Characterization of Gangliosides from Bovine Erythrocyte Membranes

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Two glucosamine-containing gangliosides, sialosylhexa-glycosylceramides, were isolated from bovine erythrocyte membranes. Both gangliosides were hydrolyzed by neuraminidase isolated from Clostridium perfringens to become neutral hexaglycosylceramides. Based on the results of sequential enzymatic hydrolysis and gas chromatography-mass spectrometry analyses of the methylated sugars, the structures of these two gangliosides were shown to be NeuAc-Out 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-ceramide and NeuGc-Out 2 → 3GalPl → 4(NeuAca1-3)Galβ1 → 4Glc-Cer; G2, NeuGc-Out 2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-ceramide, respectively. In addition, N-acetyl- and N-glyco-lynueraminiosyllacto-N-neotetraosylceramides, and N-acetyl- and N-glycolynueraminiosyllactosylceramides were also found in bovine erythrocytes. The predominant fatty acids in these two gangliosides were C22:0 and C24:0. C18 sphingosine was the major base detected.

The major gangliosides of neural tissues contain N-acetyl-galactosamine in their oligosaccharide chain. In 1965, Wiegandt and Baschang (1) detected the presence of glucosamine-containing gangliosides in bovine erythrocytes. The sialic acid in this ganglioside was identified as N-glycolynueraminic acid. The same ganglioside with N-acetylneuraminic acid was subsequently isolated from human muscles, erythrocytes, leukocytes, brain, and peripheral nerve (2–6). In spite of their wide occurrence, the glucosamine-containing gangliosides have not been carefully studied. This report presents a simple procedure for the isolation of gangliosides from bovine erythrocytes and the structural elucidation of these gangliosides.

EXPERIMENTAL PROCEDURES

Materials — Fresh bovine blood was obtained from a slaughter- house in Covington, La. Precoated silica gel plates were the products of Brinkmann Instruments Inc., Westbury, N. Y. Fatty acid methyl esters, sphingosines, 10% DEGS PS on 80/100 Supelcoport, 3% OV-17 on 100/120 Gas-Chrom Q, 3% OV-210 on 100/120 Chromosorb W, 3% SE-30 on 80/100 Supelcoport, and Silica Gel G were purchased from Supelco, Inc., Bellefonte, Pa. Cellex D was obtained from Bio- rad Laboratories, Richmond, Calif., and Sephadex LH-20 from Pharmacia Fine Chemicals, Piscataway, N. J. The following glyco- sphingolipids were isolated in this laboratory: galactosylceramide, hematoside (G2, NeuGc-Out 2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-ceramide, and globoside from human erythrocytes; lactosylceramide and pentaglycosylceramide from bovine erythrocytes. Neuraminidase from C. perfringens (type V) was purchased from Sigma Chemical Co., Saint Louis, Mo. β-Galactosidase, and β-N-acetylgalactosaminidase were isolated from jack bean meal (7, 8).

Extraction of Glycosphingolipids from Bovine Erythrocytes — Bovine stroma was prepared according to the method of Grochowska and Koscielak (9). The acetone-dried stroma (200 g) was swelled and homogenized with 4 volumes of 0.1 N KCl solution. Four volumes of tetrahydrofuran was added to the homogenate (10), and the mixture was stirred overnight at room temperature. After being filtered through a Buchner funnel, the residue was reextracted three more times with 1.6 liter of tetrahydrofuran: H2O (8:1). The combined filtrate was dried in a rotary evaporator. To the same flask, 200 ml of 0.6 N NaOH in methanol was added and the mixture was incubated at 37°C for 6 h. After exhaustive dialysis, the content was dried completely in a rotary evaporator.

DEAE-cellulose Column Chromatography — DEAE-cellulose (Cel- lex D, CI form) was converted to the acetate form according to the method of Rouese et al. (11) with modification. The cellulose was washed twice with 5 volumes of 0.1 N NaOH. After removing the fine particles and rinsing with H2O, we added 1 volume of 1 N acetic acid (12). The sediment renewed was washed three times with H2O, then with methanol. The washed cellulose was suspended in chloroform: methanol (2:7) and packed into a column (2.9 x 33 cm) that was equilibrated overnight with the same solvent. The dialyzed lipid extract was dissolved in 25 ml of chloroform:methanol (2:1) and applied to the column. Neutral lipids were eluted from the column with 1.2 liter of chloroform:methanol (2:7) and gangliosides with the same solvent containing 0.01, 0.02, and 0.05 M sodium acetate successively. The 0.01 M sodium acetate effluent (Fraction I) which contained about 94% of the total gangliosides was further purified by Silica Gel G column chromatography.

