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QUARTERLY

The levels of glycosphingolipids, ceramides, sialic acid and glycogen are changed in plasma membranes of rat platelets harvested during recovery from immune-mediated thrombocytopenia*

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Plasma membranes of rat platelets produced at normal platelet counts and during early recovery from immune-mediated thrombocytopenia were examined for the contents of carbohydrates, lipids and glycosphingolipids. Glucosylceramide, two monosialo-gangliosides and one disialo-ganglioside were found to be the major glycosphingolipids of platelets. During thrombocytopenia the contents of these glycosphingolipids as well as of ceramides were several fold elevated. Among carbohydrate constituents of platelets and platelet plasma membranes, glycogen content was increased and that of sialic acid decreased. These results are discussed in the light of literature data on relevant biochemical characteristics of megakaryocytes at different stages of maturation and on thrombopoiesis during acute experimental thrombocytopenia.

Platelet count in blood is maintained through a feedback control whereby thrombocytopenia stimulates thrombopoiesis. Platelets are released by mature, polyploid megakaryocytes which differentiate through endomitosis from diploid progenitors [1–3]. A great deal about thrombopoiesis has been learned from studies on small rodents serving as a model of acute, experimental thrombocytopenia. In the immune-mediated model, animals are injected with anti-platelet serum, which leads to a rapid destruction of most of the circulating platelets [4]. During the recovery phase, both thrombopoiesis and the maturation rate of megakaryocytes are accelerated and larger than normal platelets are produced [4–7]. Polyploidization of megakaryocytes is increased but only after a lag period of about 48 h [6, 7]. In contrast, an increase of MPV occurs as early as a few hours after induction of thrombocytopenia [4–7]. The latter finding was interpreted to mean that large platelets could originate from megakaryocytes with incompletely formed demarcation membranes [8]. This view is not universally accepted [9]. Biological effects of thrombocytopenia are mediated to a large ex-

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Abbreviations: CPD, citrate-phosphate-dextrose; GLC, gas liquid chromatography; GSL(s), glycosphingolipid(s); HPLC, high pressure liquid chromatography; MPV, mean platelet volume: PMSF, phenylmethylsulfonyl fluoride; Fuc, fucose; GalN, galactosamine; GlcN, glucosamine; Gal, galactose; Glc, glucose; Man, mannose; Sial, sialic acid; Sphi, sphingoid base; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

tent by thrombopoietin, a recently cloned thrombopoietic hormone [10].

Glycosphingolipids and other cell surface glycoconjugates are markers of cell differentiation [11] yet they have been seldom investigated in studies on thrombopoiesis [12–14]. Application of flow cytometry with the use of antibodies and lectins against carbohydrate antigens brought about rather disappointing results because of a close similarity of surface characteristics of megakaryocytes and platelets [15]. In the present study we have quantitated GSLs, lipids and carbohydrates in control and immuno-thrombocytopenic plasma membranes of rat platelets. The results of this study reveal that contents of these constituents in thrombocytopenic platelets are changed, most likely due to the accelerated maturation of megakaryocytes in course of thrombocytopenia.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 250–300 g were used in all experiments. Rabbits were cross-breeds of New Zealand and Belgian Giant strains.

Isolation of rat platelets. Blood samples from hepatic vein of the animals lightly anaesthetized with chloroform/ether were collected into CPD-adenine 1 anticoagulant (6:1, v/v). The samples were further processed exactly as in [16] except for the last two steps. In the original method it consisted of two successive centrifugations of platelets in citrate buffer, pH 6.5, at $750 \times g$ for 15 min. Under these conditions platelets and other blood cells sediment together. Therefore, at the last two steps of our procedure platelets were suspended in 10 vol. of 0.9% NaCl containing 1 mM PMSF and centrifuged at $80 \times g$ for 4 min to deposit residual erythrocytes and leukocytes. The platelets remaining in the supernatant were then sedimented by centrifugation at $750 \times g$ for 15 min. Final preparations of platelets were of 99.9% purity, according to light microscopy. For single analyses platelets from three rats were pooled.

Anti-rat-platelet serum. This was prepared essentially by the method of Rolovic *et al.* [17]. The only difference was that rabbits were given five intravenuos injections of 10^{10} platelets every fourth day instead of ten injections of 2×10^9 platelets in the foot pad in the complete Freund's adjuvant over a six-week period.

Induction of immune-mediated thrombocytopenia. Rats were given single intraperitoneal injections of 0.5 ml of anti-rat-platelet serum. Blood was collected 48 h later from hepatic vein.

