Gangliosides of Human Erythrocytes. A Novel Ganglioside with a Unique *N*-Acetylneuraminosyl-(2→3)-*N*-acetylgalactosamine Structure†

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ABSTRACT: A novel ganglioside having both *N*-acetylgalactosamine and *N*-acetylglucosamine was isolated from human erythrocyte membranes. Its structure was characterized by successive exoglycosidase treatment, methylation analysis, and direct-probe mass spectrometry of permethylated intact and desialylated glycolipid. The core structure of the ganglioside was found to be *N*-acetylgalactosaminyl paragloboside with sialosyl substitution by 2→3 linkage at the terminal GalNAc residue: NeuNAcα2→3GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer. The ganglioside was characterized by the presence of a ceramide with myristic acid as the major component.

Our recent studies on gangliosides of human erythrocyte membranes established the presence of nine monosialosyl gangliosides (G_1–G_9). These are hematoside (G_1), two sialosyl paraglobosides having sialosyl2→3Gal (G_2) and sialosyl2→6Gal (G_4), two sialosyllacto-*N*-norhexaosylceramides having sialosyl2→3Gal (G_6) and sialosyl2→6Gal (G_7), GM_{1b} ganglioside (G_5), and those having a branching structure with blood group I and H activities (G_8 and G_9) (Watanabe et al., 1978, 1979). One ganglioside, previously isolated and designated as G_3, has now been identified as having both *N*-acetylgalactosamine and *N*-acetylglucosamine. This ganglioside, characterized by a novel sialosyl2→3GalNAc structure, is described in this paper.

Materials and Methods

The monosialosyl gangliosides were isolated and purified from human type O erythrocyte membranes according to the methods as previously described (Watanabe et al., 1978, 1979). The gangliosides from the Folch's upper phase (Folch-Pi et al., 1951) were separated from neutral glycolipids by chromatography on DEAE-Sephadex A-25 (Yu & Ledeen, 1972). The monosialosyl gangliosides were separated into components by chromatography on silicic acid (Bio-Sil-A, Bio Rad Laboratories, CA), followed by preparative TLC[1] on silica gel H according to the methods previously described (Watanabe et al., 1978, 1979). The structure of purified ganglioside G_3 was determined by degradation with exoglycosidases (Hakomori et al., 1971), methylation analysis (Hakomori, 1964; Björndal et al., 1970; Stellner et al., 1973), and direct-probe mass spectrometry of permethylated glycolipid (Karlsson et al., 1974; Ledeen et al., 1974; Watanabe et al., 1975) before and after desialylation as previously described (Watanabe et al., 1979).

Results

The carbohydrate composition of the ganglioside was found to be quite unique; it contained both glucosamine and galactosamine. The molar ratio of Gal/Glc/GlcNAc/GalNAc/NeuNAc/sphingosine was found to be 2.2:1.0:0.8:0.7:0.9:0.7. The purified ganglioside G_3 showed the same R_{spg} value as brain GM_1 ganglioside (0.75 for G_3 and 0.72 for GM_1). However, the sialosyl residue of G_3 was readily hydrolyzed by sialidase. The desialylated core glycolipid, produced by sialidase or weak acid hydrolysis, migrated slower than asialo-GM_1 (R_{pg} value was 0.35 for asialo-G_3 and 0.51 for asialo-GM_1) (Table I). The asialo-G_3 was then hydrolyzed by jack bean β-*N*-acetylhexosaminidase and was converted to a compound having a similar mobility on TLC (R_{pg} = 0.96) as paragloboside. The paragloboside-like product was further degraded by jack bean β-galactosidase and was converted to a ceramide trisaccharide having a terminal *N*-acetylglucosamine (lacto-*N*-triosylceramide GlcNAcβ1→3Galβ1→4Glc→Cer). This glycolipid was degraded by beef kidney β-*N*-acetylglucosaminidase (Boehringer Manheim Biochemicals, IN) to a lactosylceramide.

Table I: Migration Rate of Enzymatic Products from Ganglioside G_3

enzyme	R_{pg} value[a]
intact G_3	0.08
sialidase	0.35
β-*N*-acetylhexosaminidase	0.96
β-galactosidase	1.50
β-*N*-acetylglucosaminidase	1.99
ganglio-*N*-tetraosylceramide (asialo-GM_1)	0.51
lacto-*N*-neotetraosylceramide	1.00
lacto-*N*-triosylceramide	1.51
lactosylceramide	1.89, 2.04[b]

[a] Rate of paragloboside is defined as 1.00, determined on a silica gel G plate developed with chloroform-methanol-water (65:25:4 v/v). [b] Double spots due to the difference in ceramide.

