Characterization of a Blood Group B Glycolipid, Accumulating in the Pancreas of a Patient with Fabry’s Disease*

JOHN R. WHITRRETT and SEN ITIHO HAKOMORI

From the Department of Medicine (Neurology), University of Toronto, Toronto 5, and Departments of Pathobiology and Microbiology, University of Washington, Seattle, Washington 38195

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SUMMARY

An accumulation of a ceramide hexasaccharide was found in the pancreas of a patient with Fabry’s disease, in addition to the accumulation of ceramide trihexoside and ceramide digalactoside. The ceramide hexasaccharide was isolated and identified as a blood group B-active glycolipid with the following structure:

\[ \text{Gal} \rightarrow 3 \text{Gal} \rightarrow 3(\text{and } 1 \rightarrow 4) \text{GlcNAc} \rightarrow 3 \text{Gal} \rightarrow 4 \text{Glc} \rightarrow \text{Cer} \]

\[ \text{Fuc} \]

The proportion of two structures, one containing \( \text{Gal} \rightarrow 3 \text{GlcNAc} \) and one containing \( \text{Gal} \rightarrow 4 \text{GlcNAc} \), is 4:1.

The lipid accumulating in the kidney of patients with Fabry’s disease was identified first by Sweeley and Klionsky (1) as ceramide trihexoside (\( \text{Gal} \rightarrow 4 \text{Gal} \rightarrow 4 \text{Glc} \rightarrow \text{Cer} \)) and ceramide digalactoside (\( \text{Gal} \rightarrow 4 \text{Gal} \rightarrow 1 \text{Cer} \)). Fabry’s disease is characterized by a deficiency of an \( \alpha \)-galactosidase (2-4), and this is in accordance with the recent assignment of an \( \alpha \) anomic linkage of the terminal galactosyl residue in both ceramide trihexoside (5-10) and ceramide dihexoside (9, 11).

In addition to accumulation of these two glycolipids, a marked accumulation of a third component, which migrated slower on thin layer chromatography than ceramide tetra- or pentaaccharide, was observed in the pancreas of a patient with Fabry’s disease. Isolation and characterization of this substance is to be described in this paper. The substance was identified as a blood group B glycolipid.

MATERIALS AND METHODS

Tissue Source—The source of tissue was a 45-year-old man who died in 1965 with classical clinical and histopathological features of Fabry’s disease (Angiokeratoma corporis diffusum). The clinical and pathological record of this patient is available.\(^1\) His blood group was type B. Extraction and preliminary analysis of glycosphingolipids were made on formalin-fixed organs and tissues, 2 years postmortem.

Isolation of Accumulating Glycolipids from Pancreas—Formalin-fixed pancreas (2.68 g) was frozen in liquid nitrogen and pulverized to a fine powder. This powder was extracted with 19 volumes of chloroform-methanol (2:1) (solvent proportions by volume), and the resulting extract washed according to Folch et al. (12) to give 203 mg of lipid. Lipid (203 mg) was used for initial quantitative analysis of glycolipids (Table I). Similar analysis was carried out on the extracts of brain, heart, liver, kidney, spleen, thyroid, and adrenal glands. The unknown glycosphingolipid accumulating in pancreas was then purified from the remaining lipid extract (180 mg). The total extract was placed on a DEAE-cellulose column prepared (13) to give a bed measuring 2 × 18 cm, which was eluted with 460 ml of chloroform-methanol (1:1), followed by 460 ml of methanol, and collected in the same flask. The column was then eluted with 600 ml of chloroform-methanol (4:1) containing 20 ml of ammonium hydroxide and 10 nmoles of ammonium acetate per liter. This fraction was free of glycosphingolipid as demonstrated by thin layer chromatography and was discarded. The combined chloroform-methanol and methanol fractions were then chromatographed on a BioSil A column (Bio-Rad, Richmond, California), whose bed measurements were 3 × 15 cm and which was eluted with the following sequence of solvents: chloroform, 600 ml; acetone, 2400 ml; acetone-methanol (9:1), 2400 ml; and methanol, 600 ml. The unknown lipid was recovered in the fraction eluted with acetone-methanol (9:1) along with small amounts of trihexosyl ceramide and two glycosphingolipids running slightly behind it on a thin layer chromatogram developed in chloroform-methanol-water (60:35:8). Further purification was obtained by repeating chromatography on another BioSil-A column. The acetone-methanol (9:1) eluate now contained the unknown lipid free of trihexosyl ceramide and the faster of the two slow-migrating bands noted above. This fraction was evaporated to dryness, taken up in a small volume of chloroform-methanol (2:1), and the glycosphingolipid was identified as a blood group B-active glycolipid.

