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Detection in human blood platelets of sialyl Lewis X gangliosides, potential ligands for CD62 and other selectins

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Activated platelets are known to express P-selectin, a lectin-like adhesion receptor (CD62), through which they bind to sialyl Lewis X (sLe^x) ligands displayed on the membranes of leukocytes. To determine whether direct platelet–platelet interactions via P-selectin/sLe^x interactions are also possible, we have examined the ganglioside extract of human blood platelets for the presence of sLe^x ligands. Using the sensitive method of high-performance thin-layer chromatography (HPTLC)–immunostaining with the monoclonal antibody (mAb) CSLEX or with sialidase followed by mAbs MC480 or PM81, eight sLe^x bands were demonstrated at R_f 0.01, 0.03, 0.05, 0.06, 0.08, 0.10, 0.14 and 0.21 in the solvent 45:55:10 chloroform–methanol–aqueous 0.02% $CaCl_2$. The sensitivity of all eight bands to sialidase or endoglycoceramidase confirmed that they were gangliosides. Comparison of the HPTLC mobilities and densities of platelet bands with those from five other human tissues (granulocytes, monoblasts, kidney, aortic endothelium and erythrocytes) in three different solvents revealed three major bands associated with platelets: 3 (R_f 0.03), 6 (0.08) and 14 (0.21). Platelet bands were demonstrated not to have resulted from granulocyte contamination. Partial purification of platelet sLe^x gangliosides by high-performance liquid chromatography and their reaction with 14 oligosaccharide-specific mAbs (FH4, FH5, LM112-161, LM119-181, A5, 1B2, BR55-2, BE2, ES4, MC631, MH04, SH34, P001 and MC813-70) revealed that band 6 is a multifucosylated neolacto ganglioside and band 14 is a branched, disialo neolacto fucoganglioside. Platelet band 3 combined the features of both bands 6 and 14, and reacted differently than granulocyte band 3. These partial structures resemble gangliosides associated with adhesion in other cell systems. It is concluded that platelets express tissue-specific sLe^x gangliosides (sLe^x ligands). Thus, it is possible that platelet–platelet binding may be mediated at least partially through P-selectin/sLe^x interactions, especially after platelet activation.

Key words: gangliosides/HPTLC–immunostaining/platelets/selectin ligands/sLe^x

Introduction

Selectins are a class of recently described cell adhesion molecules which act to localize spatially and temporally the binding of specific leukocytes to certain vascular endothelial cells (McEver, 1991; Springer and Lasky, 1991; Lasky, 1992; Bevilacqua and Nelson, 1993; Rosen and Bertozzi, 1994). Structurally, all selectins possess a similar arrangement of protein motifs which include: (i) an amino-terminal, calcium-dependent (C-type) lectin domain; (ii) an epidermal growth factor repeat; and (iii) a discrete number of sequence modules similar to those found in complement-binding proteins. Selectins are classified (Bevilacqua and Nelson, 1993) according to the cell type on which each was originally identified: E-selectin (endothelium), P-selectin (platelets) and L-selectin (lymphocytes).

The lectin domain of selectins mediates cell adhesion via recognition and binding of tissue/cell-specific oligosaccharide ligands displayed on a variety of glycoconjugates (glycoproteins, glycosphingolipids and glycosaminoglycans). Although each selectin may have its own optimum carbohydrate ligand (Brandley *et al.*, 1993), a generally acceptable ligand (Foxall *et al.*, 1992, and references cited therein) appears to be sialyl Lewis X (sLe^x) or structurally related Lewis blood group oligosaccharides (Table I) present on the membranes of myeloid cells (Fukuda *et al.*, 1984; Macher and Beckstead, 1990) and a small percentage of blood lymphocytes (Munro *et al.*, 1992). Binding of selectin receptors to these carbohydrate ligands plays a critical early role in inflammation (Mulligan *et al.*, 1991, 1992), lymphocyte recirculation (Watson *et al.*, 1991) and possibly tumour metastasis (Lauri *et al.*, 1991).

Selectins may also play a role in thrombosis via P-selectin (CD62, where CD is cluster designation), formerly called GMP-140, a 140 kDa transmembrane glycoprotein expressed on both activated platelets and endothelium. Although other adhesion systems are known to play an important role in platelet agglutination and aggregation (e.g. the integrin glycoproteins IIb/IIIa and Ib-IX which serve as receptors for fibrinogen and von Willebrand factor), it is possible that P-selectin/sLe^x interactions may occur, particularly after platelet activation and during the early phases of platelet agglutination. Such interactions would be analogous to those occurring during the early phase of leukocyte/endothelial cell interactions. Evidence supporting a role for P-selectin in thrombosis includes the finding that antibodies against P-selectin bind to platelets after, but not before, their activation with thrombin (Hsu-Lin *et al.*, 1984; Stenberg *et al.*, 1985), the first step of the process of platelet aggregation and thrombus formation. However, in such platelet processes the membrane-bound sLe^x ligand needed for possible P-selectin binding has been presumed to be provided by thrombus-associated leukocytes. This is because it has been assumed that platelets do not express sLe^x ligands. This assumption is based on antibody binding studies by Mollicone *et al.* (1988) who did not observe sLe^x epitopes on human

