigen after pertrimethylsilylation produced a strong m/z 375 corresponding to ion



which with a loss of HCOOTMS produced m/z 257. These ion structures were confirmed by mass spectral analysis of the compounds after pertrimethylsilylation with deuterated silylating reagent.

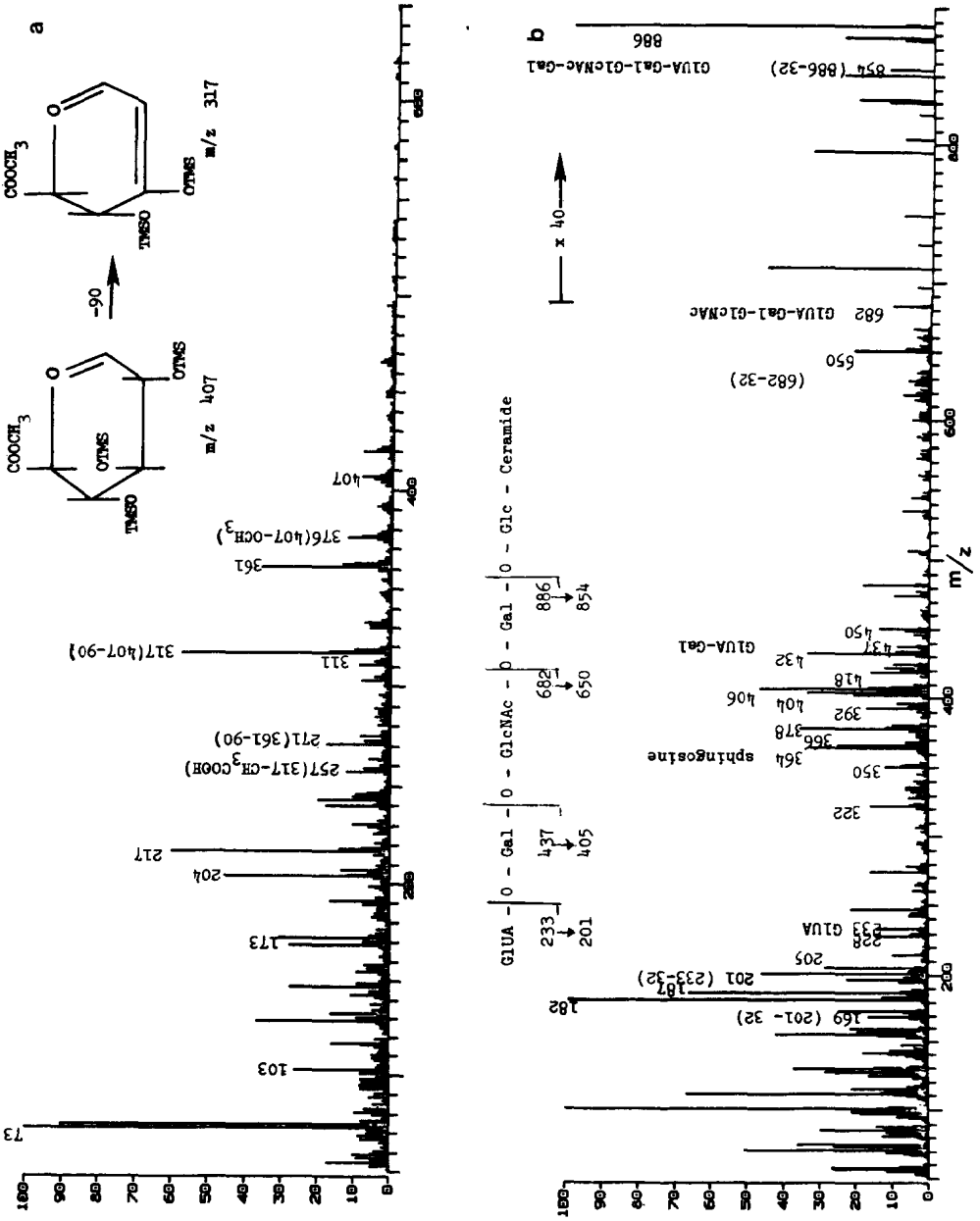


Fig. 2. (a) Mass spectrum of the acid treated antigen as pertrimethylsilylated derivative in 20 ev electron impact mode.
(b) Mass spectrum of the acid treated antigen as permethylated derivative in 20 ev electron impact mode.