\(^1\) B. R. Fast and H. M. Ross, University of Manitoba, Faculty of Medicine, unpublished data.
TABLE I
Estimation of glycosphingolipid in Fabry pancreas

Glycosphingolipids were isolated by column chromatography as described in the text. Individual classes were separated by thin layer chromatography and hexose content determined by gas chromatography (14).

<table>
<thead>
<tr>
<th>Glycosphingolipid</th>
<th>Micromoles per g wet tissue</th>
<th>Galactose/glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceramide monohexoside</td>
<td>0.22</td>
<td>5.3</td>
</tr>
<tr>
<td>Ceramide dihexoside</td>
<td>0.83</td>
<td>56.6</td>
</tr>
<tr>
<td>Ceramide trihexoside</td>
<td>2.5</td>
<td>21.4</td>
</tr>
<tr>
<td>Unknown</td>
<td>1.44</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Calculated from glucose or assumed molar ratio of hexose to ceramide.

was precipitated with 10 volumes of acetone at 4°C for 15 hours, in order to remove a yellow contaminant which remained soluble in acetone. The yield of dried precipitate was 12.98%. Final purification to yield a substance giving a single spot on thin layer chromatograms developed in the above solvent was achieved through preparative thin layer chromatography on Silica Gel G using chloroform-methanol-water (60:35:8) for development and a water spray for detection.

The lipid was recovered from the silica gel by extracting the gel obtained from a single plate five times with 15 ml of chloroform-methanol-water (60:35:8) in a centrifuge tube. Pure lipid (2.76 mg) was obtained from one-third of the acetonemethanol fraction from the second column.

Analytical Procedures Homogeneity of the glycosphingolipid was determined by thin layer chromatography on Silica Gel G using the solvent system chloroform-methanol-water (60:35:8). Spots were detected with a spray of 0.2% orcinol in 2 M sulfuric acid. The carbohydrate composition was determined by gas chromatography according to the modified method of Sweeley and Walker (14) and the modified method of Sawardeker et al. (15), as described by Yang and Hakomori (16). Detector response factors for trimethylsilyl ethers of methylglycosides in the Schedley method were calculated using mannitol as an internal standard. Fatty acid and sphingosine base were determined essentially according to the combination of the methods of Gaver and Schedley (17) and Carter and Gaver (18), as described by Yang and Hakomori (16). Fatty acids were identified by comparison with standard mixtures run under the same conditions on columns of SE-30 and ethylene glycol succinate. To determine whether or not hydroxy fatty acids were present, a portion of the total fraction of methyl esters was chromatographed on a plate of Silica Gel H using hexane-diethyl ether (85:15). Normal and hydroxy fatty acid methyl esters were identified by spraying with water and eluted from the gel with diethyl ether. A known amount of C18:1 fatty acid methyl ester standard was added to each of the normal and hydroxy fractions, and they were then analyzed by gas chromatography on ethylene glycol succinate.

Degradation and Determination of Carbohydrate Sequence—Enzymatic hydrolysis of the glycosphingolipid was carried out by sequential treatment of the glycolipid and its hydrolysis product with enzymes. The hydrolysis products, extracted with chloroform-methanol, were examined by thin layer chromatography. Details of this method are essentially the same as described previously (5, 19). The glycolipid (500 µg) was first hydrolyzed with Li’s fig α-galactosidase (0.6 units in 0.2 ml; p-nitrophenyl-α-galactoside as substrate) by the method described (5). The H-active glycolipid (about 350 µg) derived from the unknown glycolipid by α-galactosidase was further degraded by hydrolysis of fucosyl residue by 0.1 N trichloroacetic acid in a sealed tube at 100°C for 2 hours (20). H-active glycolipid was also degraded by α-L-fucosidase preparation from liver of Haliotis (abalone), donated by Professor Toshiaki Osawa, Tokyo University, College of Pharmaceutical Sciences. The enzyme was prepared by the method of Iijima et al. (21). It was degraded in 0.1 ml of 0.05 M citrate buffer, pH 4.0, containing 100 µg of sodium taurocholate at 37°C for 18 hours. The degradation product (ceramide tetrasaccharide) of H-active glycolipid resulting from trichloroacetic acid hydrolysis was further degraded by 0.2 units of jack bean β-galactosidase (EC 32.1.23) (22), and the resulting ceramide trisaccharide was further degraded by 0.4 units of jack bean β-N-acetylhexosaminidase (EC 32.1.30) (23) by the same conditions as previously described (5).

