Sphingolipid composition of human platelets

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Abstract Total lipid extracts from washed trypsinized human platelets were fractionated into neutral lipids, glycosphingolipids, and phosphclipids by silicic acid chromatography. The concentrations and chemical structures of the neutral and acidic glycosphingolipids were then studied in detail. On the basis of sugar molar ratios, studies of permethylation products, and the action of stereospecific glycosidases on the lipids, identifications were made of four neutral glycosphingolipids. Lactosylceramide was the most abundant type and accounted for 64% of the total neutral glycolipid mixture. The major fatty acids of the lactosylceramide were 20:0, 22:0, 24:0, and 24:1; the major long-chain base was 4-sphingenine. The platelets were surprisingly rich in a ceramide fraction, which represented 1.3% of the total platelet lipids. It had a different fatty acid composition than the neutral glycosphingolipid and ganglioside fractions. Hematoside was also isolated from the total lipid fraction of platelets; the neuraminic acid component was *N*-acetylneuraminic acid. Treatment of platelets with trypsin, chymotrypsin, or thrombin increased the yield of hematoside as compared with a control, while the level of ceramides was not changed. It was concluded that the platelets are similar to leukocytes, liver, and spleen in that lactosylceramide and hematoside are the principal neutral and acidic glycosphingolipids. The presence of a relatively high proportion of ceramide in platelets may be a unique characteristic of this cellular fraction of blood.

Supplementary key words ceramide · glycosphingolipid · hematoside · fatty acids · long-chain bases · neuraminic acid · methyl glycosides · alditol acetate · thin-layer chromatography · gas–liquid chromatography

Extensive studies have been made of the neutral lipid and phospholipid composition of human platelets (1–3). Details are lacking, however, about the occurrence of sphingolipids, except for some relatively brief reports (4–7). Free ceramides were identified recently in lipid extracts of human platelets (8). To complement previous studies on the glycosphingolipids of normal human plasma (9), erythrocytes (9), and leukocytes (10–12), as well as studies of these lipids in patients with lipid storage diseases (13–15) and leukemia (12), the present investigation was undertaken to determine the major sphingolipid constituents of human platelets.

Abbreviations: Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; NANA, *N*-acetylneuraminic acid; ceramide, 2-*N*-acylsphingosine; G_{M3} is the standard Svennerholm nomenclature for gangliosides; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography and mass spectrometry.

MATERIALS

Platelets

Human platelet concentrates were prepared according to a previously established procedure (16) which involved repeated differential centrifugation to remove erythrocytes. Residual erythrocyte contamination was estimated to be less than 0.2% (v/v) based on spectrometric assay (540 nm) of the hemoglobin content present in the supernatant solution. The pellet, obtained after centrifuging at 10,000 rpm (18,000 *g*) at 0°C for 5 min, was used for lipid extraction. Preliminary studies were performed on the total lipid extract from the residues of 73 platelet units after the isolation of platelet membrane glycopeptides by brief trypsin treatment (17). In addition, lipid extracts from nontreated platelets were compared with those obtained after incubations with trypsin, chymotrypsin, and thrombin.

Other materials

All solvents were of analytical grade and were redistilled before use. The hexane (Nanograde) used for extraction of fatty acid methyl esters was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. The methanol used in methanolysis (acid-catalyzed) of purified glycosphingolipids was dried over magnesium turnings. Methanol–HCl was prepared by bubbling gaseous hydrochloric acid into dry methanol at room temperature. Pyridine was redistilled from barium oxide

before use and was stored over KOH pellets after the distillation. A Hewlett-Packard model 402 gas–liquid chromatograph was used for GLC analyses. An LKB 9000 single-focusing mass spectrometer was employed to record mass spectra of GLC peaks.

Chemicals

Hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories, State College, Pa., and Anspec Co., Ann Arbor, Mich. *N*-Acetylneuraminyllactose and *N*-acetyl- and *N*-glycolylneuraminic acids were purchased from Sigma Chemical Co., St. Louis, Mo. Analytical grade powdered silver carbonate was obtained from Mallinckrodt. Unisil (200–325 mesh, Clarkson Chemical Co., Williamsport, Pa.) was utilized in column chromatography for the separation of glycosylceramides. Precoated plates (20 × 20 cm) of silica gel G from Quantum Industries, Fairfield, N.J., and Analtech, Newark, Del., were employed for TLC separations. Reference glycosphingolipids (glucosylceramide, lactosylceramide, trihexosylceramide, and globoside) were isolated from human and porcine erythrocytes, and hematoside was isolated from equine erythrocytes. The stereospecific glycosidases used in the anomerity studies were generous gifts from Dr. Y.-T. Li.