Table I. Monoclonal antibodies used to identify and structurally characterize the oligosaccharide moieties of sialylated Le^x gangliosides

Epitope		mAb		Reference
Grouping/name	Structure	Clone[a]	Subclass	
1. Le^x-containing neolacto series Lewis X (Le^x)	Galβ1→4GlcNAcβ1→3Gal | 1,3 Fucα	MC480	IgM	Kannagi *et al.*, 1982
		PM81	IgM	Magnani *et al.*, 1984
		534F8	IgM	Huang *et al.*, 1983
		CSLEX	IgM	Fukushima *et al.*, 1984
Sialyl Lewis X (sLe^x)	Galβ1→4GlcNAcβ1→3Gal | 2,3 | 1,3 NeuAcα Fucα			
Difucosyl Lewis X	Galβ1→[4GlcNAcβ1→3Galβ→]3Gal | 1,3 $n = 2$ Fucα	FH4	IgG	Fukushi *et al.*, 1984a
Trifucosyl Lewis X	Galβ1→[4GlcNAcβ1→3Galβ→]3Gal | 1,3 $n = 3$ Fucα	FH5	IgM	Fukushi *et al.*, 1984a
Lewis Y (Le^y)	Galβ1→4GlcNAcβ1→3Gal | 1,2 | 1,3 Fucα Fucα	BR55-2	IgG	Blaszczyk-Thurin *et al.*, 1987
2. Other neolacto series Blood group i (lactosamine)	Galβ1→4GlcNAcβ1→	1B2	IgM	Young *et al.*, 1981
	Galβ1→4GlcNAcβ1→3Gal	A5	IgM	Fenderson *et al.*, 1986
H, type 2 chain	Galβ1→4GlcNAc | 1,2 Fucα	BE2	IgM	Young *et al.*, 1981
3. Lacto series Lewis A (Le^a)	Galβ1→3GlcNAcβ1→3Gal | 1,4 Fucα	LM112-161	IgM	Gamma Biologics
Lewis B (Le^b)	Galβ1→3GlcNAcβ1→3Gal | 1,2 | 1,4 Fucα Fucα	LM119-181	IgM	Gamma Biologics
Sialyl Lewis A (sLe^a)	Galβ1→3GlcNAcβ1→3Gal | 2,3 | 1,4 NeuAcα Fucα	19-9	IgM	Magnani *et al.*, 1982
Blood group A	GalNAcα1→3Galβ1→3/4GlcNAc | 1,2 Fucα	MH04	IgM	Ortho Diagnostic Systems
Blood group B	Galα1→3Galβ1→3/4GlcNAc | 1,2 Fucα	ES4	IgM	Gamma Biologics
4. Ganglio series Asialo-GM1	Galβ1→3GalNAcβ1→4Galβ1→4Glc	SH34	IgM	Solomon *et al.*, 1987
5. Globo series Galabiose	Galα1→4Gal	P001	IgM	Brodin *et al.*, 1988
SSEA-3	GalNAcβ1→3Galα1→4Gal	MC631	IgM	Kannagi *et al.*, 1983b
6. Ganglio or globo series SSEA-4	Galβ1→3GalNAc | 2,3 NeuAcα	MC813-70	IgG	Kannagi *et al.*, 1983a

[a]All clones were mouse hybridoma.

platelets using immunofluorescence microscopy, and on GMP-140 binding studies by Moore *et al.* (1991) who interpreted their negative findings as suggesting that homotypic interactions between platelets do not occur.

On the other hand, Weinfeld *et al.* (1985) reported that 'sialosyl 3-fucosyl-lactosamine' or sLe^x epitopes were present in the ganglioside extract of human platelets, based on high-performance thin-layer chromatography (HPTLC)–immunostaining and autoradiography. In order to resolve the question of whether or not platelets express sLe^x ligands (epitopes) and are possibly capable of direct platelet–platelet interactions via P-selectin/sLe^x interactions, we have re-examined the ganglioside extract of human platelets for the presence of sLe^x ligands. This time, three new monoclonal antibodies (mAbs) specific

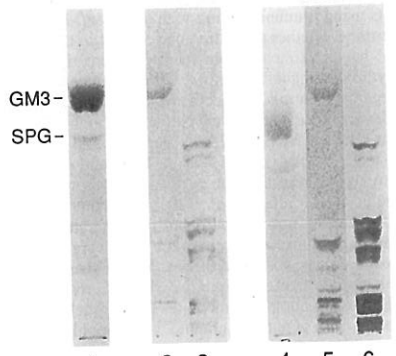

Fig. 1. Demonstration of sLe^x-containing gangliosides in human platelets via HPTLC–immunostaining. Total ganglioside extracts of human platelets (lanes 1, 2, 4 and 5) are compared with those of human granulocytes (lanes 3, 6). Lane 1, chemically stained with diphenylamine reagent to reveal non-specifically all gangliosides (for reference); lanes 2 and 3, immunostained with CSLEX mAb to reveal sLe^x-containing gangliosides; lane 4, immunostained with MC480 to reveal Le^x-containing gangliosides; lanes 5 and 6, first treated with the enzyme sialidase, then immunostained with MC480 to reveal the cryptic Le^x epitopes of sLe^x-containing gangliosides. Abbreviations: GM3, ganglioside GM3; SPG, ganglioside sialoparagloboside.

for Le^x-containing epitopes, as well as many other relevant mAbs (Table I), have been used and the sensitive method of HPTLC–immunostaining and avidin–biotin enzymatic detection has been employed. The cellular purity of our platelet preparation has also been an important consideration in this investigation. This study is part of a series on the structure and function of human platelet glycosphingolipids (GSLs) (Koerner *et al.*, 1986, 1989b; Cunningham *et al.*, 1993).