Isolation of platelet plasma membranes. The membranes were isolated by centrifugation in Percoll (Pharmacia, Sweden) gradient according to Perret *et al.* [18]. Platelets were suspended in cold 10 mM Tris/HCl lysis buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and sonicated three times at 0°C with 10 s and 20 s breaks between pulses and 25 W output power using a Labsonic U model sonifier (Brown, U.S.A.). Platelet lysate was then centrifuged at $1000 \times g$ for 10 min and the supernatant mixed with appropriate volume of Percoll, five fold concentrated lysis buffer and water to obtain a starting density of 1.05 g ml^{-1} and osmolality similar to that of the lysis buffer. Samples of 18.5 ml were subsequently centrifuged in the TV 865B vertical rotor of a Sorvall model Combi plus (Du Pont Co., U.S.A.) ultracentrifuge at $32000 \times g$ for 20 min at 20°C. The membrane band was aspirated from the top. Percoll and residual non-membrane proteins were then removed by filtration through a 90 mm × 300 mm column packed with Sepharose 2B (Sigma Chem. Co., U.S.A.) in lysis buffer. Membranes were eluted with the lysis buffer in the void volume of the column.

Platelet counting and sizing. Samples were diluted to give a final count between 100000 and 300000 platelets μl^{-1}. Concentrations of platelets and MPV were determined using a Cell-Dyn 1500 Hematology Analyser made by Sequoia-Turner Co. (U.S.A.).

Fractionation of plasma membranes. The membranes (0.5–1.0 mg) were suspended in 0.2–0.3 ml water and extracted with 5 ml of chloroform/methanol (2:1, v/v) at room temperature for 24 h and centrifuged. The residue was reextracted with chloroform/methanol (1:2, v/v) for 5 h and centrifuged again. The organic solvent extracted fractions of the membranes were dried *in vacuo* and used directly for carbohydrate analysis.

Chloroform/methanol, 2:1 and 1:2, extracts were pooled and evaporated to dryness under a stream of nitrogen. The residue was sub-

sequently dried *in vacuo* over phosphorus pentoxide, acetylated and fractionated as acetates on SPE Florisil (6 ml) columns (Baker JT, Holland) into cholesterol, GSLs and phospholipids as described by Saito & Hakomori [19]. GSLs were subsequently deacetylated, neutralized with ethyl acetate, partitioned twice with 7.5 ml of chloroform/methanol/water (8:4:3, by vol.), and the materials soluble in the lower phase separated into ceramides and GSLs by chromatography on 1 ml SPE Supelclean LC-Si columns (Supelco Inc., U.S.A.) [20]. After initial washing of the column with 15 ml of chloroform, ceramides were eluted with 15 ml chloroform/methanol (98:2, v/v) and GSLs with 15 ml of acetone/methanol (9:1, v/v) and 15 ml of acetone/methanol (8:2, v/v) [20]. For characterization and quantitation of gangliosides, control and thrombocytopenic platelets (1.5×10^{10} cells from 6 rats and 4.5×10^{9} cells from 8 rats, respectively) were separately extracted with successive portions of chloroform/methanol (2:1, v/v), chloroform/methanol (1:1, v/v), chloroform/methanol (1:2, v/v) and finally with chloroform/methanol/water (30:60:8, by vol.) solvent mixture. The extracts from control and thrombocytopenic platelets were then separately pooled, dried under vacuum, and partitioned in chloroform/methanol/water (8:4:3, by vol). The upper phases were then evaporated to dryness, degraded in 0.6 M NaOH in methanol for 20 h at 4°C, dialysed and finally separated on 1 ml columns packed with DEAE-Sephadex A-25 (Pharmacia, Sweden), in acetate form. Neutral GSLs were eluted with chloroform/methanol/water (30:60:8, by vol.) and gangliosides with the same solvent mixture but containing in addition 0.2 M ammonium acetate. Individual ganglioside bands were scraped from TLC plates that had been developed with chloroform/methanol/aqueous 0.02% $CaCl_2$ (60:40:9, by vol.). Ganglioside bands were visualized with 0.005% primulin in acetone/water (4:1, v/v) under an UV lamp.