Methylation Analysis—Positions of the linkages between monosaccharides were determined by permethylation (24) followed by acetylation with 0.5 N sulfuric acid in 93% acetic acid, reduction, and acetylation. The partially methylated alditol acetates and 2-deoxy-2-N-methylacetamidohexoses were identified by gas chromatography using a 6-foot glass column packed with 3% ECNSS-M operated isothermally at 150°C and 180°C, respectively. Peaks were analyzed by mass spectrometry using a Finnigan mass spectrometer (model 4000) with quadrupole all glass separator. Identification of partially O-methylated hexitol acetates was carried out according to the methods and the mass spectra established by Lindberg’s group (25, 20). Identification of partially O-methylated 2-deoxy-2-N-methylacetamidohexoses was carried out according to the methods and the mass spectra established in our laboratory, and gas chromatography mass spectrometric patterns of partially O-methylated 2-deoxy-2-N-methylacetamidohexoses were determined by the use of methylated products of partially O-(1-methoxy)methylated 2-deoxy-2-N-acetamidohexoses. Various kinds of O-methylated hexitol acetates were prepared by the procedure previously described (19).

Determination of Antigenic Properties—Blood group B activity was determined by inhibition of hemagglutination. Five fold quantities of cholesterol and lecithin were added to the glycosphingolipids as auxiliary lipids (27), and titrations were carried out with four hemagglutinating doses of anti-B serum using microtiter plates. Appearance of H activity after α-galactosidase degradation was determined using double diffusion in a thin film of agarose (28) to detect the precipitin reaction between glycosphingolipids and anti-H lectin (Ulex europeus). As reference compounds in both inhibition of hemagglutination and

1 Similar results were obtained using a more highly purified preparation of the same enzyme provided by Dr. P. Okuyama of Seikagaku Chemical Co., Tokyo. This enzyme preparation is commercially available.

2 Permethylation of complex carbohydrates containing aminosugars resulted in N-methylation of aminomethylidohexamannose; the hydrolysis of a glycosidic linkage of N-methylidohexamannose is generally resistant to aqueous acid because of a strong basic property of methyl mido group. The yield of 2-deoxy-2-N-methylacetamidohexoses under a number of different degradation conditions have been compared, and the previously mentioned condition was recorded as the best and reproducible (K. Stellner, H. Saito, and S. Hakomori, in preparation).


4 Blood group H-specific glycoproteins gave a very faint H-specific precipitin reaction with Ulex europeus, but H-specific glycolipid gave fairly strong precipitin reaction with a crude extract of Ulex europeus.
The purified glycosphingolipid ran as a single spot on a thin layer chromatogram developed in chloroform-methanol-water (60:35:8) (Fig. 3). It migrated slower than rabbit ceramide pentasaccharide (Gal α 1 → 3 Gal 1 → 4 GlcNAc 1 → 3 Gal 1 → 4 Glc → Cer) or an H-active ceramide pentasaccharide (Fuc α 1 → 3 Gal β 1 → 4 GlcNAc β 1 → 3 Gal β 1 → 4 Glc → Cer), whose structure has been elucidated recently (20), suggesting that the glycolipid might be a ceramide hexasaccharide. The results of sugar analysis indicated that the glycolipid contained fucose, galactose, glucosamine, and glucose in a molar ratio of 1:3:1:1 (glucosamine to galactose to glucose ratio determined by Sweeney’s procedure 0.8:2.0:1.0; glucosamine to glucose to fucose ratio determined by Yang and Hakomori procedure 1.0:1.0:0.9). The results again suggested that the glycolipid is a ceramide hexasaccharide.