Ceramide (normal fatty acids) was a generous gift from Dr. Karin Samuelsson. A ceramide fraction containing hydroxy fatty acids was obtained from Sigma. DL-Sphingosine and dihydrosphingosine were purchased from Miles Laboratories, Elkhart, Ind., and tripalmitin was acquired from Supelco, Bellefonte, Pa. Fatty acid methyl ester standards for GLC were purchased from Applied Science Laboratories, and a polyunsaturated fatty acid standard (PUFA No. 1) was secured from Supelco. 3% EGSS-X on Gas-Chrom Q (100–120 mesh) was obtained from Applied Science Laboratories; 15% ethylene glycol adipate on Chromosorb WHP (80–100 mesh), 3% GC grade SE-30 on Supelcoport (100–120 mesh), 16% ethylene glycol succinate on Gas-Chrom P (80–100 mesh), and 3% ECNSS-M on Supelcoport HD (100–120 mesh) were acquired from Supelco. Hydrogen chloride (lecture bottle) was secured from Matheson Gas Products, East Rutherford, N.J.

METHODS

Extraction of total lipids

The trypsinized platelet pellet was extracted with chloroform–methanol 2:1 (v/v) according to the method of Folch, Lees, and Sloane Stanley (18), using a Waring blender to prepare the chloroform–methanol 2:1 homogenate. The mixture was filtered through a sintered-glass filter, and the extraction was repeated three times with chloroform–methanol 2:1, after which 0.2 vol of 0.75% NaCl was mixed with the combined filtrates (19). The biphasic mixture was allowed to stand overnight. The upper phase was then removed by aspiration, and the lower organic phase was washed with Folch's "pure solvents upper phase" (chloroform–methanol–water 3:48:47 [v/v/v]) (20) three times. The combined lower phases were evaporated to dryness in vacuo to yield a fraction of crude total lipids.

Isolation of glycosphingolipids

A portion (200–400 mg) of crude total lipids was fractionated into neutral lipids, glycosphingolipids and ceramides, and phospholipids by silicic acid chromatography as described previously (9). In the study in which the effects of protease or thrombin treatment was evaluated, a ceramide fraction was obtained by elution of the column with chloroform–methanol 98:2 (v/v) (21), and the glycosphingolipids were then eluted with acetone–methanol 9:1 (v/v). The crude glycosphingolipids (acetone–methanol 9:1 [v/v] fraction) were subjected to mild alkaline hydrolysis (9) to remove phospholipid contaminants, after which the crude glycosphingolipids were further purified by preparative TLC on 250-μ precoated plates developed in a paper-lined tank with chloroform–methanol–water 70:30:5 (v/v/v) as solvent (single development).

Acid-catalyzed methanolysis

The TLC plate was exposed briefly to iodine vapor, and individual bands were marked and scraped with a razor blade. The neutral glycosphingolipids were eluted from the gel (0.2–0.4 g) with 40–50 ml of chloroform–methanol–water 100:50:10 (v/v/v) at room temperature. The elution time was 1–2 hr. Hematoside was eluted under the same conditions with 40–50 ml of methanol–chloroform–water–pyridine 56:40:12:2 (by volume) (22), and ceramides were eluted with ethyl acetate. The recovered glycosphingolipids were subjected to acid-catalyzed methanolysis, using 3 ml of 0.75 N hydrochloric acid in anhydrous methanol for the neutral glycosphingolipids and 3 ml of 0.5 N methanolic HCl for the acidic glycosphingolipids. The glycosides of liberated galactosamine and methyl neuraminate were converted to *N*-acetyl derivatives by reaction with 0.2 ml of acetic anhydride at room temperature overnight. Details of the procedures for methanolysis and *N*-acetylation have been reported elsewhere (23).

Identification of fatty acid methyl esters

Methyl esters of fatty acids were recovered from the acidic methanolysate by hexane extraction. The methyl esters were then purified by preparative TLC on silica gel G with hexane–diethyl ether 85:15 (v/v) as the developing solvent (24), using methyl esters of palmitic

and α-hydroxy palmitic acids as markers. Bands were made visible with bromothymol blue and subsequently scraped from the plate within an hour to avoid extensive losses of short-chain esters by evaporation. A suspension of the silica gel in diethyl ether was packed into a small glass column, and the esters were eluted with diethyl ether (40 ml/g of silica gel) (25). Purified methyl esters were analyzed by GLC at 190°C on a glass column (6 ft by $^1/_8$ inch I.D.) packed with 15% ethylene glycol adipate. The hydroxy acids were analyzed as their trimethylsilyl methyl ester derivatives (26). The methyl esters were identified by comparing their retention times with those of standards and by coinjection of the unknown with an appropriate standard. Plots of relative retention times vs. carbon numbers were employed for the identification of fatty acids not represented in the standard. Areas were calculated from peak heights and widths at half height and the compositions were expressed as percentages of uncorrected total area.

Identification of methyl glycosides

Methyl glycosides were separated by GLC as *O*-trimethylsilyl derivatives at 160°C in a glass column (6 ft by $^1/_8$ inch I.D.) packed with 3% SE-30 on Gas-Chrom Q (100–120 mesh). Alternatively, the temperature was increased from 160°C initially to an upper limit of 230°C at a rate of 2°C/min. Peak areas were measured as before and quantitated by methods described previously (9, 23), using mannitol (100–200 μmoles) as an internal standard.