Determination of carbohydrates. For determination of sialic acid, samples were hydrolysed with 0.2 M TFA for 1 h at 80°C. To release neutral sugars and hexosamines, the organic solvent extracted fractions of platelets or platelet membranes or whole platelets were hydrolysed with 2 M TFA for 4 h at 100°C. GSLs were hydrolysed under the same conditions but only for 2 h. Monosaccharides, sialic acid and maltose were determined by HPAEC-PAD with a Dionex series 4500i system (Dionex Co., Sunnyvale, CA, U.S.A.), a Carbopac PA-1 analytical column (4 mm × 250 mm) and a Carbopac PA-1 guard column (4 mm × 40 mm). The PAD had electrode potentials set on $E_1 = +0.05$ V, $E_2 = +0.6$ V, $E_3 = -0.6$ V with pulses of 300, 120 and 60 msec durations, respectively. Neutral sugars and hexosamines were eluted with 16 mM NaOH, and sialic acid and maltose with 150 mM NaOH. Separations were achieved isocratically at ambient temperature. Samples were injected on a 25 µl sample loop. Flow rate was 1 ml min^{-1}. Standards containing Fuc, GlcN, GalN, Gal, Glc and Man were from Dionex. Internal standard (3-*O*-methyl glucose) and sialic acid and maltose were from Sigma (U.S.A.).

Determination of fatty acids. Fatty acid methyl esters, prepared by heating samples in 1.5 M methanolic HCl at 80°C for 20 h, were determined by GLC with a Philips gas chromatograph, model PU 4410 equipped with a flame ionization detector. The carrier gas was helium and the column (CP-Sil 88, 2 m in length and 4 mm in diameter) was from Chrompack International (Holland). The column was heated at 170°C for 10 min and then programmed to 220°C at 1°C min^{-1}.

Other chemical analyses. Cholesterol was determined as in [21], lipid P as in [22], sphingoid base as in [23], protein as in [24].

Enzyme activities. Acid phosphatase (EC 3.1.3.2), 5'-nucleotidase (EC 3.1.3.5), and β-galactosidase (EC 3.2.1.23) were determined as in [25]; phosphodiesterase (EC 3.1.4.1) as in [26]; lactic acid dehydrogenase (EC 1.1.1.27) as in [27]; glucose 6-phosphatase (EC 3.1.3.9) as in [28].

Identification of glycogen. This was done by treatment of 1 mg portions of the organic solvent extracted fractions of platelets or platelet plasma membranes with α-amylase (Sigma). Samples were first suspended and briefly homogenized in 100 µl aliquots of 0.02 M phosphate buffer, pH 6.9. Portions of α-amylase (5 U in 100 µl of the phosphate buffer) were added and the samples incubated at 37°C for 1 h. Maltose, the reaction product, was determined by HPAEC-PAD.

Statistical analysis. Statistical comparisons were made using independent *t* tests.

RESULTS

Counts and volumes of control and thrombocytopenic platelets

Figure 1 shows platelet counts and MPV after induction of thrombocytopenia in rats. Platelet count was found to drop to less than 30000 at 2 h and returned to about 1/3 of the normal count at 48 h. In preliminary experiments we isolated control platelets and those harvested at 24 h and 48 h after induction of thrombocytopenia. We noticed that, due to the large size of platelets harvested at 24 h, it was difficult to separate them from other blood cells by differential centrifugation. Therefore we limited our studies to the platelets collected 48 h after induction of thrombocytopenia.

Isolation of neutral GSLs and lipids

For isolation of neutral GSLs we have selected the method of Saito & Hakomori [19]. The procedure involves an acetylation-deacetylation step and is more laborious than conventional techniques. Yet it allows to obtain a fraction of total neutral GSLs that is free of sphingomyelin. Thus, neutral GSLs in this fraction, after separation from ceramides, can be quantitated by independent determinations of glucose and sphingoid base. Saito & Hakomori [19] did not measure recoveries of cholesterol and phospholipids. According to our determinations cholesterol was recovered in 85.3 ± 2.1% (n = 3) and phospholipids (lipid P) in 83.0 ± 2.6% (n = 3). Calculations were referred to cholesterol and lipid P contents in the crude organic solvent extracts of the membranes. Recoveries of pure cholesterol and phosphatidylcholine, subjected to the isolation procedure, were higher and amounted to about 90%.

Neutral GSLs and carbohydrates contents in control and thrombocytopenic platelets

Analysis of neutral GSL fractions of both control and thrombocytopenic platelets revealed that glucose was its major carbohydrate component. This fraction contained also sphingoid base at 1:1 molar ratio with glucose strongly suggesting that glucosylceramide was the major glycosphingolipid of rat platelets. This was confirmed by TLC (see Fig. 2). A significant rise of glucosylceramide in platelets occurred during thrombocytopenia from 1.6 nmol per 10^9 control platelets to 9.4 nmol per 10^9 thrombocytopenic platelets and was 2.6 times greater than the concomitant increase of MPV (in this experiment from 3.5 fmol to 8.0 fmol). These results were obtained with pooled control and pooled thrombocytopenic platelets from four rats each. The content of gangliosides, quantitated in terms of lipid bound sialic acid, amounted to 0.44 nmol per 10^9 control platelets. In thrombocytopenic platelets, the increase of gangliosides was by 65% larger than the increase of MPV. The molar ratio of gangliosides, expressed as lipid bound sialic acid, to glucosylceramide in control and thrombocytopenic platelets was 0.3 and 0.18, respectively.