The carbohydrate moiety of this glycosphingolipid was identified as O-α-galactosyl(1 → 3)-[O-α-L-fucosyl(1 → 2)]O-β-galactosyl(1 → 3 and 1 → 4)-O-β-N-acetyl glucosaminyl(1 → 3)-O-β-galactosyl(1 → 4)-O-glucosylceramide on the following evidence. (c) The purified glycosphingolipid was a potential inhibitor of blood group B specific hemagglutination. Nine nanograms of the pure material inhibited completely four hemagglutinating doses of anti-B serum. (b) The permethylated glycosphingolipid, after acetylation, hydrolysis, reduction, and acetylation, gave five peaks of methylated alditol acetate at 150° and two peaks of methylated 2-deoxy-2-N-acetamidoalactitol at 180° on gas chromatography. The five peaks of neutral sugars were identified on gas chromatography-mass spectrometry as acetates of 2,3,4-trimethylfucitol, 2,3,4,6-tetramethylgalactitol, 2,4,6-trimethylgalactitol, 2,3,6-trimethylhexitol, and 4,6-dimethylalactitol. The two peaks of methylated aminosugars (Peaks 6 and 7) were separated, and “Peak 6” and “Peak 7” were identified, respectively, as 3,6-di-O-methyl-2-deoxy-2-N-acetamidoalactitol and 4,6-di-O-methyl-2-deoxy-2-N-methylacetamidoalactitol. The ratio of 3,6-di-O-methyl and 4,6-di-O-methyl derivative was 1 to 4 (Fig. 2). (e) The terminal galactosyl residue was found to be hydrolyzed readily by Li’s α-galactosidase from fein, and the glycolipid was quantitatively converted to a faster-migrating glycolipid (ceramide hexasaccharide), whose migration rate was identical to that of H1-glycolipid of Stellner et al. (20) (Fig. 3). (d) The glycolipid resulting from hydrolysis with Li’s α-galactosidase (5, 6) reacted strongly with the Ulex europaeus anti-H reagent (Fig. 4). (e) The H-active glycolipid formed as the hydrolys product with α-galactosidase was further degraded to a ceramide tetrasaccharide by hydrolysis with weak acid (0.1 N trichloroacetic acid). (f) The H-active glycolipid was also degraded by the α-fucosidase of abalone liver obtained through Dr. Toshiaki Osawa of Tokyo University. The enzyme preparation, however, contained β-galactosidase and β-N-acetylhexosaminidase and therefore the following degradation products were given: ceramide tetrasaccharide, ceramide trisaccharide, and lactosylceramide. (g) The ceramide tetrasaccharide obtained by acid hydrolysis of H-active glycolipid was further hydrolyzed by β-galactosidase to a ceramide trisaccharide. The resulting ceramide trisaccharide was then specifically degraded by jack bean β-N-acetylhexosaminidase. The hydrolysis product showed chromatographic behavior identical with lactosylceramide.

The composition of ceramide moiety is shown in Table II; C22:0 fatty acid and C18 sphingosines were the major components. No hydroxy fatty acids were detected. Based on these findings the structure of the accumulated glycolipid is shown in Fig. 5.
Fabry pancreas. Peaks 1 to 6 are partially O-methylated bixinol acetates; gas chromatogram-mass spectra of these neutral sugar tri-O-methyl-2-deoxy-2-A-acetamidoglucitol 1.0.

of sugar composition, pre-methylation and gas chromatography-mass spectrometric identification of methylated sugars to de-termed by double diffusion, it could be concluded that the terminal trisaccharide was identical with the above structure. It should be noted that the presence of multiple lines of precipitation and the absence of a line of identity joining the two precipitates between Ulex and II-active glycosphingolipid in Fig. 4 does not indicate that the antigens differ. This behavior is characteristic of glycosphingolipid antigens and is dependent, in part, on the state of aggregation of the antigen (40). In the blood group substances derived from glycoproteins, oligosaccharide chains were found in which the linkages of the sub-terminal galactose to the next sugar in the chain N acetylglucosamine were (1 \rightarrow 3) and (1 \rightarrow 4) and are referred to as “type 1” and “type 2” chains, respectively (39). The results of methylation study indicated that the majority (80%) of the glycosphingolipid accumulating in pancreas had a type I chain. This contrasts with Hr-glycolipid (20) and all “variants” of blood group A glycolipids of human erythrocytes which have type 2 chains (41). The Lewis a-active glycosphingolipid from human adenocarcinoma (42) had a type 1 chain, whereas a blood group inactive analogue from the same source (16) had a type 2 chain.