Results

The distribution of platelet gangliosides

Shown in lane 1 of Figure 1 is the HPTLC analysis of the total platelet ganglioside extract in which lipid-bound carbohydrate has been stained with diphenylamine reagent. All gangliosides are demonstrated non-specifically by this reagent. The pattern observed by densitometry is predominantly that of gangliosides GM3 (NeuAcα2→3Galβ1→4Glcβ1→1′ceramide; 93 mol %) and sialoparagloboside (SPG, 4 mol %), as expected from previous studies (Weinfeld and Koerner, 1985; Koerner *et al.*, 1989a). Similar results were obtained with resorcinol or a charring reagent. The distribution and quantitation of total platelet gangliosides thus observed in lane 1 serve as a useful reference in comparisons with the following immunostaining experiments.

Platelets possess sialyl Lewis X gangliosides

Shown in lane 2 of Figure 1 is the HPTLC–immunostaining of platelet gangliosides using CSLEX, an antibody which recognizes the sLe^x epitope (Table I). For comparison, granulocyte gangliosides are shown (lane 3) since they are known to contain abundant sLe^x gangliosides (Fukuda *et al.*, 1985; Spitalnik *et al.*, 1986; Macher and Beckstead, 1990) and serve as a positive control. In these experiments, five sLe^x-positive bands were observed in the platelet ganglioside extract and eight bands in granulocyte ganglioside extracts; however, the staining is weak for both cell types. Non-specific binding of CSLEX mAb to the GM3 ganglioside band was also noted, which occurred for this mAb as well as others because of the great abundance of this ganglioside in the platelet ganglioside extract.

In order to confirm these CSLEX findings, immunostaining was repeated with two other mAbs that recognize the Le^x epitope, i.e. the asialo form of sLe^x-containing gangliosides. These mAb were MC480 and PM81 (Table I). As shown in Figure 1, lane 4, HPTLC–immunostaining directly with mAb MC480 showed that no free Le^x epitopes were present in the platelet ganglioside extract; a finding that was also confirmed with mAb PM81 (data not shown). For both antibodies, only a very faint non-specific binding was observed associated with the major ganglioside bands at GM3 and SPG. However, after treatment with sialidase, which enzymatically removes masking sialic acid residues, HPTLC–immunostaining with mAb MC480 revealed eight distinct Le^x-positive ganglioside bands (Figure 1, lane 5). Similarly, the granulocyte ganglioside extract (positive control) showed numerous Le^x-positive bands after prior sialidase treatment (Figure 1, lane 6). HPTLC–immunostaining with mAb PM81 also showed strongly reacting Le^x-positive bands for both platelet and granulocyte ganglioside extracts after sialidase treatment (data not shown).

A comparison of CSLEX and sialidase/MC480-reactive bands in platelet ganglioside fractions showed experimentally identical mobility of all five CSLEX bands with five of eight sialidase/MC480-reactive bands (R_f difference < 0.02). Thus, both immunochemical methods appeared to have the same specificity, but the sialidase/MC480 procedure was more sensitive. Consequently, the sialidase/MC480 procedure was used in all subsequent experiments for the detection of sLe^x-positive gangliosides.

Comparison of sLe^x ganglioside bands (Figure 1, lane 5) with the total ganglioside distribution of platelet ganglioside, as revealed by diphenylamine reagent (lane 1), shows that sLe^x gangliosides are minor components of the human platelet. Each band represents <1 mol % of the total ganglioside diphenylamine positivity (densitometrically scanned) and in aggregate represents <3 mol % of the total.

Confirmation that platelet sLe^x bands are gangliosides

Heretofore, sLe^x-reactive bands were assumed to be gangliosides based on their appearance in 'acidic lipid fraction 2' of the Ledeen and Yu isolation procedure (1982) and their sensitivity to sialidase treatment (see above). In order to confirm that the aglycon of sLe^x-reacting bands was indeed ceramide (*N*-acyl sphingosine), the platelet extract containing these bands was treated with endoglycoceramidase, an enzyme with a well-established specificity for the R-Glcβ1→1′ceramide glycosidic linkage (Ito and Yamagata, 1986). Exposure of the platelet extract to the enzyme for <5 min (zero time) revealed a diphenylamine reactivity and sialidase/MC480 reactivity identical to lanes 1 and 5 of Figure 1, respectively, whereas, after 48 h exposure to endoglycoceramidase (0.5 mU), diphenylamine reactivity showed >85 mol % destruction of the GM3 ganglioside and sialoparagloboside bands (positive control), and sialidase/MC480 treatment showed >99 mol % destruction of all eight sLe^x-containing bands (by densitometry). Thus, all