The sequence of carbohydrate was further confirmed by direct-probe mass spectrometry of the permethylated intact glycolipid and that of the permethylated desialylated compound (see parts A and B of Figure 1). The ions *m/e* 376 and 344 (376 − 32) assigned for *N*-acetylneuraminic acid were detected as a nonreducing terminal sugar from a permethylated intact ganglioside. Detection of the ions *m/e* 621 and 589 (621 − 32) indicated the presence of NeuNAc–HexNAc structure as a nonreducing terminal disaccharide residue,[2] and the ions

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[1] Abbreviations used: TLC, thin-layer chromatography; R_{spg} value, rate of TLC mobility as compared to that of sialosyl paragloboside as 1.00; R_{pg}, rate of TLC mobility as compared to that of paragloboside as 1.00.

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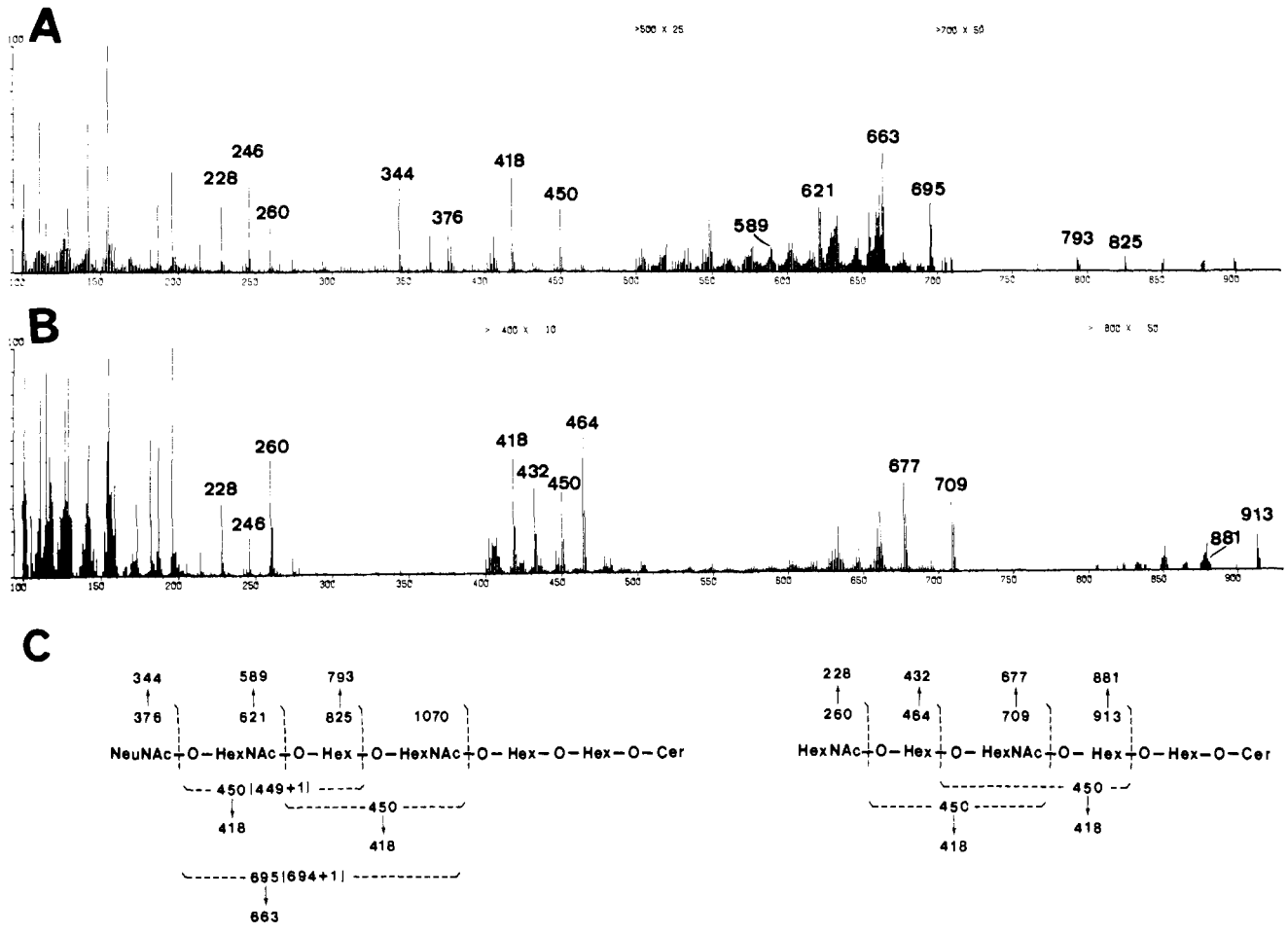