Identification of sphingosine bases

Purified glycosphingolipids were subjected to methanolysis by the method of Gaver and Sweeley (27), using 1 N aqueous methanolic HCl. After methanolysis, the reaction mixture was extracted with hexane three to five times to remove methyl esters, and the lower phase was neutralized with silver carbonate. The mixture was centrifuged, and the supernatant fraction was evaporated to dryness under nitrogen. The residue was dissolved in chloroform and applied to a column containing about 1 g of Unisil in chloroform. The column was eluted with 10–15 ml of chloroform (discarded), and the long-chain bases were then recovered with 10–15 ml of methanol. The methanol eluate was evaporated to dryness, and bases were *N*-acetylated with 50 μl of methanol–acetic anhydride 4:1 (v/v) at room temperature overnight. To facilitate the removal of excess acetic anhydride, butanol was added and the mixture was coevaporated under a stream of nitrogen. The acetylated bases were then converted to *O*-trimethylsilyl derivatives for GLC at 230°C on 3% SE-30. Reference *N*-acetyl sphingosines were used as standards.

A crude mixture of phospholipids (140 mg), eluted from the silicic acid column with methanol, was subjected to mild alkali-catalyzed methanolysis, using 1 ml of 0.6 N methanolic NaOH for each 10 mg of lipid. The solution of lipids was allowed to stand overnight at room temperature. Sphingomyelin was separated from alkali-stable lipids by TLC in chloroform–methanol–water 100:42:6 (v/v/v) (28). Long-chain bases were liberated from the sphingomyelin (22 mg) by acid-catalyzed methanolysis in 10 ml of the modified aqueous methanolic HCl (27). Selective *N*-acetylation of the free bases and conversion of the *N*-acetylated derivatives into 1,3-di-*O*-trimethylsilyl ethers was accomplished as outlined above, except that trimethylsilylation was carried out as described by Carter and Gaver (29). The derivatized bases were analyzed by GLC at 230°C on 3% SE-30 and 3% OV-17 columns. Reference samples of *N*-acetylated sphingosines were employed as standards. Plots of relative retention times vs. carbon numbers were used for identifications of bases not represented in the standard. The structures were further confirmed by GLC–MS of the trimethylsilyl derivatives on both polar and nonpolar columns, and the aldehydes derived from periodate oxidations of the free long-chain bases (30) were analyzed by GLC and GLC–MS on a 3% EGSS–X column at 130°C.

Identification of *N*-acylneuraminic acid

N-Acylneuraminic acid was liberated from the acidic glycosphingolipid under mild conditions with 0.03 N aqueous hydrochloric acid. After partitioning the hydrolysate with chloroform, the aqueous phase was evaporated to dryness and the product was further purified and identified by column and thin-layer chromatography according to the method of Puro (31). Authentic *N*-acetyl- and *N*-glycolylneuraminic acids were used as standards. The *N*-acylneuraminic acid was also characterized by GLC and GLC–MS as the trimethylsilylated derivative at 220°C on 3% SE-30.

Identification of ceramide

The ceramide fraction, isolated by TLC in the neutral solvent system, was further purified on a 500-μ silica gel G Uniplate (Analtech) in chloroform–methanol–glacial acetic acid 192:5:8 (v/v/v) (32). In other instances, ceramides obtained from silicic acid chromatography (98:2 fraction) were further purified on a 250-μ silica gel G Quantum plate with chloroform–methanol 95:5 (v/v) as the developing solvent (32). Authentic ceramides containing normal and hydroxy fatty acids were used as standards. After exposure to iodine, the band that corresponded to the standard was eluted from gel with chloroform–methanol 2:1 (v/v) and ethyl acetate after the iodine had sublimed. The fraction was

evaporated to dryness in vacuo and the residue was methanolized (27). Fatty acid methyl esters were recovered by hexane extraction and the extract was divided into two equal portions. Ester groups were estimated quantitatively by the method of Rapport and Alonzo (33) on one aliquot and the distribution of fatty acids was analyzed by GLC as described above. The sphingosine content was determined by a modification (34) of the method of Lauter and Trams (35), and the long-chain bases were identified by GLC and GLC–MS of the *N*-acetyl derivatives as 1,3-di-*O*-trimethylsilyl ethers.

Linkage studies

Neutral glycosphingolipids or gangliosides (1.0 mg) were dissolved in 0.5 ml of dry dimethyl sulfoxide and methylated according to the method of Hakomori (36), using 0.5 ml of the carbanion solution. The contents were sealed in small vials with Teflon-lined caps and sonicated for 30 min, and the reaction was then allowed to proceed for 6 hr, after which methyl iodide was added (36). After washing the contents with water and chloroform, the chloroform layer was reduced to dryness under a gentle nitrogen flow. The residue was dissolved in 1 ml of 2 N methanolic HCl and methanolysis was carried out at 120°C for 5 hr. Fatty acid methyl esters were extracted into petroleum ether. The permethylated methyl glycoside fraction was further hydrolyzed to free sugars with 1 ml of 2 N aqueous HCl at 100°C for 3 hr. The hydrolysate was neutralized, the methylated sugars were reduced with sodium borohydride, and alditol acetates were prepared with acetic anhydride–pyridine 1:1 (v/v) according to the method of Pepper and Jamieson (37). The alditol acetates were identified by GLC (38) and GLC–MS (39, 40) on 3% ECNSS-M at 175°C. The products from reference compounds of lactose, *N*-acetylneuraminyllactose, Fabry kidney trihexosylceramide, and globoside (porcine red cells) were employed as standards.