The Fabry tissue provided an opportunity to confirm the structure of a group B-active glycosphingolipid proposed previously on the basis of compositional analysis of glycosphingolipids isolated from human red cells (43, 44). Because of extremely low yield and extreme difficulty in purification of blood group A and B substances of human erythrocyte membrane, pure material sufficient for structural study has not been obtained. Further confusion and difficulty in chemical characterization of blood group glycosphingolipids was caused by the presence of multiple components carrying the same blood group specificities (variants), although some chemical distinctions between these variants have been recently explored (41). An additional group B-active glycosphingolipid with a larger oligosaccharide was isolated from red cells, and there is evidence for additional group B-active components in the Fabry pancreas. In the
Fig. 3 (left). Treatment of Fabry pancreas glycosphingolipid with α-galactosidase. Samples: Lane 1, globoside, 10 μg; Lane 2, Fabry pancreas glycosphingolipid following purification by preparative thin layer chromatography; Lane 3, Fabry pancreas glycosphingolipid, 20 μg; Lane 4, Fabry pancreas glycosphingolipid, 20 μg, following incubation with α-galactosidase (5) showing conversion to ceramide pentasaccharide; Lane 5, H-active ceramide pentasaccharide (20), 10 μg; Lane 6, rabbit ceramide pentasaccharide, 10 μg; Lane 7, ceramide trihexoside, 10 μg; Lane 8, ceramide trihexoside, 10 μg, following incubation with α-galactosidase (5) and showing conversion to ceramide dihexoside; Lane 9, ceramide lactoside, 10 μg. The plate was developed in chloroform, methanol-water (60:35:8) and spots visualized with Man II. Fatty acids and sphingosines in blood group B glycosphingolipid accumulating in Fabry's disease.

TABLE II

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Total</th>
<th>Sphingosines</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>1.9</td>
<td>C16:0</td>
<td>0</td>
</tr>
<tr>
<td>C18:1</td>
<td>5.5</td>
<td>C16:1</td>
<td>73</td>
</tr>
<tr>
<td>C18:2</td>
<td>4.0</td>
<td>C18:2</td>
<td>16</td>
</tr>
<tr>
<td>C18:2</td>
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<td>Phytosphingosine</td>
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<tr>
<td>C20:0</td>
<td>16.7</td>
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<td>C20:2</td>
<td>30.7</td>
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<td>C22:0</td>
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<td>16.0</td>
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Acetone-methanol and methanol eluates from the silicic acid columns at least two further glycosphingolipid bands were detected on thin layer chromatograms which migrated more slowly than the group B-active lipid. The fastest-migrating substance was present in a sample treated with α-galactosidase, and it was also degraded to a faster-running component. It is possible that the content of these higher variants of B-active glycosphingolipid in the lipid extract might have been greatly reduced during the process of Folch's washing.

The finding of increased blood group-active glycosphingolipid in an inherited disease characterized by deficiency of α-galactosidase demonstrates an interesting interaction between two genetic traits. The ABO gene determines the substrate specificity of the glycosyltransferase which transfers a sugar (galactose, N-acetylgalactosamine, or no sugar) to H substance to form complete oligosaccharide chains in glycoproteins or glycosphingolipids. In Fabry's disease the mutation affects an hydrolase demonstrates an interesting interaction between two genetic traits. The ABO gene determines the substrate specificity of the glycosyltransferase which transfers a sugar (galactose, N-acetylgalactosamine, or no sugar) to H substance to form complete oligosaccharide chains in glycoproteins or glycosphingolipids. In Fabry's disease the mutation affects an 

4 J. R. Wherrett, unpublished experiments.
enzyme (2-4) which would be expected to degrade blood group B substance by catalyzing the hydrolysis of the glycosidic linkage conferring B activity. Therefore, it is only in Fabry patients with blood group B that an accumulation of B blood group substances is to be expected. The blood group active glycosphingolipids are found normally in glandular epithelial tissues such as pancreas and intestine (44, 45), and only trace quantities are found in other parenchymatous organs. It is in the epithelial tissues and blood that one might expect accumulation of these lipids in Fabry’s disease. In one previous study only have these tissues been analyzed. Schibanoff et al. (34) analyzed formalin-fixed pancreas from a patient who died with Fabry’s disease but did not report increased amounts of more complex glycosphingolipids. We assume that this patient was not blood group B. We may also speculate whether an accumulation of additional substance in a blood group B patient will have an adverse influence on the clinical state (46).

Accumulation of a blood group B-active glycosphingolipid rather than a ceramide tetraxose free of fucose in a disease substrate? that catabolism of these blood group lipids involves the addition of additional substance in a blood group B patient will hypothetically influence the clinical state (46)

Acknowledgments—Dr. B. B. Fast and Dr. H. M. Ross of the University of Manitoba Faculty of Medicine kindly provided the tissue for this study. Dr. Yu-Teh Li of Tulane University kindly donated purified a galactosidase and b-galactosidase for this study. Gas chromatographic-mass spectrometric analysis of methylated neutral sugars and aminosugars were carried out by Dr. Klaus Stellner and Mr. Randy Jenkins of our laboratory.

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