ACCUMULATION OF GLYCOLIPIDS CONTAINING N-ACETYLGLUCOSAMINE IN ERYTHROCYTE STROMA OF PATIENTS WITH CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II (HEMPAS)

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Ceramide trihexoside from Congenital Dyserythropoietic Anemia Type II (HEMPAS) erythrocyte stroma contains approxomately equimolar amounts of galactoseα1→4 galactoseβ1→4 glucosyl ceramide and N-acetylglucosamineβ1→3galactoseβ1→4 glucosyl ceramide. The latter compound is not chemically detectable in normal stroma. 25% of the ceramide tetrahexoside fraction of HEMPAS stroma is composed of a glycolipid containing N-acetylglucosamine, presumably paragloboside (galactoseβ1→4*N*-acetylglucosamineβ1→3galactoseβ1→4 glucosyl ceramide).

Congenital Dyserythropoietic Anemia Type II (HEMPAS) is characterized by ineffective erythropoiesis, erythroblastic multinuclearity, and shortened survival time of the erythrocyte in the peripheral circulation (1). HEMPAS erythrocytes are immunologically reactive with anti-i sera and IgM antibody present in many normal sera can cause complement mediated lysis resulting in a false positive Ham test. Ceramide dihexoside, ceramide trihexoside (CTH), and ceramide tetrahexoside are significantly elevated in HEMPAS stroma relative to normal stroma (2). Further investigation has now revealed the presence of large quantities of N-acetylglucosamine in both HEMPAS CTH and ceramide tetrahexosides. The structures and amounts of HEMPAS stromal glycolipids is the subject of the present report.

Abbreviations: GLC, gas liquid chromatography; Glc, glucose; Gal, galactose GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; CTH, ceramide trihexoside.
Lacto-N-tetraose is the trivial name for galactoseβ1→3N-acetylglucosamineβ1→3galactoseβ1→4glucose

MATERIALS AND METHODS

Blood from two clinically affected HEMPAS siblings (P.M., F. M.) was drawn on two separate occasions several months apart. Blood drawn from normal volunteers was used as controls. Handling of blood, purification of stroma, and subsequent isolation of glycolipids were performed as previously described (2).

Hexosamine was measured colorimetrically with the Elson-Morgan assay (3). Glycolipids were quantitated and their carbohydrate composition determined by the method of Esselman *et al* (4). Relative response factors for individual hexosides on gas liquid chromatography (GLC) were determined using lacto-N-tetraose as a standard. The lacto-N-tetraose was isolated from human milk (5) and twice crystallized.

Lacto-N-tetraose and HEMPAS CTH were methylated with methyl sulfinyl carbanion in dimethyl sulfoxide according to Hakomori (6). Acetolysis and acid hydrolysis of permethylated compounds, monosaccharide reduction, and subsequent acetylation were adapted from standard procedures (4, 7). Partially methylated alditol acetates were separated by GLC on 3% OV-225 on Gas Chrom Q (Applied Sciences State College, Pa.) at 180° C for neutral alditols and 210° C for hexosaminitol derivatives. Structures were confirmed by mass spectrometry and comparison with standards derived from lacto-N-tetraose and published spectra (7, 8).

Beef brain sphingomyelin (Applied Sciences, State College, Pa.) was used to provide standard sphingosine (erythro C_{18}-sphingosine). Long chain base from HEMPAS CTH was analyzed by the method of Carter and Gaver (9) as modified by Yang and Hakomori (10). Octacosane was used as an internal standard. Mass spectral analysis after separation by GLC on 3% OV-225 on Gas Chrom Q at 210° C confirmed structural assignments.

Anomeric linkages and carbohydrate sequences were determined with fig ficin α-galactosidase (11), and jack bean meal β-N-acetylhexosaminidase and β-galactosidase (12). Routinely 50 µg of glycolipid was incubated with appropriate enzyme at 37° C in 0.05 M citrate buffer pH 4.0 containing sodium taurochlolate (Nutritional Biochemical Company) and toluene as a bacteriocide (13). Following overnight incubation the glycolipids were extracted into chloroform-methanol (2/1) and analyzed by thin layer chromatography. Glycolipids were visualized with the orcinol sulphuric acid spray (13).