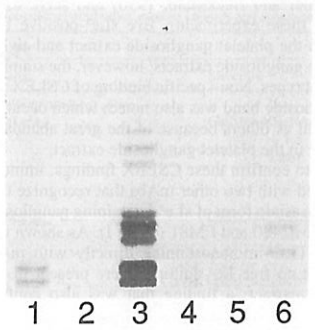

Fig. 2. Tissue specificity in the patterns of sLe^x-containing gangliosides revealed via HPTLC–immunostaining with sialidase/MC480. The same amount of total ganglioside extract (100 μg) from the following human tissues was compared: platelets (lane 1), aortic endothelium (lane 2), granulocytes (lane 3), monoblasts (lane 4), red cells (lane 5) and kidney (lane 6).

eight sLe^x bands have a general sialyl-glycan-Glcβ1→1′ceramide structure which is the structural definition of a ganglioside.

Tissue-specific distribution of sLe^x gangliosides

Both Le^x and sLe^x have been isolated or immunohistochemically identified in several human tissues, including haematopoietic tissues. Therefore, total ganglioside extracts from erythrocytes, granulocytes and monoblasts [acute myelocytic leukaemia (AML) M5b] were compared to the extract from platelets using immunostaining with sialidase/MC480 in order to determine whether any difference existed in the distribution of sLe^x bands extracted from these different tissues. Kidney and erythrocyte gangliosides were studied to serve as positive and negative controls, respectively. Endothelial gangliosides were also included as they were extracted from haemostatically relevant tissues (McEver, 1991; Springer and Lasky, 1991; Lasky, 1992; Bevilacqua and Nelson, 1993) and also because of previous studies reporting the presence of sLe^x gangliosides in endothelial cells (Gillard *et al.*, 1991; Sawada *et al.*, 1993). As shown in Figure 2, this experiment revealed striking quantitative and qualitative differences in the expression of sLe^x gangliosides in the six tested tissues. Monoblasts had only two very faint bands near the origin. No sLe^x-reactive gangliosides were detected in human erythrocytes or endothelium.

A comparison of platelet, granulocyte, monoblast and kidney revealed a distinct pattern of shared and tissue-specific sLe^x-reactive bands (Table II). An initial comparison of relative mobilities (R_f) in Solvent A showed two major bands (R_f 0.01 and 0.03) and one additional band (R_f 0.11) common to both platelets and granulocytes. To further establish the identity and similarity or dissimilarity of these bands, sialidase/MC480 HPTLC–immunostaining was repeated following HPTLC development of platelet and granulocyte gangliosides in two additional solvents (Solvents B and C) of increasing polarity. Based on mobilities in these three solvents, a total of 18 sLe^x-containing gangliosides could be resolved for all four tissues (Table II). Eight distinct sLe^x platelet ganglioside bands were identified, of which four, bands 4, 8, 11 and 14, appeared to be platelet specific based on their mobilities. Granulocyte-specific bands include bands 9, 12, 13, 15, 16, 17 and 18. Two bands, 3 and 5, appear to be common to both platelet and granulocyte. Two additional platelet bands, 2 and 6, do not statistically differ from granulocyte bands 1 and 7, but may represent platelet associated sLe^x gangliosides.

Table II. Tissue-specific detection of sLe^x-positive ganglioside bands and their mobilities in three solvents[a]

Band	Band mobility (R_f)			Tissues studied[b]			
	Solvent A	Solvent B	Solvent C[c]	PLTS	GRAN	MONO[d]	KID[d]
1	≤ 0.01	≤ 0.01	0.01 ± 0.005		+		
2	≤ 0.01	0.01	0.02 ± 0.004	+			
3	≤ 0.01	0.03	0.05 ± 0.004	+	+	+	
4	≤ 0.01	0.05	0.07 ± 0.005	+			
5	0.03	0.06	0.10 ± 0.005	+	+	+	
6	0.03	0.08	0.13 ± 0.006	+			
7	0.03	0.08	0.14 ± 0.004		+		
8	0.03	0.10	0.17 ± 0.009	+			
9	0.03	0.12	0.19 ± 0.004		+		
10[d]	0.05	ND	ND				+
11	0.08	0.14	0.22 ± 0.007	+			
12	0.06	0.16	0.24 ± 0.003		+		
13	0.07	0.19	0.26 ± 0.005		+		
14	0.11	0.21	0.29[e]	+			
15	0.09	0.23	0.32 ± 0.004		+		+
16	0.11	0.27	0.35 ± 0.008		+		
17	0.20	0.40	0.49 ± 0.009		+		
18	0.24	0.43	0.52 ± 0.004		+		

[a]Bands were detected as MC480 or FH4 positivity following sialidase digestion, and were tentatively assigned by the pattern of their relative mobilities and of their mAb binding densities.
[b]Human aortic endothelium and erythrocytes contained no detectable sLe^x bands. Symbols used: PLTS, platelets; GRAN, granulocytes; MONO, monoblasts; KID, kidney.
[c]Multiple measurements of R_f were made in this solvent (n = 3) and standard deviations are reported.
[d]Analysed in Solvent A only; ND, not determined.
[e]Broadly reacting band between R_f 0.29 and 0.30.