Anomeric configurations of the major platelet glycosphingolipids were determined by the use of specific glycosidases according to the methods described by Hakomori et al. (41) and Laine et al. (42).

RESULTS

Lipid composition

The relative proportions (wt %) of neutral lipids, glycosphingolipids plus ceramide, and phospholipids from the total lipid extract of trypsinized human platelets are given in Table 1. Neutral lipids constituted 25% of the total lipid fraction, whereas the sphingolipids and phospholipids accounted for 8% and 65%, respectively.

TABLE 1. Total lipid composition of platelets

Sample	Total	Neutral Lipids		Glycosphingolipids[a]		Phospholipids	
		Wt	% of Total Lipid	Wt	% of Total Lipid	Wt	% of Total Lipid
	mg	*mg*		*mg*		*mg*	
1	372	88	24	31	8	244	66
2	279	74	26	22	8	180	65
Mean			25		8		66

[a] Includes ceramide and G_{M3}. The yield is for crude glycosphingolipids before mild alkali-catalyzed methanolysis; phospholipid accounted for 50–60% of the weight.

Thin-layer chromatography

Commercially available precoated TLC plates of silica gel G were used for the separation of glycosylceramides. Uniplates gave a better separation of the globoside (R_F = 0.16) and hematoside (R_F = 0.07) than did Quantum plates (Fig. 1), as previously observed (43). After development in chloroform–methanol–water 70:30:5 (v/v/v), four neutral glycosphingolipids (Fig. 1, *I*, *II*, *III*, and *IV*) were located in the same areas as glucosylceramide, lactosylceramide, Fabry trihexosylceramide, and porcine globoside, respectively, and an additional band (*V*), which migrated between the origin and globoside in this solvent system, was shown by resorcinol spray to contain sialic acid.

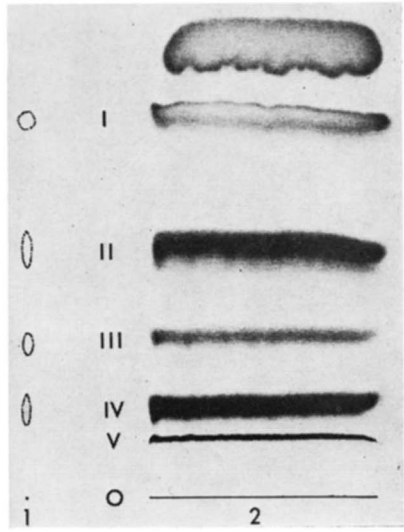

FIG. 1: TLC (on 500-μ precoated silica gel G plates from Analtech) of glycosphingolipid fraction from washed normal human platelets treated with trypsin. In lane *1*, reference standards from top to bottom are glucosylceramide, lactosylceramide, Fabry trihexosylceramide, and porcine globoside. In lane *2*, major neutral glycosphingolipids (*I–IV*) and acidic glycosphingolipid (*V*) are separated from crude total glycosphingolipid fraction of platelets. Unlabeled zone at top contains primarily ceramides along with methyl esters that were released from contaminating phospholipid by mild alkali-catalyzed methanolysis. The solvent system used was chloroform–methanol–water 70:30:5.

Gas–liquid chromatography

Representative gas–liquid chromatograms of the *O*-trimethylsilylated methyl glycosides of the neutral glycosphingolipids are shown in Fig. 2. The calculated average molar ratios of galactose to glucose were 1.1, 2.2, and 2.1 for bands II, III, and IV, respectively (Table 5). Glucose was the only component found in band I. *N*-Acetylgalactosamine was also present in IV, and the molar ratio of *N*-acetylgalactosamine to glucose was 0.8. The sialic acid-containing compound (V) had a galactose to glucose ratio of 1.2, and a molar ratio of *N*-acylneuraminic acid to glucose of 0.54, as shown in Fig. 3. These results agree with the assignment of bands I–V as monohexoside, dihexoside, trihexoside, tetrahexoside, and sialo-lactoside, respectively.