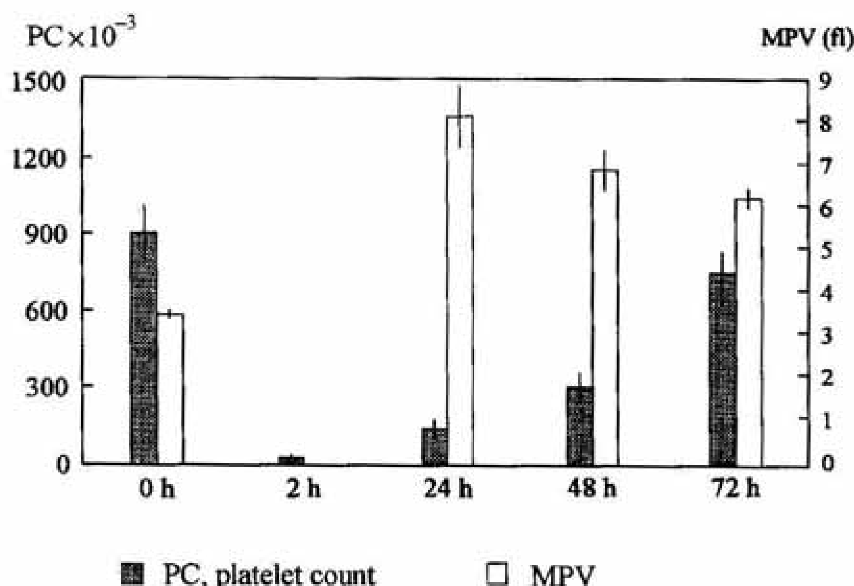

Fig. 1. Platelet counts (PC) in blood and mean platelet volume (MPV) after administration of anti-platelet serum.

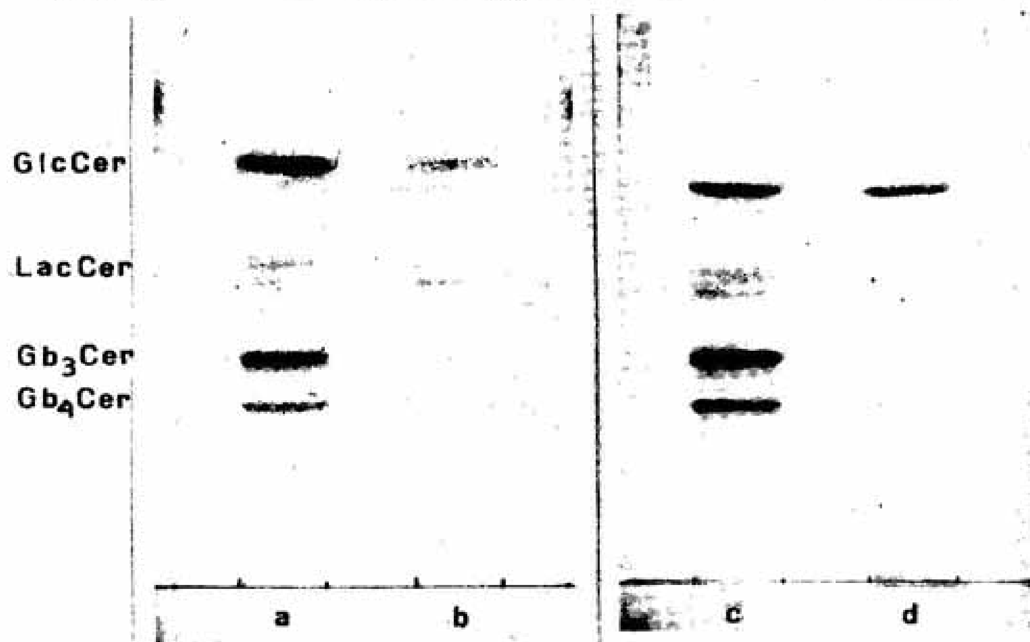

Fig. 2. Thin-layer chromatography of glycosphingolipids from rat platelets. Lanes a, c show orcinol stained GSLs standards from human erythrocytes; lanes b and d show equivalent aliquots of GSLs from control and thrombocytopenic platelets, respectively. Solvent: chloroform/methanol/water (65:35:8, by vol.).