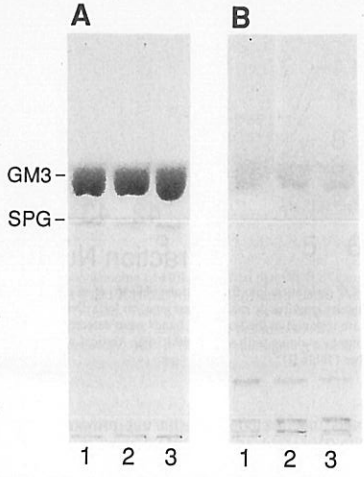

Fig. 3. Effect of leukodepletion on the HPTLC patterns of platelet gangliosides, as detected by diphenylamine reagent (**A**) or by immunostaining with sialidase/MC480 (**B**). All lanes were spotted with 60 μg of total ganglioside and run in Solvent A. States of leukodepletion were as follows: after centrifugal purification (lane 1), after first leukofiltration (lane 2), and after second leukofiltration (lane 3).

Determination of cellular purity of platelet preparations

Because sLe^x bands were a minor component of the total gangliosides from platelets, it was possible that these bands were derived from granulocytes which might have contaminated the platelet preparations that were used for extraction. This possibility was addressed first by assessing the actual leukocyte contamination present in our platelet preparations. Using the most sensitive method available, the Naegotte counting chamber (Brandwein and Dickstein, 1991; Masse *et al.*, 1992; Rebulla *et al.*, 1993), it was found that our standard platelet purification procedure (differential centrifugation) yielded a platelet preparation that contained 0.003 ± 0.001% (n = 4) leukocytes/total cells. This was a number of leukocytes that was at the lower limit of what can be experimentally detected using current technology. When these trace leukocytes present in our platelet preparations were morphologically differentiated, only 3% of the total were granulocytes, the rest being mononuclear cells. Thus, there was no more than 1 p.p.m. granulocytes present in our platelet preparations.

Effect on platelet sLe^x bands of repeated leukofiltration

We next considered the effect of further leukodepletion of our platelet preparations on the total and sLe^x-positive ganglioside patterns of the platelet. In these experiments, we utilized newly developed polyester filters (PL-100 HF) designed to specifically and exhaustively eliminate leukocytes from blood products (Brandwein and Dickstein, 1991; Masse *et al.*, 1992; Rebulla *et al.*, 1993). Shown in Figure 3A is the effect on the total ganglioside pattern of leukofiltration of our platelet preparation prior to ganglioside extraction and HPTLC (lanes 1–3). Even more important is the effect on the sLe^x-positive ganglioside pattern of leukofiltration (Figure 3B, lanes 1–3). In both cases, there is no detectable difference in the patterns observed either after one or two leukofiltrations. Thus, there is no detectable granulocyte contamination present in our platelet preparation using the most sensitive methods available.

Quantitation of platelet and granulocyte sLe^x gangliosides

In order to determine the relative proportions of the sLe^x-containing gangliosides, the immunostained bands were scanned densitometrically. The quantitation of sialidase/MC480-positive bands, as well as CSLEX-positive bands, is shown for platelets in Figure 4A (lane 1) and Table III, and for granulocytes in Figure 4B (lane 6). Scanning densitometry identified bands 3, 6 and 14 to be the major sLe^x-reactive bands of the platelet, comprising 64% of the total MC480 binding (Table III). Platelet-specific bands (2, 4, 6, 8, 11 and 14) represented 71% of the total MC480 binding. Particularly interesting were bands 6 and 14, which were both major bands

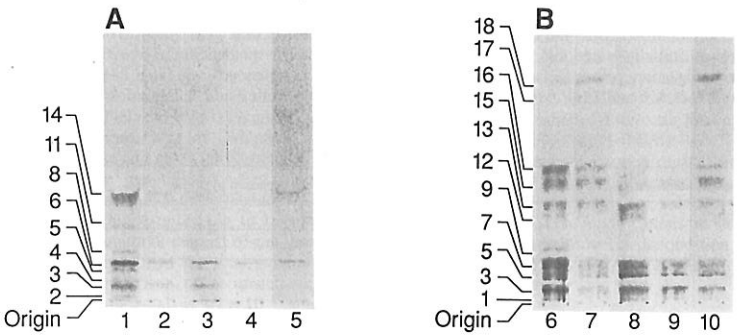

Fig. 4. Immunological characterization of the structures of sLe^x bands present in the total ganglioside extract of platelets (**A**) and granulocytes (**B**). Each lane was spotted with a comparable mass of ganglioside [100 μg in (A) and 10 μg in (B) of total extract mass]. Bands were detected via HPTLC–immunostaining after incubation with sialidase and each of the following mAbs: MC480 (lanes 1 and 6), FH4 (lanes 3 and 8), FH5 (lanes 4 and 9) and LM112161 (lanes 5 and 10), except for lanes 2 and 7 which were incubated with the mAb CSLEX only. Vertical numbering refers to band numbers.