The ratio of *N*-acylneuraminic acid to glucose was somewhat lower than the theoretical value. The lower recoveries of neuraminic acid are not uncommon (44),

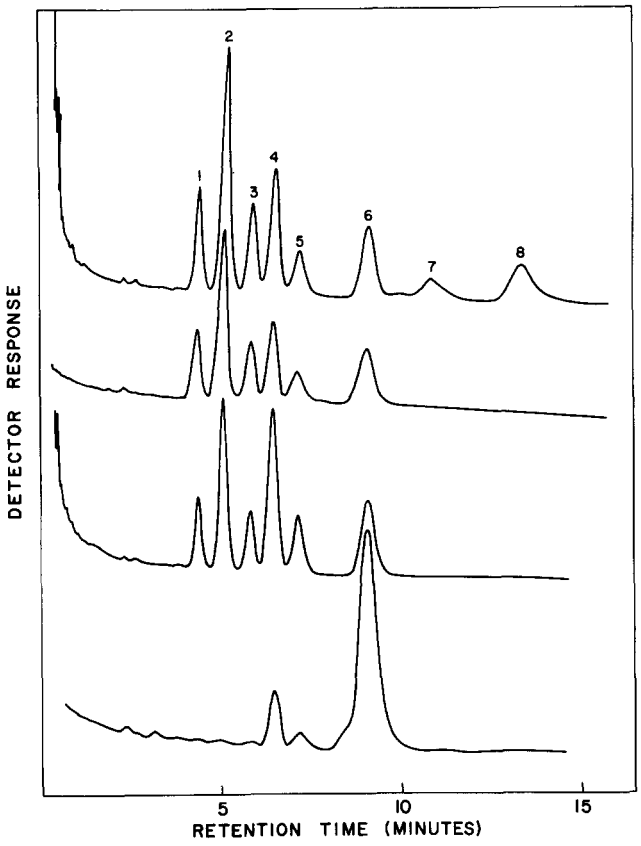

FIG. 2. Gas–liquid chromatograms of *O*-trimethylsilyl derivatives of methyl glycosides from major neutral glycosphingolipids of normal human platelets treated with trypsin. Peaks correspond to the following sugars: galactose, *1*, *2*, *3*; glucose, *4*, *5*; mannitol, *6*; and *N*-acetylgalactosamine, *7*, *8*. From top to bottom: GL-4 (IV), GalNAc(1→3)gal(1→4)Gal(1→4)Glc(1→1′)ceramide containing Gal:Glc:GalNAc 2.1:1.0:0.8; GL-3 (III), Gal(1→4)Gal(1→4)Glc(1→1′)ceramide containing Gal:Glc 2.2:1.0; GL-2 (II), Gal(1→4)Glc(1→1′)ceramide containing Gal:Glk 1.1:1.0; and GL-1 (I), Glc(1→1′)ceramide containing only glucose.

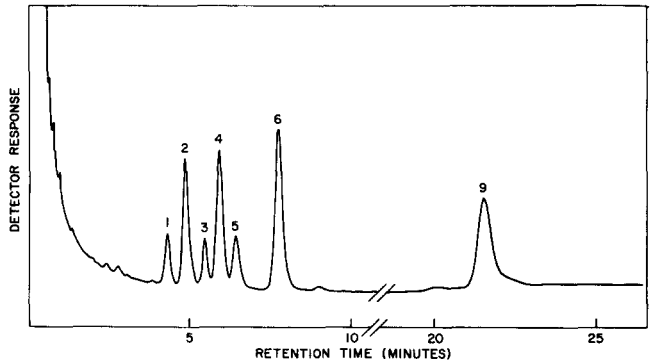

FIG. 3. Gas–liquid chromatogram of *O*-trimethylsilyl derivatives of methyl glycosides from G_{M3} (lower phase), NANA(2→3)-Gal(1→4)Glc(1→1′)ceramide containing NANA:Gal:Glc 0.6:1.1:1.0, of washed normal human platelets treated with trypsin. Peaks correspond to the following sugars: galactose, *1*, *2*, *3*; glucose, *4*, *5*; mannitol, *6*; and *N*-acetylneuraminic acid, *9*. Analysis was done on 3% SE-30, programmed from 165 to 230°C at 2°C/min with a carrier gas flow rate of 30 ml/min (nitrogen).

however, and may be the result of incomplete *N*-acetylation, partial acid destruction during methanolysis, overexposure to pyridine during the preparation of the trimethylsilyl derivatives (45), or loss during silicic acid chromatography (46). It is possible to obtain better relative values for neuraminic acid by using 0.5 N methanolic HCl during methanolysis and exposing to pyridine in the trimethylsilylation reaction for a short period of time, since the neuraminic acid values decrease slowly with time (45).

Linkage studies of the glycosphingolipids

The gas–liquid chromatographic retention behavior of the partially methylated alditol acetates from the three major neutral glycosphingolipids (bands II, III, and IV) were compared with the retention times of corresponding reference compounds (Table 2). Band I was

TABLE 2. Retention times of partially methylated alditol acetates from platelet glycosphingolipids[a]

	GLC Peaks			
	(a)	(b)	(c)	(d)
	min			
Lactose	15.9			32.9
Lipid II	16.3			32.7
Fabry kidney trihexosylceramide	16.3		31.7	33.2
Lipid III	16.3		31.7	32.9
Porcine globoside		29.9	31.5	33.0
Lipid IV		30.1	31.8	33.8
N-Acetylneuraminyllactose		30.1		33.3
Lipid V		29.7		32.9

[a] Peaks of partially methylated alditol acetates are represented as (a), 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; (b), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; (c), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol; and (d), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol.

not analyzed in this way because of insufficient material. Band II revealed two peaks with the same retention times as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol. Three peaks were found for III, with retention times identical with those of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol. Three peaks having the same retention times as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, were obtained from IV. Finally, V gave two peaks which corresponded to the retention times of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol.