In a separate experiment we tentatively characterized and quantitated individual gangliosides of whole platelets. Both control and thrombocytopenic platelets gave on TLC the same three ganglioside bands I, II, III with R_F values relative to bovine brain GM1 ganglioside of 1.07, 0.93 and 0.67, respectively. After desialylation with 1 M formic acid at 100°C for 45 min all three gangliosides either from control or thrombocytopenic platelets were converted to materials migrating on TLC with the R_F value of 1.04 relative to desialylated GM1 standard. Yields (nmoles per 10^9 platelets) and molar composition of gangliosides isolated from thrombocytopenic platelets were as follows: ganglioside I, 0.23 nmol, GalN 1.0, Gal 2.2, Glc 0.7, Sial 0.8, Sphi 1.0; ganglioside II, 0.24 nmol, GalN 1.0, Gal 2.3, Glc 1.0, Sial 0.7, Sphi 0.9; ganglioside III, 0.36 nmol, GalN 1.0, Gal 2.1, Glc 3.0, Sial 2.1, Sphi 1.1. High glucose content in ganglioside III resulted most likely from a contamination with this ubiquitous carbohydrate. This conclusion is supported by an almost theoretical sphingosine content in ganglioside III. Thus gangliosides I, II, and III were assigned to the "ganglio" series tetraglycosylceramides with either one or two sialic acid residues per mole. All three gangliosides had identical fatty acid compositions with C16:0, C18:0 and C18:1 being the major constituents (C16:0, 21–25%; C18:0, 39–41%; C18:1 18–19.5%). Yields of gangliosides from control platelets (nmol per 10^9 platelets) were much lower and therefore gangliosides I and II were analysed together: ganglioside I+II, 0.05 nmol, GalN 1.0, Gal 2.3, Sial 1.1; ganglioside III, 0.07 nmole, GalN 1.0, Gal 2.2, Sial 2.5. Due to small amounts of the materials available, the results of glucose determinations were unreliable; sphingosine was not determined.

We have also measured carbohydrates in whole platelets. The results of these determinations will be published elsewhere. Data that are relevant to this study concern sialic acid and glucose. Interestingly, in spite of a large difference in MPV, sialic acid content in thrombocytopenic platelets was decreased in comparison to control platelets by 10% and amounted to 12.0 nmol per 10^9 cells. Glucose that was released by acid hydrolysis rose from 52.2 nmol to 95.4 nmol per 10^9 cells and thus kept level with the increase in cell volume. The glucose was subsequently identified as glycogen-derived because after treatment of the organic solvent extracted fractions of platelets with α-amylase about 80% of the total acid-hydrolysable glucose was converted to maltose. With liver glycogen this figure amounted to 65%.

Plasma membranes

Platelets produced in the course of immune-mediated thrombocytopenia are not only larger than normal but also richer in endoplasmic reticulum and the Golgi complex [29]. Both structures are known to contain glycoconjugates. It was therefore of interest to measure GSLs in comparable cellular structures, i.e. plasma membranes. The identity of platelet

plasma membranes was ascertained by activities of the enzymes characteristic of the plasma membranes (see Table 1). In comparison to platelet lysate the activities of plasma membrane associated enzymes, i.e. of acid phosphatase, phosphodiesterase and 5′-nucleotidase were increased. On the other hand, the activities of lysosome associated β-galactosidase and cytosol associated lactic acid dehydrogenase were decreased.

Table 1
Specific activities of enzymes in platelet lysate and plasma membranes

	Lysate	Membranes	Ratio to lysate
	nmol substrate/mg protein per h		
Acid phosphatase	4190	15385	3.7
Phosphodiesterase	100	1200	12.0
5′-Nucleotidase	29	172	5.9
β-Galactosidase	381	60	0.2
Lactic acid dehydrogenase	8290	400	0.06

GSLs and carbohydrates in control and thrombocytopenic platelet membranes

Table 2 presents the contents in plasma membranes of neutral GSLs and carbohydrates. The latter were determined in the organic solvent insoluble fractions of the membranes. Like in intact platelets, glucose was a major component of the GSL fraction of platelet plasma membranes, confirming that glucosylceramide was the predominant neutral glycolipid of rat platelets. This conclusion was supported by TLC (not shown). Glucosylceramide content was significantly elevated in the membranes of thrombocytopenic platelets. Elevation of glucosylceramide was accompanied by a significant rise of ceramides. Thrombocytopenic and control platelet membranes were examined for gangliosides by TLC under the same conditions as applied for gangliosides of whole platelets. The same three gangliosides I, II, and III as in whole platelets were observed in plasma mem-

Table 2
Carbohydrate and lipid composition of neutral glycosphingolipids (GSLs) and the organic solvent insoluble fractions (OSIF) of platelet membranes from control^{C} and thrombocytopenic^{T} rats.
Means and standard deviations refer to platelets from the four groups of animals of three rats each.