Silica Gel G Column Chromatography — Silica Gel G was activated overnight at 110°C and packed into a column (2.9 x 33 cm) with chloroform. Fraction I from the DEAE-cellulose column was dialyzed thoroughly against distilled water and concentrated in a rotary

† The abbreviations used (for glycosphingolipids) are: G2, Galβ1 → 3GlcNAcβ1 → 4(NeuAc-Out 2 → 3Galβ1 → 4Glc-ceramide; G2, NeuGc-Out 2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-ceramide, respectively. The abbreviated names are abbreviated as described in the text.

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evaporator. The residue was then dissolved in chloroform:methanol (2:1) and applied to the column. The eluting solvents were 1 liter each of A, chloroform:methanol:H₂O:concentrated NH₄OH (125:45:5:3); B, chloroform:methanol:H₂O:concentrated NH₄OH (125:55:6:4); and C, chloroform:methanol:H₂O:concentrated NH₄OH (120:65:10:4). Fractions of 8 ml were collected, and 50-μl aliquots were checked for ganglioside by thin layer chromatography. Gangliosides A and B were eluted by Solvent A, and gangliosides C and D by Solvent B. These four gangliosides were obtained in pure form directly from the column (Fig. 1). Gangliosides E and F were eluted by Solvent C, however, were not well separated. These two gangliosides were further purified by preparative thin layer chromatography using solvent system chloroform:methanol:0.25% KCl (55:40:5).

Permethylation Studies—The glycolipid (about 1 mg) was treated with dimethylsulfonium carbonate and then methyalted with methyl iodide (13). The methylated glycolipid was passed through Sephadex LH-20 as described by Yang and Hakomori (14). The combined glycolipid fractions were hydrolyzed with 0.7 N H₂SO₄ in 80% aqueous acetic acid (15) and then reduced and acetylated according to Bjorndal et al. (16). The partially methylated alditol acetates were analyzed with a Finnigan model 3300 gas chromatography-mass spectrometer. Neutral sugars were separated isothermally at 180°C in a column packed with 3% OV-275 Supelcoport (100 to 120 mesh). Amino sugars were separated by using a 3% OV-17 Supelcoport (100 to 120 mesh) column with a temperature increment programmed at 2°C/min from 180°C to 200°C (17, 18). The methyl esters of fatty acids were extracted with hexane from the methanolysate (1.5 ml anhydrous methanolic HCl, 80°C, 24 h) and analyzed at 190°C on a 10% DEGS column. Sphingosine was determined on a 3% SE-30 column after trimethylsilylation (19).

Incubation of Glycosphingolipids with Various Glycosidases—For the hydrolysis of sialic acid from gangliosides, 30 μg of the ganglioside was dissolved in 150 μl of 0.05 M sodium acetate buffer, pH 5.0, and incubated with 4 milliunits of neuraminidase from Cl. perfringens overnight at 37°C. One unit of enzyme releases 1 wmol of N-sides -Fig. 1 shows the mobilities of the ganglioside preparations isolated from bovine erythrocyte membranes in three solvent systems. The yields of these gangliosides from 200 g of acetone-dried stroma were: A, 1.5 mg; B, 4.2 mg; C, 6.0 mg; D, 40.6 mg; E, 2.2 mg; and F, 9 mg. Gangliosides A and B were identified as N-acetyl- and N-glycolylneuraminosyllacto-N-neotetraosylceramide, respectively. Both gangliosides were hydrolyzed by neuraminidase from Cl. perfringens to become lactosylceramide. The two bands observed with ganglioside B are probably the result of differences in fatty acid composition (Table I). Gangliosides C and D contained steric acid, galactose, glucosamine, glucose, and sphingosine in a molar ratio of 0.9:1:1.9:2:0.8:9:9:9:1 and 1.17:2:1.31:16:0:9:2:1, respectively. They were identified as N-acetyl- and N-glycolylneuraminosyllacto-N-neotetraosylceramide, respectively. The structures of gangliosides A to D were further confirmed by enzymatic