The identifications of all alditol acetates were confirmed by GLC–MS (31, 39, 40) by comparing fragmentation patterns (Table 3) with those of corresponding reference compounds. These data indicate that II, III, and IV are structurally similar to erythrocyte lactosylceramide (GL-2), trihexosylceramide (GL-3), and globoside (GL-4), and that V is a hematoside.

Sphingosine bases

4-Sphingenine (75–85%) and sphinganine (5–8%) were the predominant bases in all neutral glycosphingolipid fractions, whereas band V contained a mixture of *erythro*- (30.6%) and *threo*-sphingenine (48.0%) as the major bases. The long-chain bases of ceramide consisted primarily of 4-sphingenine (83.2%) and sphinganine (9.3%). The molar ratios of bases to esters were 0.77:1.00 and 0.80:1.00 in duplicate analyses. 4-Sphingenine was also the major long-chain base in sphingomyelin and accounted for 75% of the total bases present. Sphinganine was a minor component (15%), along with hexadecasphingenine (5%), heptadecasphingenine (2%), and octadecasphingadienine (3%).

Fatty acid distribution

The fatty acid compositions of platelet glycosphingolipids are listed in Table 4. The lipids contained primarily 20:0, 22:0, 24:0, and 24:1, although 16:0 and 18:0 were also present. 2-Hydroxy acids were not found. Long-chain fatty acids (> 20:0) constituted 73–77% of the total normal fatty acids in GL-2, GL-3, GL-4, and G_{M3}, whereas 9–14% of normal acids were dominated by 16:0 and 18:0. GL-1 showed a high percentage of 18:0 (64.0%) and 18:1 (21.9%), with less of 16:0 (3.0%) and long-chain acids. The fatty acids in ceramide were mainly 20:0, 22:0, and 24:0.

N-Acylneuraminic acid

N-Acylneuraminic acid was liberated from band V under mild acid conditions. The R_F value of the sialic acid (0.40) was identical with that of authentic *N*-acetylneuraminic acid by TLC. It was further confirmed that the G_{M3} contained *N*-acetylneuraminic acid by GLC of the liberated neuraminate as the trimethylsilylated derivative. After mild acid hydrolysis the glycolipid product, which behaved on TLC like lactosylceramide, was recovered and subjected to acid-catalyzed methanolysis. The methyl glycosides were trimethylsilylated and analyzed by GLC, and the only components found

TABLE 3. Mass spectrometric identification of partially methylated alditol acetates from platelet glycosphingolipids[a]

	Intensities of Individual Peaks[b]									
	GL-2		GL-3			GL-4			G_{M3} (lower phase)	
m/e	I	IV	I	III	IV	II	III	IV	II	IV
43	100	100	100	100	100	100	100	100	100	100
45	32	16	34	17	17	24	18	14	24	17
71	8	5	7	6	4	6	5	6	7	5
75	5	3	4	3	3	2	4	3		3
87	14	16	14	14	15	9	14	13	7	16
99		9		12	12	5	9	10	3	12
101	30	13	32	15	16	15	16	13	17	17
113	6	21	5	20	19		21	20		19
117	27	41	29	38	42	46	40	45	52	42
127						5			4	
129	20	6	22	7	6	26	8	4	31	5
145	16		17							
161	14	4	15	4	4	14	4	3	15	2
205	7		8							
233		11		9	12	5	9	12	8	15

[a] Mass spectra and peak intensities of reference compounds from lactose, Fabry kidney GL-3, porcine GL-4, and *N*-acetylneuraminyllactose were in agreement with those given above for the corresponding peaks of the platelet lipids. Intensities are expressed relative to the intensity at *m/e* 43.

[b] Peaks of partially methylated alditol acetates are represented as (I), 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; (II), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; (III), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol; (IV), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol.