	$GSLs^{C}$	$GLSs^{T}$	$OSIF^{C}$	$OSIF^{T}$
	nmol/100 nmol cholesterol			
Fuc	trace	0.23 ± 0.1	2.9 ± 0.5	3.3 ± 0.7
GalN	0.02 ± 0.01	0.13 ± 0.07	3.8 ± 0.4	4.1 ± 1.6
GlcN	0.02 ± 0.02	0.11 ± 0.1	22.8 ± 4.1	16.8 ± 5.8
Gal	0.06 ± 0.02	0.36 ± 0.1	19.2 ± 3.5	21.0 ± 5.6
Man	0.0	0.0	13.1 ± 2.6	11.1 ± 3.5
Glc	0.59 ± 0.1	1.63 ± 0.17	3.3 ± 1.5	12.9 ± 2.4**
Sial	nd	nd	3.1 ± 0.1	2.4 ± 0.3**
Sphi	0.72 ± 0.08	2.03 ± 0.2*	nd	nd
Ceramides	0.6 ± 0.04	2.3 ± 0.5	nd	nd
Lipid P	91.1 ± 10.7	89.0 ± 11.6		
Protein[1]	nd	nd	123.5 ± 5.4	110.3 ± 26.2

*$P < 0.001$; **$P < 0.005$; [1]µmol/100 nmol cholesterol

branes. On visual inspection of TLC plates the contents of the gangliosides appeared to be higher in thrombocytopenic than in control platelets but precise determinations were not performed. The neutral GSL fraction of both control and thrombocytopenic platelet membranes contained also minor amounts of galactose and other carbohydrates. These minor components were also elevated in thrombocytopenic platelet membranes but interpretation of this observation requires caution (see Discussion).

Among carbohydrate constituents of the organic solvent extracted fractions of the membranes, glycogen-derived glucose and sialic acid were the only sugars that were, respectively, significantly increased and decreased. Except for glucose, carbohydrates of the organic solvent extracted fractions can be expected to represent the total content of glycoproteins and proteoglycans minus the amount of the protein bound carbohydrate that became dissolved in organic solvents and subsequently was retained on silicic acid columns. According to our estimate the dissolved portion does not exceed 22% of the total membrane carbohydrate. Protein-bound sialic acid dissolved to the extent of 14%.

DISCUSSION

Our results show that glucosylceramide is the predominant neutral GSL of rat platelets whereas gangliosides are represented by two monosialo- and one disialo-tetraglycosylceramides, presumably belonging to the "ganglio" series of GSLs. All these GSLs are clearly elevated in platelets and platelet plasma membranes under conditions of thrombocytopenia. Other neutral GSLs may also be present in thrombocytopenic platelets as suggested by the presence of small amounts of galactose and other carbohydrates in the neutral GSL fraction of the platelet plasma membranes. At this point, however, caution is required since the error in quantitation of small carbohydrate peaks by HPLC may be quite large. Also, even though our platelet preparations were of a purity exceeding 99.9%, a contamination with GSLs from other blood cells cannot be entirely excluded as the latter are very much larger than platelets. Apart from GSLs also ceramides were elevated in platelet plasma membranes.

In addition to GSLs we have also determined carbohydrates in the organic solvent extracted fractions of the platelet plasma membranes. Only the glycogen-derived glucose and sialic acid contents of these materials from thrombocytopenic platelets were significantly different from those of control platelets. Similar changes i.e. lower content of sialic acid and higher of glycogen-derived glucose were observed in whole thrombocytopenic platelets.