RESULTS

Chromatographic Behavior of Bovine Erythrocyte Gangliosides—Fig. 1 shows the mobilities of the ganglioside preparations isolated from bovine erythrocyte membranes in three solvent systems. The yields of these gangliosides from 200 g of acetone-dried stroma were: A, 1.5 mg; B, 4.2 mg; C, 6.0 mg; D, 40.6 mg; E, 2.2 mg; and F, 9 mg. Gangliosides A and B were identified as N-acetyl- and N-glycolylneuraminosyllacto-N-neotetraosylceramide, respectively. Both gangliosides were hydrolyzed by neuraminidase from Cl. perfringens to become lactosylceramide. The two bands observed with ganglioside B are probably the result of differences in fatty acid composition (Table I). Gangliosides C and D contained steric acid, galactose, glucosamine, glucose, and sphingosine in a molar ratio of 0.9:1:1.9:2:0.8:9:9:9:1 and 1.17:2:1.31:16:0:9:2:1, respectively. They were identified as N-acetyl- and N-glycolylneuraminosyllacto-N-neotetraosylceramide, respectively. The structures of gangliosides A to D were further confirmed by enzymatic

TABLE I

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<th>C</th>
<th>D</th>
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2 We thank Dr. R. K. Yu of Yale University for his help in the determination of steric acid species by gas-liquid chromatography.
Gangliosides of Bovine Erythrocyte Membranes

Hydrolysis and methylation analysis. Gangliosides E and F were the sialosylhexaglycosylceramides. The ratio of sialic acid, galactose, glucosamine, glucose, and sphingosine in these two gangliosides were 0.92:2.83:1.92:0.90:1 and 0.95:2.70:1.83:0.94:1, respectively. Using thin layer chromatography and gas-liquid chromatography, gangliosides C and E were found to contain N-acetylneuraminic acid while gangliosides D and F contained N-glycolyneuraminic acid as their sialic acid constituent. As shown in Fig. 1A, with the solvent system chloroform: methanol: H₂O: concentrated NH₄OH (60:35:6:2), gangliosides A and B were well separated from each other. Gangliosides C and D migrated very closely to brain G₁ and G₁₁, respectively. Gangliosides E and F were not well separated with this solvent system. When the solvent system chloroform: methanol: H₂O: concentrated NH₄OH (55:40:6:2) was used, gangliosides A and B were no longer separated from each other; however, a very good separation was obtained among gangliosides C, D, E, and F (Fig. 1B). Under this condition, ganglioside C migrated only slightly behind G₁₁ but very much ahead of G₁₁₁; ganglioside D, slightly ahead of G₁₁₁; and gangliosides E and F, behind G₁₁₁. As Fig. 1C shows, in the neutral solvent system chloroform: methanol: H₂O (60:35:8), no separation could be obtained between gangliosides A and B. Gangliosides C, D, and E migrated closely together with G₁₁, while ganglioside F showed the same mobility as G₁₁₁.

Sequential Arrangement and Anomeric Configuration of

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Fig. 2. Enzymatic hydrolysis of bovine stroma sialosylhexaglycosylceramide. Lanes 1 and 9 are the standards: from top, glucosylceramide, lactosylceramide, ceramide trihexoside (CTH), globoside, and pentaglycosylceramide. 2, sialosylhexaglycosylceramide; 3, 2 + neuraminidase; 4, 3 + β-galactosidase; 5, 4 + β-hexosaminidase; 6, 5 + β-galactosidase; 7, 6 + β-hexosaminidase; 8, 5 + β-galactosidase + β-hexosaminidase.

Fig. 3. Gas chromatogram and mass spectra of partially methylated alditol acetates from bovine stroma sialosylhexaglycosylceramide. Separation was carried out on 3% OV-275 column as described in the text. Mass Spectra 1 and 2 were obtained from Peaks 1 and 2, respectively. The authentic standards were shown on the left.
Sugar Units — Fig. 2 shows the results of the enzymatic hydrolysis of ganglioside F. The intact ganglioside was resistant to α- and β-galactosidase and β-N-acetylhexosaminidase. Incubation of this ganglioside with neuraminidase produced a neutral glycosphingolipid with mobility slower than the pentaglycosylceramide. β-Galactosidase subsequently converted this glycolipid into a compound with mobility slightly ahead of the pentaglycosylceramide. As shown in Fig. 2, by treating the newly formed glycolipid with β-N-acetylhexosaminidase and β-galactosidase alternatively, we converted the pentaglycosylceramide stepwise into a tetra-, tri-, di-, and glycosylceramide. From the result of the sequential enzymatic hydrolysis, one can deduce the structure NeuGcα → Galβ → GlcNAcβ → Galβ → GlcNAcβ → Galβ → Glc → Cer for the anomeric configuration and sequential arrangement of saccharide units in this ganglioside. Identical results were obtained with ganglioside E.