TABLE 4. Fatty acid compositions of human plasma, erythrocyte, and platelet glycopshingolipids[a]

	Ceramide	GL-1		GL-2		GL-3		GL-4		G_{M3} (lower phase)		
	Platelet	Platelet	RBC	Platelet	RBC	Platelet	RBC	Platelet	RBC	Platelet	RBC	Plasma
						% of total fatty acids						
14:0	0.5	0.6		1.3		0.8		1.0		tr		0.7
15:0	tr	1.0		0.6		0.8		1.1		tr		0.8
16:0	13.4	3.0	4.9	9.7	9.9	6.6	2.4	5.4	3.2	8.6	5.5	10.7
17:0	0.7	1.8		0.7		1.3		1.9		1.7		1.4
18:0	14.7	64.0	6.6	3.3	3.5	4.3	2.3	3.4	1.6	5.5	4.6	10.9
18:1	1.4	21.9	4.1	1.5	tr	1.1	tr	1.2	1.0	1.1	tr	3.5
18:2		3.4		0.7		1.1		1.7		1.4		
20:0	12.9	2.2	7.0	7.0	2.8	7.1		6.2	1.3	5.8	4.5	5.3
22:0	29.2	tr	7.5	34.7	13.5	35.7	14.4	37.6	20.6	33.9	21.1	23.0
22:1	tr	tr	14.6	4.2	3.3	4.2	2.6	tr		4.1	5.3	
23:0	4.0		5.4	3.2		3.0		3.5	4.0	3.4	3.8	9.9
24:0	22.4		27.0	20.1	40.9	20.3	48.1	29.6	49.1	21.3	38.1	18.9
24:1	tr		12.8	11.8	23.4	13.0	28.2	6.7	18.8	10.5	5.1	9.0
Others	0.7	3.0	10.0	1.0	2.5	0.6	2.0	0.6	0.4	3.2	12.0	5.8

[a] Fatty acids are denoted as chain length:number of double bonds. The amounts found are given as percentages of total area on gas chromatograms.

were galactose and glucose in a molar ratio of 1:1. It was therefore concluded that platelet band V was *N*-acetylhematoside (G_{M3}).

Concentrations of platelet glycosphingolipids

The composition of the platelet glycosphingolipid fraction is shown in Table 5, in which the averages from duplicate analyses are reported. It is evident that lactosylceramide (GL-2) was the major neutral glycosphingolipid in the platelet lipids and accounted for 64% of the total neutral glycosphingolipid fraction. There was an appreciable amount of hematoside, GL-3, and GL-4 in platelets too, but glucosylceramide was present in rather small amounts as compared with other tissues. Ceramide (washed platelets) was surprisingly high and accounted for 55% of the observed total sphingolipids including G_{M3}.

An attempt was made to determine whether there was a difference in the glycosphingolipid content derived from trypsin-treated and nontreated platelets. As shown in Table 5, the concentration of hematoside was much lower in the platelets that were not treated with trypsin; otherwise, the composition was not changed by trypsin treatment. A more detailed investigation of the effect of proteolytic enzymes on the G_{M3} and ceramide levels in platelets is summarized in Table 6. The concentration of G_{M3} was increased in all of the treated platelets, the greatest increase resulting from an incubation with thrombin. Ceramide levels were unaffected by pretreatment of the platelets with the proteolytic enzymes.

TABLE 6. Concentration of ceramide and hematoside in platelets treated with proteolytic enzymes[a]

Lipid Component	Control	Trypsin	Chymotrypsin	Thrombin
		μmoles/g TL		
G_{M3}	0.26	0.46	0.73	1.25
Ceramide[b]	9.85	9.44	8.59	9.08

[a] These analyses were carried out on separate batches of approximately 3 units of platelet concentrate (1 platelet unit is from 450 ml of whole blood) incubated with trypsin (1 mg/unit), thrombin (100 NIH units/unit), or chymotrypsin (1 mg/unit) at 37°C for 30 min (except chymotrypsin, which was incubated for 60 min) in 0.85% saline at pH 6.7 before extraction of the lipids. The control was incubated in the same way without added proteolytic enzyme.

[b] Averages of two determinations, each in duplicate.

TABLE 5. Concentration of glycosphingolipids in human platelets

Sample[a]	Lower Phase GL-1	GL-2		GL-3		GL-4			G_{M3}		
	μmoles/g TL[b]	*Gal/Glc*[c]	*μmoles/g TL*	*Gal/Glc*	*μmoles/g TL*	*Gal/Glc*	*GalNAc/Glc*	*μmoles/g TL*	*Gal/Glc*	*NANA/Glc*	*μmoles/g TL*
1	0.22	1.1	4.81	2.1	1.31	2.1	0.85	1.17	1.1	0.55	1.32
2	0.21	1.1	5.03	2.2	1.33	2.1	0.80	1.31	1.2	0.53	1.40
Mean	0.22	1.1	4.92	2.2	1.32	2.1	0.83	1.24	1.2	0.54	1.36
3	0.35	1.0	4.29	1.9	1.70	1.9	0.60	1.48	1.0	0.52	0.38

[a] Samples 1 and 2 are different weights and were analyzed at different times (about 2 months apart); they represent the lipid extract from trypsin-treated platelet residues. Sample 3 is the lipid extract from washed human platelets that were not pretreated with trypsin.

[b] TL, total lipids.

[c] Molar ratios of sugars as determined by GLC.