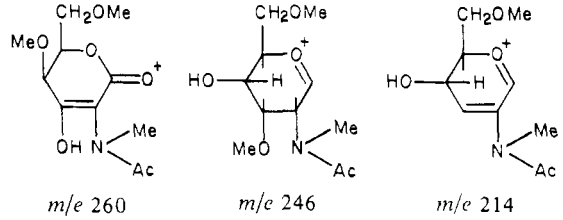
FIGURE 1: Mass spectra and simplified formula for interpretation of the fragment ions derived from permethylated intact and desialylated ganglioside G_3. Panel A: mass spectrum of permethylated intact ganglioside. Panel B: after desialylation. Mass spectrometry was performed with a Finnigan 3300 mass spectrometer with 6110 data system under the following conditions: electron energy, 30 V; ion energy programmed from +3.5 V; extractor, +8.0 V; lens, 35 V; emission, 0.5 mA; electron multiplier, 2220 V; sensitivity, 10^{-7} A/V. Ordinate, relative intensity (percent); abscissa, *m/e*. Panel C: simplified formula for interpretation of the fragment ions derived from carbohydrate moieties of permethylated intact and desialylated ganglioside. NeuNAc, *N*-acetylneuraminic acid; Hex, hexose; HexNAc, hexosamine; Cer, ceramide.

m/e 825 and 793 (825 − 32) are assigned to the terminal trisaccharide as NeuNAc–HexNAc–Hex. These ions are very unique for this glycolipid and have never been detected in other gangliosides. In addition to the ions *m/e* 450 and 418 (450 − 32), the ions for the internal disaccharide HexNAc–Hex or Hex–HexNAc, *m/e* 695 (694 + 1) and 663 (695 − 32), were detected, which were assigned to the internal trisaccharide HexNAc–Hex–HexNAc. The detection of these ions clearly indicated the presence of two hexosamines in one molecule. From the desialylated glycolipid, on the other hand, ions representing the nonreducing terminal hexosamine (*m/e* 260 and 228) were detected in addition to the ions *m/e* 464, 432 (HexNAc–Hex), 709, 677 (HexNAc–Hex–HexNAc), 913, and 881 (HexNAc–Hex–HexNAc–Hex). Thus, the ganglioside G_3 should have the carbohydrate sequence as follows: NeuNAc–HexNAc–Hex–HexNAc–Hex–Hex–Cer.

The results of the methylation analysis gave details of the carbohydrate linkages. As shown in Figure 2, the intact ganglioside, after methylation and hydrolysis, gave both 3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol and 4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol, in addition to 2,4,6-tri-*O*-methylgalactitol and 2,3,6-tri-*O*-methylglucitol. Desialylated ganglioside (asialo-G_3) gave, on methylation and hydrolysis, 3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol but did not give 4,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxygalactitol (Figure 2B). It also gave 2,4,6-tri-*O*-methylgalactitol and 2,3,6-tri-*O*-methylglucitol. No tetra-*O*-methylhexitol was detected in the hydrolysate of permethylated desialylated G_3 ganglioside. These data clearly indicated that sialosyl substitution is at the GalNAc residue with a 2→3 linkage.

The ganglioside failed to react with the following antibodies by double-diffusion precipitation on agarose gel plate with antibrain GM_1, anti-asialo-GM_1, anti-asialo-GM_2, anti-Forssman glycolipid, and antigloboside antibodies. The ganglioside after removal of sialic acid reacted weakly with anti-

[2] The permethylated intact G_3 gave an ion *m/e* 260 on direct-probe mass spectrometry. This ion has been assigned and regarded as the diagnostic value for the nonreducing terminal hexosamine (Karlsson et al., 1974; Ledeen et al., 1974; Watanabe et al., 1975). Obviously, an intact G_3 had no terminal hexosamine as judged on methylation analysis. The presence of *m/e* 260 in the direct-probe mass spectrometry is difficult to explain. It may be that NeuNAc2→3GalNAc may be cleaved and the sialic acid linked GalNAc could be converted to a structure as shown below (left) which has *m/e* 260 (259 + 1). The origin of *m/e* 246 and 214 (246 − 32) could be as shown below (center and right).