Aqueous phases remaining after chloroform-methanol extraction were deionized over a mixed bed column of Ag3X4A(OH^-) and AG50WX8(H^+). Eluates were dried to a convenient volume and spotted on Whatman No. 1 paper along with appropriate standards and developed in ethyl acetate/pyridine/H_2O(12/5/4). Silver nitrate reagent was used to visualize the monosaccharides (14).

RESULTS AND DISCUSSION

HEMPAS CTH and ceramide tetrahexoside gave positive colorimetric reactions for hexosamine. GLC confirmed the presence of N-acetylglucosamine in HEMPAS CTH (Fig. 1). The four samples of HEMPAS CTH analyzed (two from each patient) had molar ratio of N-acetylglucosamine to glucose varying from 0.4 and 0.66. GLC analysis of three samples of CTH from normal stroma were negative for

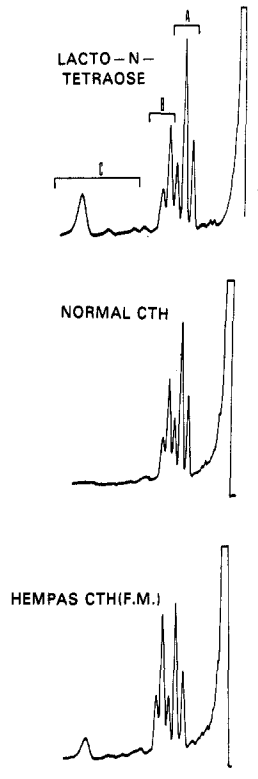

FIG. 1. GLC of trimethyl silyl derivatives of methyl glycosides obtained from methanolysis of appropriate compounds was performed on 3% SE-30 on Gas Chrom Q (Applied Sciences, State College, Pa.) at 180° C. Top: lacto-N-tetraose. Peaks under A, B, and C are methyl galactosides, methyl glucosides, and methyl N-acetyl glucosaminides respectively. Middle: derivatives from normal stroma CTH Bottom: derivatives from HEMPAS stroma (F.M.) CTH.

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hexosamine. Hexosamine present at the 0.03 level of glucose would have been detected.

Methylation analysis of the HEMPAS CTH indicated the presence of non-reducing terminal N-acetylglucosamine and galactose, 4-O substituted galactose and glucose, and 3-O substituted galactose (Fig. 2). Mass spectrometry confirmed assigned structures.

After acetylation (15) HEMPAS CTH could be separated into two distinct glycolipids by thin layer chromatography (Fig. 3). Carbohydrate ratio in the upper band was Gal/Glc 2/1 and in the lower GlcNAc/Gal/Glc 1/1/1. After elution