Table III. Relative binding of anti-Lewis mAb to platelet sLe^x-positive ganglioside bands

Band[b]	mAb reactivity[a]				
	MC480	CSLEX	FH4	FH5	LM112–161
2	0.09		0.06		
3	0.31	0.34[c]	0.36	0.67	0.11
4			0.15		
5	0.07	0.10	0.12		
6	0.26	0.25	0.31	0.33	0.44
8	0.05				
11	0.06	0.05			
14	0.16	0.26	[d]		0.44

[a]Measured as the fraction of total mAb binding density associated with a particular band. Binding density (mm^2) was determined by densitometric scanning, total area = 1.00, SD < 0.05, unless otherwise noted. All mAbs were applied after sialidase digestion, except for CSLEX. Bands for which no densitometric density is reported were undetectable. HPTLC was carried out in Solvent B.
[b]Bands numbered according to Table II.
[c]SD = 0.09.
[d]Rare faint binding at < 0.10.

and platelet specific. Scanning densitometry of granulocyte sLe^x-reactive gangliosides showed eight granulocyte-specific bands (1, 7, 9, 12, 13 and 16–18) comprising 55% of the total. Granulocyte bands 3, 5 and 7 were the three major bands, accounting for ~57% of the total. Particularly interesting was band 7 which was both a major band and granulocyte specific. Although fewer bands were detected, the quantitation of both platelet and granulocyte sLe^x bands was generally similar to the above when CSLEX mAb was used (Figure 4; Table III).

Immunocharacterization of platelet and granulocyte sLe^x gangliosides

In order to obtain preliminary structural information, platelet sLe^x-reactive gangliosides were reacted with several mAbs with known oligosaccharide specificities (Table I). The results of these HPTLC–immunostaining experiments are shown in Figure 4A. Granulocyte gangliosides were also examined for comparison (Figure 4B). Quantitative immunostaining with mAbs FH4 and FH5, which recognize repeating Le^x epitopes, showed FH4 reactivity to platelet bands 2–6 with weak FH5 binding to bands 3 and 6 only (Figure 4A, lanes 3 and 4; Table III). No staining was initially noted for mAb LM112-161; however, after prior digestion with sialidase, weak reactivity was observed with bands 3, 6 and 14 (Figure 4A, lane 5). This finding was unexpected based on our previous studies with mAb 19-9 (Weinfeld *et al.*, 1985). However, this result indicates that LM112-161 is either a more sensitive mAb than 19-9 or that LM112-161 cross-reacts with both sLe^x and sLe^a epitopes due to their structural similarity (Table I). Immunochemical studies reveal that the latter explanation is correct (Oriol *et al.*, 1987). No binding of platelet gangliosides was noted for mAbs LM119-181, BR55-2, BE2, ES4, MC631 or P001.

Granulocyte bands 3 and 7, like platelet bands 3 and 6, were reactive with mAbs FH4, FH5 and LM112-161 (Figure 4B). However, band 5 demonstrates a tissue-specific immunoreactivity, binding to FH5 and LM112-161 after sialidase treatment in the case of the granulocyte, but not in the case of the platelet. Thus, band 5 may have a different structure in granulocytes than in platelets.

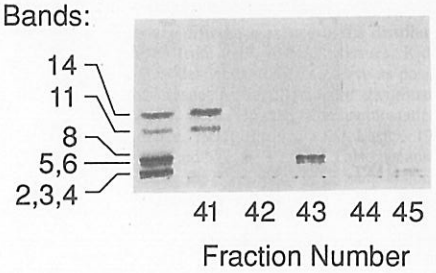

Fig. 5. HPLC separation into five fractions (41–45) of sLe^x bands present in the total platelet ganglioside extract (first lane, on left). Details of the HPLC procedure are reported in the text. sLe^x bands were detected in Solvent A via HPTLC–immunostaining with sialidase/MC480. Vertical numbering refers to band number (Table II).

Partial separation of platelet sLe^x gangliosides

In order to better study individual sLe^x gangliosides, the total platelet ganglioside extract was separated by HPLC (see Materials and methods) and fractions screened for sLe^x activity with sialidase/MC480 immunostaining in Solvent A. As shown in Figure 5, all eight platelet sLe^x bands were detected in five HPLC fractions (numbers 41–45) which eluted at a chloroform–methanol ratio of ~76:24 (v/v). Thus, bands 11 and 14 were separated from band 8; 8 from 5 and 6; 5 and 6 from 4; and 4 from 2 and 3. Immunostaining of isolated or partially separated sLe^x gangliosides with CSLEX, MC480, FH4, FH5 or LM112-161 mAb showed a pattern of immunoreactivity that confirmed the identity of each band as observed in the total extract (Table IV). Platelet bands 14, 11, 6 and 3 also demonstrated apparent immunoreactivity with mAbs 1B2 and A5 (prior sialidase digestion). Repeat immunostaining with mAb MC480, A5 and 1B2 following HPTLC development in Solvents B and C showed 1B2- and A5-reactive bands coincident to platelet bands 14 and 3 in all three solvent systems tested (Solvents A, B and C) and in two separate HPLC fractionations. In contrast, bands 6 and 11 (Solvent A), when run in the more polar Solvent C, dissociated into sub-bands, some with 1B2 and A5 reactivity, and some with MC480 activity (Figure 6).