The carbohydrates in the glycolipid antigen were characterized and quantitated after acid methanolysis followed by high resolution capillary GLC-mass spectrometric analysis of the trimethylsilylated derivatives of the sugars released (19). The analysis showed the presence of Glc:Gal:GlcNAc:GlcUA in the proportion of 1:2:1:1, respectively. The fatty acid composition of the antigenic glycolipid was 16:1 (2.4%), 16:0 (13.2%), 18:1 (16.1%), 18:0 (11.4%), 20:1 (4.3%), 20:0 (5.3%), 21:1 (0.2%), 21:0 (2.0%), 22:1 (8.1%), 22:0 (1.4%), 23:0 (4.0%), 24:1 (12.5%), 24:0 (15.3%), 25:0 (3.3%) and 26:1 (1.0%).

Mass spectrum of the acid treated and permethylated antigen is shown in Fig. 2b. The spectrum did not have m/z 219 corresponding to a terminal hexose (20) but contained m/z 233 corresponding to terminal GlcUA. Loss of methanol (mass unit 32) produced strong m/z 201. Internal hexoses as expected produced m/z 187. The m/z 182 is common to all permethylated N-acetyl-hexosaminyl residues with no substitution at C-3 position indicating that N-acetyl glucosamine residue has substitution at C-4 position. The presence of m/z 228 also confirmed this result. Presence of m/z 437 should be due to a disaccharide GlcUA-hexose residue, m/z 682 and 650 should be related to a trisaccharide GlcUA-hexose-hexosaminyl residue, whereas m/z 886 and 854 should be related to a tetrasaccharide GlcUA-hexose-hexosaminyl-hexose residue. Ion at m/z 364 is due to C_{18}-sphingenine whereas m/z 366 and 392 are possibly due to C_{18}-sphinganine and C_{20}-sphingenine, respectively. Ion at m/z 392 could also be due to a fragment of ceramide residue with 23:0 fatty acid. Other ceramide fragments corresponding to 18:0 (m/z 322), 20:0 (m/z 350), 22:0 (m/z 378), 24:1 (m/z 404), 24:0 (m/z 406), 25:1 (m/z 418) and 26:1 (m/z 432) are also found in the spectrum.

Based on the chemical as well as mass spectrometric evidence it appears that the native antigenic compound is an acidic sulfated glycosphingolipid with five sugars. Part of the acidic character of the lipid is due to a terminal glucuronic acid in the structure. Mild acid methanolysis of the native antigen removes the sulfate group and the terminal glucuronic acid is converted to its methylester. The methylester is converted back to the free acid by mild alkaline hydrolysis and the product migrates slightly ahead of the native antigen on TLC plate. The carbohydrate analysis and mass spectrometry of the acid treated antigen provided good evidence to suggest the following possible structure: GlcUAβ1$\rightarrow$3Galβ1$\rightarrow$4GlcNAcβ1$\rightarrow$3Galβ1$\rightarrow$4Glcβ1$\rightarrow$ 1 ceramide. However, the exact sugar linkages and the position of the sulfate group remains to be determined.

The results also provide some information about the nature of the epitopes reacting with the IgM paraprotein and HNK-1. The epitopes must be somewhat different since, reactivity with both antibodies is abolished by mild acid treatment however, subsequent alkali treatment at least partially

restores reactivity with the human IgM but not with HNK-1. This indicates that the epitope for the human IgM involves a free carboxyl group on the GlcUA, but the acid labile sulfate group is required for reactivity with HNK-1. Whether HNK-1 also requires the free carboxyl group, and whether full reactivity with the human IgM requires sulfate group remains to be determined.

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