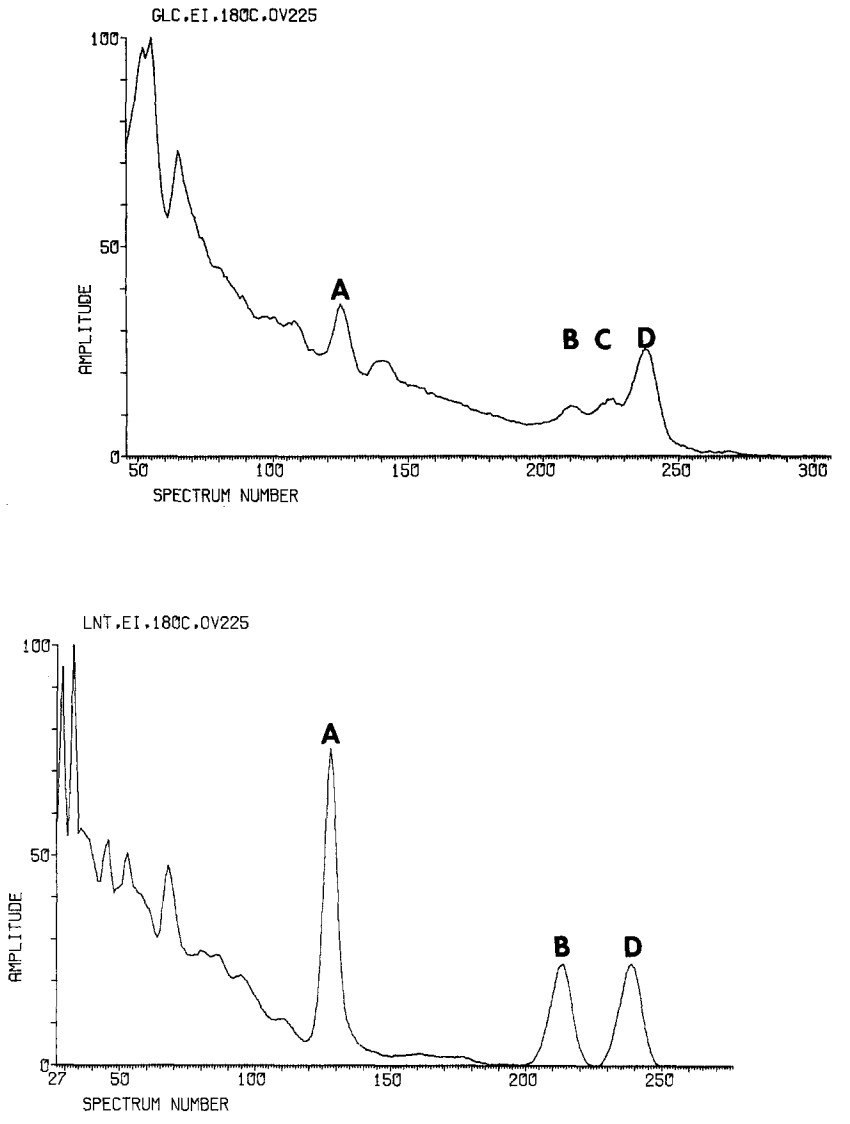

FIG. 2. GLC of partially methylated neutral alditol acetates of Lacto-N-Tetraose (bottom) and HEMPAS CTH (top) on 3% OV-225 on Gas Chrom Q at 180° C.
Peak A: 2,3,4,6-tetra-O-methyl-1,5-di-O-acetylgalactitol.
Peak B: 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl-galactitol.
Peak C: 2,3,6-tri-O-methyl-1,4,5-tri-O-acetyl-glactitol.
Peak D: 2,3,6-tri-O-methyl-1,4,5-tri-O-acetyl-glucitol.
When run at 210° C one additional peak corresponding 3,4,6-tri-O-methyl-1,5-di-O-acetyl-2-deoxy-2-N-methylacetamidoglucitol was seen with the HEMPAS CTH (data not shown).

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of the bands and deacetylation, vigorous periodate oxidation (8) destroyed all hexose in the upper band, but only N-acetylglucosamine and glucose in the lower.