Table IV. Immunological characterization of platelet sLe^x-positive ganglioside bands partially separated by HPLC

Band[b]	Mobility (R_f)	HPLC fraction	mAb reactivity[a]						
			MC480	CSLEX	FH4	FH5	LM112-161	A5	1B2
2, 3, 4	0.01	45	+	+	+	+		+	+
4	0.02	44	+		+				
5, 6	0.03	43,44	+	+	+	+	+	+	+
8	0.04	42	+						
11	0.08	41	+	+				+	+
14	0.11	41	+	+	±		±	+	+

[a]All mAbs were applied after sialidase digestion, except CSLEX. HPTLC was carried out in Solvent A.
[b]Bands numbered according to Table II.

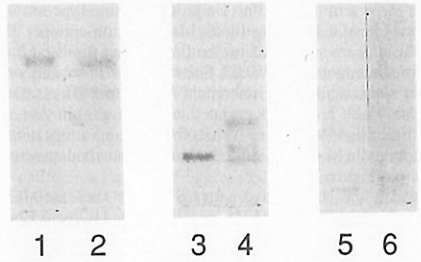

Fig. 6. HPTLC–immunostaining of HPLC-purified platelet sLe^x ganglioside bands. Band 14 (lanes 1 and 2, HPLC fraction 41), band 6 (lanes 3 and 4, fraction 43) and band 3 (lanes 5 and 6, fraction 45) were reacted with sialidase/MC480 (lanes 1, 3 and 5, respectively) or sialidase/1B2 (lanes 2, 4 and 6, respectively). All HPTLC–immunostaining was in Solvent C.

Discussion

We describe here the identification, purification and partial characterization of several sLe^x-reactive gangliosides from the human blood platelet. Several tissues have previously been identified which express Lewis X (CD15) or its sialylated derivative (McEver, 1991; Springer and Lasky, 1991; Lasky, 1992; Bevilacqua and Nelson, 1993; Rosen and Bertozzi, 1994); however, this is the first report of the isolation of sLe^x-reactive glycoconjugates from platelets. Eight sLe^x-reactive platelet glycosphingolipids were identified by HPTLC–immunostaining of total platelet gangliosides using several mAbs specific for Lewis X and sialylated Lewis X oligosaccharides, including CSLEX, MC480 and PM81 (Figure 1). These findings confirmed our preliminary study (Weinfeld *et al.*, 1985) which employed sialidase and mAb 534F8 treatment.

The pattern of these eight sLe^x gangliosides, when compared to five other human tissues, was shown to be distinctive, with bands 4, 8, 11, 14 and possibly 6 appearing to be platelet specific (Table II). The total amount of these sLe^x gangliosides was small, representing <3 mol % of the total ganglioside. Using ganglioside GM3 for comparison, the estimated number of sLe^x gangliosides (at 2% of the total) is 3.2×10^{-41} amol or ~2000 molecules/platelet. This is a number comparable to that observed (Koerner *et al.*, 1989b) for the blood group A-reactive neutral glycosphingolipids of the human platelet.

The possibility that our platelet preparations were contaminated with granulocytes, a known source of sLe^x gangliosides (Figure 2), was considered. This possibility may be dismissed because: (i) less than one cell in a million in our platelet preparation was found to be a granulocyte; (ii) the patterns of sLe^x bands in platelets and granulocytes differ qualitatively (note band 14 especially); (iii) two successive leukofiltrations of our platelet preparation caused no change in the total ganglioside pattern; and (iv) two successive leukofiltrations of our platelet preparation caused no change in the sLe^x ganglioside pattern (Figure 3). In fact, it is far more likely that contamination of granulocytes with platelets has been unappreciated in reported studies of granulocytes (Fukuda *et al.*, 1985; Spitalnik *et al.*, 1986; Macher and Beckstead, 1990), since it is known now that the platelet contamination in standard granulocyte products is 10-fold greater than the actual number of granulocytes (Strauss *et al.*, 1991). Thus, those bands that are common to both platelets and granulocytes (e.g. bands 3 and 5, and possibly 1/2 and 6/7) are far more likely to have originated from platelets.

The finding that platelets possess multiple sLe^x gangliosides confirms our early, preliminary immunostaining/autoradiography study (Weinfeld *et al.*, 1985), but appears to contradict the findings of Mollicone *et al.* (1988) who did not detect any sLe^x ligands on the platelet cell surface using the CSLEX mAb and immunofluorescence microscopy. However, when other findings by these French investigators are taken into account, and interpreted using more recent data, the apparent contradiction may be explained. Mollicone *et al.* observed that a majority of the platelets studied showed no immunofluorescence with an antibody that reacts with sialyl Lewis^a antigens (i.e. CA-50), but that a small subpopulation (~5%) of the same platelet samples did react very strongly with the sLe^a antibody. It is now known (Ault *et al.*, 1989) that between 3 and 4% of platelets are activated during their collection, as described by Mollicone *et al.* In addition, it is known (Magnani, 1991) that sLe^a and sLe^x show great structural similarity (Table I), and antibodies to them may cross-react. When these findings of others are taken into account, one may conclude that Mollicone *et al.* (1988) were observing sLe^x ligands on platelets, but only for the subpopulation of activated platelets. This reasoning leads us to consider the possibility that the activation of platelets could correlate with the surface expression of sLe^x ligands.