Methylation Studies — Analysis by gas chromatography-mass spectrometry revealed that ganglioside F contained 2,4,6-tri-O-methylgalactitol-1,3,5-triacetate, 3,6-di-O-methyl-2-deoxy-2-N-methylacetamidoglucitol-1,4,5-triacetate (Fig. 3), and 3,6-di-O-methyl-2-deoxy-2-N-methylacetamidoglucitol-1,4,5-triacetate (Fig. 4). No 2,3,4,6-tetramethylgalactitol-1,5-diacetate was detected. The same partially methylated sugars were obtained with ganglioside F.

Fatty Acid and Sphingosine Compositions — As shown in Table I, the fatty acids of N-acetyl- and N-glycolylneuramino-

SYNTHETIC STUDIES ON GANGLIOSIDES OF BOVINE ERYTHROCYTE MEMBRANES

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syhexaglycosyleramides were composed predominantly of behenic and lignoceric acids while C-18 sphingosine was the major long chain base detected.

Based on the above results the structure for both gangliosides E and F was shown to be NeuAc (or NeuGc)α2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-Cer.

DISCUSSION

In order to appreciate the structure and function of the cell membrane, it is imperative to fully understand the nature of its constituents. Erythrocyte membrane has served as a model in studying cell membranes, mainly because erythrocytes are readily available in large quantities and their plasma membrane can be easily prepared. Since the discovery of globoside and hematoside in human erythrocytes by Klenk and Lauenstein (25) and Yamakawa and Suzuki (26), the glycosphingolipids in erythrocyte membranes have been the subject of great interest. Besides simple glycosphingolipids such as glucosylceramide, lactosylceramide, and ceramide trihexoside, the glycosphingolipids of mammalian erythrocytes can be classified into two types: (a) those of human, pig, guinea pig, and sheep, which contain N-acetylgalactosamine, and (b) those of rabbit and bovine, which contain N-acetylgalactosamine. Despite their common occurrence, galactosamine-containing glycosphingolipids have not been extensively studied.

Wiegandt (27) reported that gangliosides of human tissues contain only N-acetylenuraminic acid, while those isolated from cattle contain both N-acetyl- and N-glycolynuraminic acid. Our studies also showed that gangliosides isolated from bovine erythrocytes contain both species of sialic acid. Recently Wiegandt (28) has identified a monosialolactoisohexosylceramide from human spleen following ozonolysis and alkaline fragmentation of the total ganglioside fraction. The structure was shown to be NeuNacaα2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-Cer. The ganglioside E reported here has the same structure while ganglioside F contains a N-glycolynuraminic acid instead of N-acetylenuraminic acid.

Careful examination of structure of these gangliosides revealed some interesting facts. Except for Gs, the major gangliosides isolated from bovine erythrocytes contain glucosamine. In these glycosphingolipids, the sialic acid residue is always linked to the sugar unit at the nonreducing terminal of the saccharide chain. The shortest neutral hexosyl unit in these gangliosides is lactose. The longer chain neutral hexosyl units are formed by the addition of 1 or more disaccharide units: Galβ1 → 4GlcNAc to the galactosyl unit of lactosylceramide through β1 → 3 linkage. Addition of one such disaccharide will form lacto-N-neotetraosylceramide. A hexaglycosylceramide is derived from this tetraglycosylceramide by the addition of another Galβ1 → 4GlcNAc unit. The nonreducing terminals of these saccharide chains are substituted by either N-acetylenuraminic acid or N-glycolynuraminic acid. The ratio between N-acetyl- and N-glycolynuraminosylhexosides, N-acetyl- and N-glycolynuraminosyltetraosylceramide, and N-acetyl- and N-glycolynuraminosylhexaglycosylceramides is 1:3, 1:7, and 1:4, respectively. We also detected a ganglioside with octahexosyl unit, which sequential enzyme hydrolysis revealed to contain 3 repeating units of Galβ1 → 4GlcNAc attached to lactosylceramide. Therefore, the general structure of the gangliosides in bovine erythrocytes can be expressed as sialosylα2 → 3(Galβ1 → 4GlcNAc)αβ1 → 3Galβ1 → 4Glc-Cer. The value of n may range from 0 to 3.

Interestingly this type of repeating unit is also present in
keratan sulfate. Therefore, the saccharide chains in these gangliosides should be susceptible to the endo-β-galactosidase isolated from *Escherichia freundii* (29). As shown in Fig. 5, incubation of sialosylhexaglycosylceramide with this endo-β-galactosidase produced both triglycosylceramide and glucosylceramide. The biological significance of the repeating unit, Galβ1 → 4GlcNAc, in the glycosphingolipids of bovine erythrocyte membranes is still not clear although different serological activities due to the difference in the number of this repeating unit have been reported in human erythrocytes (30).

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