In our experimental design, platelets from thrombocytopenic rats were harvested 48 h after administration of anti-platelet serum. According to literature data, at this time megakaryocytes already have responded by endomitotic divisions but, most likely, have not yet fragmented into platelets [30]. Thus, our results should be discussed in the context of maturation rate of megakaryocytes rather than of their polyploidization. Maturation occurs in each principal ploidy class of megakaryocytes [6] and it is assumed that each class gives rise to platelets. During maturation megakaryocytes acquire cytoplasmic granules, vesicles, organelles, and demarcation membranes but lose the ability to synthesize DNA [31]. Schick & He [14] studied biosynthesis of GSLs and of ceramides in guinea pig megakaryocytes at different stages of maturation. The biosynthesis of these substances from palmitate occurred primarily in the most immature megakaryocytes whereas formation from acetate predominated in the cells of intermediate maturity. Palmitate was incorporated into both sphingoid base and fatty acids but acetate only into fatty acids. Thus, the biosynthesis of GSLs occurs predominantly in immature megakaryocytes. Interestingly, a label from radioactive galactose was incorporated only into glucose of glucosylceramide and not into more complex GSLs that were identified in guinea pig megakaryocytes and platelets. Platelets retain to some extent the capacity to synthesize ceramides but not GSLs. Admittedly, the GSL biosynthesis may not be necessarily correlated with GSLs contents in immature and mature megakaryocytes. In view of the fact, however, that the maturing megakaryocytes undergo a major increase in size [6, 32, 33] with a concomitant accumulation of phospholipids and cholesterol [34] it is likely that

the ratio of GSLs to protein, cholesterol, and phospholipids would decrease during normal maturation. Thus, during accelerated maturation one would expect a higher ratio of GSLs to other membrane components and thus higher contents of GSLs in thrombocytopenic platelets. Therefore, the elevation of glycosphingolipids in platelets during thrombocytopenia should be considered as evidence that they originated from less mature megakaryocytes. The low sialic acid content in thrombocytopenic platelets is also in keeping with this conclusion. According to Schick & Filmyer [13] a proportion of immature megakaryocytes of stage I and II that were labeled with sialic acid reactive-wheat germ lectin was lower than that of mature megakaryocytes of stage III and IV. Lectin binding ability of these cells was largely eliminated by pretreatment with neuraminidase. Thus, the lower content of sialic acid in thrombocytopenic platelets and platelet plasma membranes would support the hypothesis that, during early recovery from acute experimental thrombocytopenia, excessively large platelets are produced from less mature megakaryocytes with incompletely formed demarcation membranes [10]. At present, however, factors that control changes in MPV immediately after the onset of thrombocytopenia are not well understood [7]. Elevated glycogen-derived glucose content in platelets during thrombocytopenia is in keeping with the report that under these conditions the glycogen content in rat megakaryocytes is increased [12].

In conclusion, we have demonstrated for the first time that contents of GSLs, ceramides, sialic acid, and glycogen in platelet plasma membranes can vary during thrombocytopenia. Our findings suggest that these changes are brought about by accelerated maturation of megakaryocytes.

REFERENCES

1. Mazur, E.M. (1987) Megakaryocytopoiesis and platelet production: A review. *Exp. Hematol.* **15**, 340–350.
2. Gewirtz, A.M. & Hoffman, R. (1990) Human megakaryocyte production: Cell biology and clinical considerations. *Hematol. Oncol. Clin. North. Am.* **4**, 43–64.
3. McDonald, T.P (1992) Thrombopoietin. Its biology, clinical aspects, and possibilities. *Am. J. Pediat. Hematol. Oncol.* **14**, 8–21.
4. Corash, L. (1989) The relationship between megakaryocyte ploidy and platelet volume. *Blood Cells* **15**, 81–107.
5. Hill, R.J. & Levin, J. (1989) Regulation of thrombopoiesis: Their biochemistry and physiology. *Blood Cells* **15**, 141–166.
6. Ebbe, S., Yee, T., Carpenter, D. & Phalen, E. (1988) Megakaryocytes increase in size within ploidy groups in response to the stimulus of thrombocytopenia. *Exp. Hematol.* **16**, 55–61.
7. Corash, L. & Levin, J. (1990) The relationship between megakaryocyte ploidy and platelet volume in normal and thrombocytopenic C3H mice. *Exp. Hematol.* **18**, 985–989.
8. Paulus, J.M., Bury, J. & Grosdent, J.C. (1979) Control of platelet territory development in megakaryocytes. *Blood Cells* **5**, 59–88.
9. Trowbridge, A. (1990) The circulating megakaryocyte, platelet volume heterogeneity and thrombopoiesis; in *Platelet Heterogeneity. Biology and Pathology* (Martin, J. & Trowbridge, A., eds.) pp. 155–183, Springer-Verlag, London.
10. Aster, R.H. (1995) What makes platelet go? The cloning of thrombopoietin. *Transfusion* **35**, 1–3.
11. Andrews, P.W., Nudelman, E., Hakomori, S. & Fenderson, B.A. (1990) Different patterns of glycolipid antigens are expressed following differentiation of TRA-2 human embryonal carcinoma cells induced by retinoic acid, hexamethylene bisacetamide (HMBA) or bromodeoxyuridine (BUdR). *Differentiation* **43**, 131–138.
12. Penington, D.G. & Streatfield, K. (1975) Heterogeneity of megakaryocytes and platelets. *Ser. Haemat.* **8**, 22–48.
13. Schick, P.K. & Filmyer, W.G., Jr. (1985) Sialic acid in mature megakaryocytes: Detection by wheat germ agglutinin. *Blood* **65**, 1120–1126.
14. Schick, P.K. & He, X. (1990) Composition and synthesis of glycolipids in megakaryocytes and platelets: differences in synthesis in megakaryocytes at different stages of maturation. *J. Lipid Res.* **31**, 1645–1654.
15. Baldus, S.E., Thiele, J., Charles, A., Hanisch, F.G. & Fischer, R. (1994) Carbohydrate antigens of human megakaryocytes and platelet glycoproteins: A comparative study. *Histochemistry* **102**, 205–211.
16. Crook, M. & Crawford, N. (1988) Platelet surface charge heterogeneity: Characterization of human platelet subpopulations separated by