m/e 260 *m/e* 246 *m/e* 214

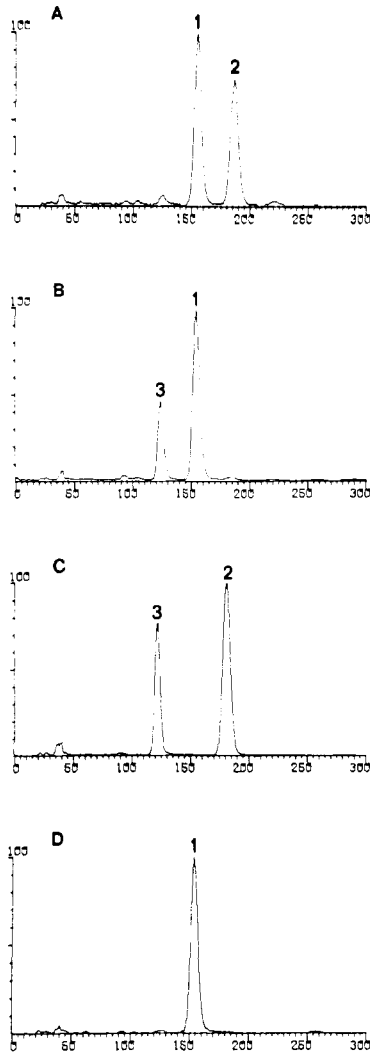


FIGURE 2: Mass fragmentogram for hexosamine (ion *m/e* 158) of the hydrolysate of permethylated intact and desialylated ganglioside. Panel A: intact ganglioside. Panel B: after desialylation. Panel C: Forssman glycolipid as the standard compound. Panel D: paragloboside as the standard compound. Identified peaks are (1) 3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol 1,4,5-tri-*O*-acetate, (2) 4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol 1,3,5-tri-*O*-acetate, and (3) 3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol 1,5-di-*O*-acetate, determined with a Finnigan 3300 mass spectrometer with 6110 data system. The permethylated sample, isolated and purified on Sephadex LH-20 column (Pharmacia Chemicals), was hydrolyzed in 90% acetic acid–0.5 N H_2SO_4 for 5 h under a nitrogen atmosphere. The partially O-methylated amino sugars in the hydrolysate were reduced and acetylated by the procedure previously described (Stellner et al., 1973). The entire modified procedure was recently described (Watanabe et al., 1978, 1979). Gas chromatography was performed on a 5 ft × 2 mm column of 3% OV-17 coated on Spectoport 80–100 mesh at 190 °C isothermal. Conditions of mass spectrometry: electron energy, 70 V; ion energy, 3.0 V; electron multiplier, 1800 V; sensitivity, 10^{-7} A/V. Ordinate, ion intensity; abscissa, scan number.

asialo-GM_2 and antigloboside antibody, respectively. This reactivity must be a cross reaction between the structures having a terminal β-GalNAc, indicating that the nonreducing terminal hexosamine of asialo G_3 should be β-*N*-acetylgalactosamine. On the basis of the above data, the chemical structure of ganglioside G_3 is proposed to be NeuNAcα2→3GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer. This ganglioside is characterized by the presence of a large proportion of myristic acid (Table II), and the sphingosine base was C_{18} sphinganine.

Table II: Fatty Acid Composition of Ganglioside G_3[a]

	%		%
12:0	0.3	unidentified	1.2
13:0	0.6	19:0	2.2
14:0	37.8	20:0	6.1
14:1	0.9	20:1	8.8
15:0	0.4	21:0	0.7
15:1	0.8	21:1	0.6
16:0	6.9	22:0	10.8
16:1	3.7	22:1	11.7
unidentified	0.6	24:0	1.0
18:0	1.3	24:1	2.4
18:1	1.2		

[a] Analyzed by gas chromatography on 3% ethyl-30 column after methanolysis according to the method of Gaver & Sweeley (1965).

Discussion

The results clearly indicate the unique structure of this ganglioside, containing (1) both *N*-acetylgalactosamine and *N*-acetylglucosamine and (2) a sialosyl2→3GalNAc structure. In all gangliosides hitherto isolated, the sialosyl residue was linked to the galactosyl residue of ganglio-*N*-tetraosylceramide or lacto-*N*-neotetraosylceramide or their analogues, and the linkage has been limited to α2→3Gal. An exception has been described which has sialosyl2→6Gal (Wiegandt, 1973; Watanabe et al., 1979). Ganglioside G_3 is now identified as having a unique sialosyl linkage, sialosyl-(2→3)-*N*-acetylgalactosamine. The ganglioside is a minor component of erythrocyte membranes, but a carbohydrate chain with the same sequence may be present in the glycoprotein. Kőscielak et al. (1978) reported that glycolipids with short fatty acid aliphatic chains may be serum-derived. Because this ganglioside also has a large amount of C_{14} fatty acid (myristic acid), it may be interesting to see if the same ganglioside is present in a larger quantity in serum.

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