DISCUSSION

The results presented in this paper were obtained with a pooled sample of trypsinized platelets from approximately 75 donors. Simultaneous analyses of the glycoprotein composition of these platelets necessitated the trypsin treatment, and a comparison was therefore made on a smaller scale of untreated platelets and platelets treated with several proteolytic enzymes to determine whether the composition of the platelet sphingolipids was changed by preincubation with these enzymes. The yield of hematoside was substantially higher when the cells were trypsinized and was higher still after incubation with thrombin, but no other measurable changes were observed. It has been suggested that proteolytic enzymes act on the platelet surface, splitting a target protein and producing an active peptide that might stimulate the release reaction (47). The source of the additional hematoside and the mechanism of action of the proteolytic enzymes on membrane glycosphingolipids are not clear at the present time.

The yields of total phospholipids (65%) and neutral lipids (25%) and their compositions (not reported in detail here) were not remarkably different from the results reported by Marcus, Ullman, and Safier (2). Phosphatidylcholine was the major phospholipid and free cholesterol accounted for most of the neutral lipid fraction. The proportion of sphingomyelin was approximately equal to that reported for erythrocytes and was considerably higher than that of normal lymphocytes and polymorphonuclear leukocytes (11).

The solvent-soluble sphingolipid fraction of platelets consisted of sphingomyelin, ceramide, a family of neutral glycosphingolipids resembling those of plasma and erythrocytes (9, 13), and G_{M3} ganglioside. The molar ratios of sugars, comparisons of mass spectra of the intact lipids with those of reference glycolipids (48), and permethylation studies coupled with use of specific glycosidases confirmed that the major neutral glycosphingolipids were galactosyl(β1 → 4)glucosylceramide (GL-2), galactosyl(α1 → 4)galactosyl(β1 → 4)glucosylceramide (GL-3), and *N*-acetylgalactosaminyl(β1 → 3)galactosyl (α1 → 4)galactosyl(β1 → 4)glucosylceramide (GL-4). The G_{M3} ganglioside consisted exclusively of *N*-acetylneuraminyl(2 → 3)galactosyl(1 → 4)glucosylceramide. The stereochemical configurations of the glycosidic linkages in GL-1 and G_{M3} ganglioside have not been determined.

The composition of the neutral glycosphingolipid fraction of human platelets was recently reported by Snyder, Desnick, and Krivit (5), who found that GL-2 (38–43%) and GL-4 (23–31%) were the major components. Our results confirm this finding, although we observed that GL-2 was relatively much more dominant (64%) and GL-3 and GL-4 were present in about the same proportions (ca. 16%). GL-2 has also been shown to be the major neutral glycosphingolipid of human leukocytes (10–12). Platelets and leukocytes differ substantially in this respect from erythrocytes, in which GL-4 is the major neutral glycosphingolipid (9). It is interesting that in porcine leukocytes the concentration of GL-3 exceeds that of GL-2 (49). We have observed that this is also the case with porcine platelets, confirming the recent findings of Heckers and Stoffel (50). Thus, there appears to be a degree of species specificity in the composition of the neutral glycosphingolipids of these cells that is not observed in the erythrocytes.

Leukocytes are able to carry out the synthesis of GL-1 and GL-2 (49, 51), as are cultured cells of bone marrow (52). To our knowledge, comparable experiments have not been done with platelets to assess their enzymatic activity for glycosphingolipid biosynthesis.

It was previously reported that platelets contain G_{M3} ganglioside (4, 5, 53), but structural studies were not included in these earlier investigations. We have not examined the water-soluble ganglioside fraction as yet, and cannot comment therefore on the relative importance of G_{M3} compared with other platelet gangliosides. On the basis of a G_{M3} partition ratio of 0.4 between the upper and lower phases of a partition system (43), it can be calculated that the total platelet concentration of G_{M3} is about 1.9 μmoles/g of total lipid, a value that agrees reasonably well with the relative yields of G_{M3} given by Snyder et al. (5). It has been suggested that this ganglioside may have an immunologic role in transformed cells (54); it may also be involved as a receptor site on the platelet membrane for serotonin (55) and in platelet aggregation (5).

Platelets may be unique among mammalian cells in the high proportion of total sphingolipids accounted for by free ceramide, which confirms recent reports by Krivit and Hammarström (8) for human platelets and by Heckers and Stoffel (50) for pig platelets. It is clear from a variety of studies that ceramides are common constituents of animal tissues, including erythrocytes (21), plasma (56), aorta (57), liver (58), spleen (59), lung (60), kidney (32), and brain (61). In none of these cases is the concentration of ceramide as high as it is in platelets, however. It is possible that the ceramide fraction represents accumulation from glycosphingolipid turnover, especially since significant activities of various glycosyl hydrolases have been reported in platelets (5, 62). The fatty acid compositions of the various sphingolipid fractions do not support this theory, however. The ceramides contained significantly more 16:0 and 18:0 than any of the glycosphingolipids, while the level of 24:1 was much lower. The ceramide fraction also differed considerably in fatty acid composition from

ceramides (56) and other sphingolipids of human plasma. These results and the high concentration of the ceramide fraction suggest that its role in platelets may not be limited to that of an intermediate in the biosynthesis and degradation of more complex sphingolipids.

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