high voltage continuous flow electrophoresis. *Brit. J. Haematol.* **69**, 265–273.

17. Rolovic, Z., Baldini, M. & Dameshek, W. (1970) Megakaryocytopoiesis in experimentally induced immune thrombocytopenia. *Blood* **35**, 173–188.
18. Perret, B., Chap, H.J. & Douste-Blazy, L. (1979) Asymmetric distribution of arachidonic acid in the plasma membrane of human platelets. A determination using purified phospholipases and a rapid method for membrane isolation. *Biochim. Biophys. Acta* **556**, 434–446.
19. Saito, T. & Hakomori, S.-I. (1971) Quantitative isolation of total glycosphingolipids from animal cells. *J. Lipid Res.* **12**, 257–259.
20. Tao, R.V.P., Sweeley, C.C. & Jamieson, G.A. (1973) Sphingolipid composition of human platelets. *J. Lipid Res.* **14**, 16–25.
21. Bhandaru, R.R., Srnivasan, S.R., Paragaonkar, P.S. & Berenson, G.S. (1977) A simple colorimetric micromethod for determination of serum cholesterol. *Lipids* **12**, 1078–1080.
22. Lowry, R.R. & Tinsley, I.J. (1974) A simple sensitive method for lipid phosphorus measurements. *Lipids* **9**, 491–492.
23. Higgins, T.J. (1984) Simplified fluorometric assay for sphingosine bases. *J. Lipid Res.* **25**, 1007–1009.
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
25. Graham, J.M. (1993) The identification of subcellular fractions from mammalian cells. *Methods Mol. Biol.* **19**, 1–18.
26. Day, H.J., Holmsen, H. & Hovig, T. (1969) Subcellular particles of human platelets. A biochemical and electronmicroscopic study with particular reference to the influence of fractionation techniques. *Scand. J. Haematol.* (Suppl.) **7**, 3–35.
27. Commission Enzymologie SFBC (1982) Recommendations pour la mesure de la concentration catalytique de la lactate dehydrogenase dans le serum humain à +30°C. *Ann. Biol. Clin. Paris.* **40**, 123–125.
28. Baginski, S., Foá, P.P. & Zak, B. (1974) Glucose-6-phosphatase; in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.) vol. 2, 876–881, Verlag Chemie and Academic Press, Weinheim, New York, San Francisco, London.
29. Stenberg, P.E., Levin, J. & Corash, L. (1990) Sustained thrombocytopenia in mice: Serial studies of megakaryocytes and platelets. *Exp. Hematol.* **18**, 124–132.
30. Odell, T.T., Jr. (1974) Megakaryocytopoiesis and its response to stimulation and suppression; in *Platelets: Production, Function, Transfusion, and Storage* (Baldini, M.G. & Ebbe, S., eds.) pp. 11–20, Grune Stratton, London-San Francisco.
31. Aster, R.H. (1977) Production, distribution, life-span, and fate; in *Hematology* (Williams, W.J., Beutler, E., Erslev, A.J. & Rundles, R.W., eds.) McGraw-Hill, New York-Toronto.
32. Ebbe, S., Stohlman, F., Jr., Donovan, J., Overcash, J. & Hovard, D. (1968) Megakaryocyte maturation rate in thrombocytopenic rats. *Blood* **32**, 787–795.
33. Odell, T.T., Murphy, J.R. & Jackson, C.W. (1976) Stimulation of megakaryocytopoiesis by acute thrombocytopenia in rats. *Blood* **48**, 765–775.
34. Schick, P.K., Williams-Gartner, K. & He, X. (1990) Lipid composition and metabolism in megakaryocytes at different stages of maturation. *J. Lipid Res.* **31**, 27–35.