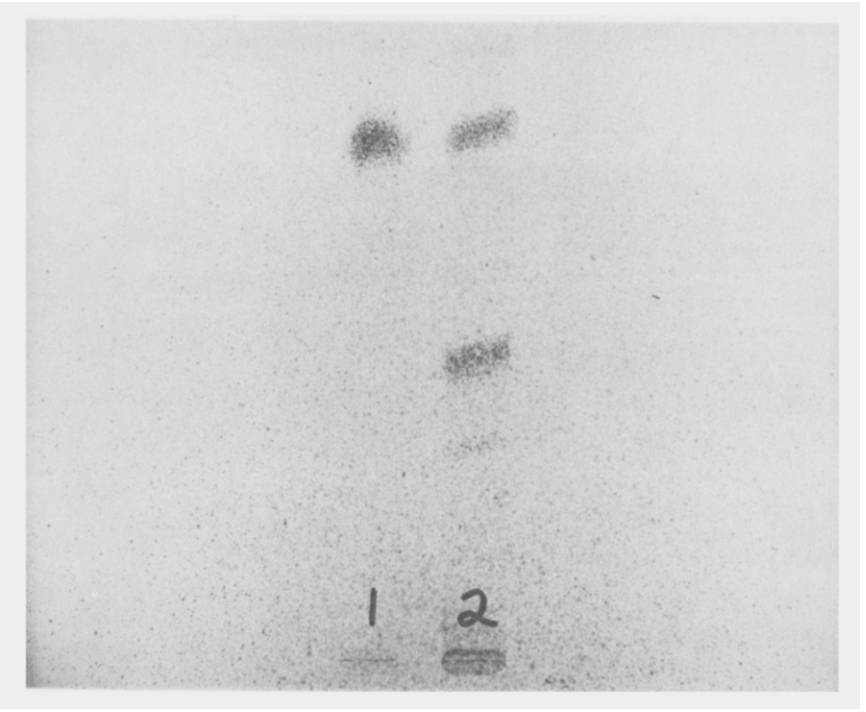

FIG. 3. Thin layer chromatograph on Silica Gel G of acetylated CTH from normal and HEMPAS stroma. Lane 1 - normal CTH. Lane 2 - HEMPAS CTH. Origin is at bottom. Developing solvent dichloroethane/methanol 90/10. Glycolipids were visualized with orcinol-sulphuric acid.

Since galactose could be quantitatively recovered in the lower band after periodate oxidation all its galactose had to be 3-O substituted.

As judged by enzymatic degradations the sequence and anomeric configuration of sugars in the upper band HEMPAS CTH component was GalαGalβGlc ceramide and in the lower band GlcNAcβGalβGlc ceramide. The hexoses released by these degradations were identified by paper chromatography. GLC mass spectrometry revealed that over 90% of the HEMPAS CTH long chain base was erythro C_{18}-sphingosine. Approximately equimolar amounts of sphingosine and glucose were present. The two HEMPAS CTH components were therefore Galα1→4Galβ1→4Glc ceramide and GlcNAcβ1→3Galβ1→4Glc ceramide. The latter compound is present as 5 μmoles/100 mls HEMPAS erythrocytes and has not been previously reported in human erythrocytes.

Four samples of HEMPAS ceramide tetrahexoside analyzed had approximate the sugar ratio of GalNAc/GlcNAc/Gal/Glc of 0.75/0.25/2/1. GlcNAc/Glu ratio varied from 0.22 to 0.29. 50% of the galactose of several samples of ceramide tetrahexoside was resistant to vigorous periodate oxidation. HEMPAS GL-4 containing GlcNAc (presumable paragloboside) is present as 3-4 μmoles/100 mls packed erythrocytes. According to Siddiqui and Hakomori (16) only 0.1-0.2 μmoles of paragloboside is present in 100 mls of normal erythrocytes.

The accumulation of several glycolipids in HEMPAS stroma (2) argues against a specific catabolic defect similar to those seen in Tay Sach's (17), Fabray's (18), or Gaucher's (19) disease. No increase of CTH occurs in Fabray erythrocytes (20) and the slight increase of glucocerebroside in Gaucher erythrocytes (20) may be due to increased absorption of elevated plasma glucocerebroside. The immunological specificity of the HEMPAS glycolipids is currently under investigation.

A possible explanation of HEMPAS glycolipid accumulation may be ineffective control of glycolipid biosynthesis during erythropoiesis. Information of glycosyl transferases in these pathways is limited, but it is interesting to speculate that the same galactosyl transferase may add the ultimate galactosyl residues of lactosyl ceramide and paragloboside analogous to the lactose-lactosamine synthetase of human milk (21).

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