Our finding of platelet sLe^x ligands also appears to contradict the GMP-140 binding studies of Moore *et al.* (1991). In their studies, platelets were first activated with thrombin, then incubated with radiolabelled GMP-140, after which very little radiolabelled P-selectin binding was observed. However, this experiment does not allow for the possibility that platelets may rapidly interact directly (homotypically) to form multimers (agglutinins) before the radiolabelled GMP-140 is added, thus blocking exogenous labelled P-selectin binding by first reacting with endogenous (unlabelled) GMP-140. Only by blocking the possible homotypic interaction during platelet activation (e.g. with soluble sLe^x or anti-P-selectin) would the platelet sLe^x ligands remain available for subsequent radiolabelled GMP-140 binding. Such modifications in the Moore *et al.* experiment would then allow for the question of a homotypic interaction between P-selectin receptors and ligands to be truly addressed.

Preliminary structures of platelet sLe^x gangliosides

The nanogram quantities of sLe^x gangliosides detected in our study precluded classical physical and chemical methods of structure analysis, at least in our hands at this time. However,

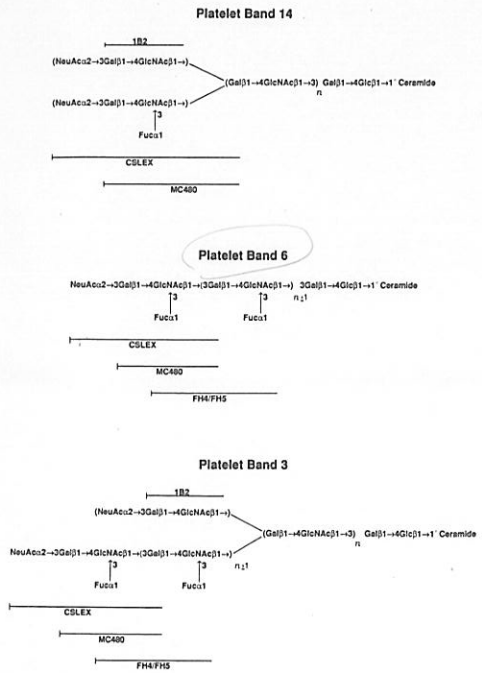

Fig. 7. Preliminary chemical structures of platelet-specific sLe^x-containing gangliosides. Also shown are epitopes for immunoreactive mAbs, as defined in Table I.

much information may be deduced from the reactions observed with the several specific mAbs that were employed in our studies. In order to minimize the possibility that multiple immunoreactivities were due to the unexpected coincidence of multiple bands, we examined the immunoreactivities of bands both in the total ganglioside mixture (Figure 4A; Table III) and as HPLC-purified or partially purified fractions (Figures 5 and 6; Table IV).

Particularly important were the major sLe^x bands of the platelet (bands 3, 6 and 14), especially those that were also platelet specific (bands 6 and 14). Band 14 was: (i) positive with CSLEX or sialidase/MC480; (ii) negative with FH4 or FH5; (iii) negative with 1B2 or A5 before sialidase, but positive with 1B2 or A5 after the enzyme digestion. Thus, band 14 must contain at least one sLe^x terminus and at least one NeuAc→Galβ1→4GlcNAc (sialyl lactosamine) terminus, but does not contain a repeating fucosyl lactosamine epitope. These conclusions are supported by the finding that the band has a fast mobility (R_f 0.11, Solvent A) and most likely only one of the two types of termini. Thus, band 14 is mostly likely a branched, disialo neolacto fucoganglioside of at least 11 oligosaccharide residues which possess one sialyl lactosamine and sLe^x terminus. Such a biantennary structure is shown in Figure 7.

Band 6 was: (i) positive with CSLEX or sialidase/MC480; (ii) positive with FH4 or FH5; (iii) negative with 1B2 or A5, both before and after sialidase. Thus, band 6 must contain only a sLe^x-type terminus and no sialyl lactosamine-type terminus, but does have a repeating fucosyl lactosamine epitope. These conclusions are supported by the finding that the band has an intermediate mobility (R_f 0.03, Solvent A) and probably only a single sialic acid in an unbranched structure. Thus, band 6 appears to be a multifucosylated neolacto ganglioside of at least nine oligosaccharide residues that contains a repeating Le^x epitope and a sLe^x terminus. Such an unbranched structure is shown in Figure 7.

Band 3 was: (i) positive with CSLEX or sialidase/MC480; (ii) positive with FH4 or FH5, negative with 1B2 or A5 before sialidase, but positive with 1B2 or A5 after the enzyme digestion. Thus, band 3 must contain at least one sLe^x terminus and at least one sialyl lactosamine terminus, as well as repeating fucosyl lactosamine epitope. These conclusions are supported by the finding that the band has a slow mobility ($R_f < 0.01$, Solvent A) and probably at least two sialic acid residues, one each for its sLe^x and sialyl lactosamine termini. Thus, band 3 appears to be a complex, long-chain fucoganglioside that combines characteristics of both bands 14 and 6, resulting in a branched, disialo, multifucosylated neolacto ganglioside of at least 14 oligosaccharide residues. Such a biantennary structure is shown in Figure 7. It should be noted that platelet band 3 must differ in structure from granulocyte band 3 because the latter was negative after sialidase/1B2 or sialidase/A5 immunostaining (data not shown).

Possible function of platelet sLe^x gangliosides

The structures deduced from immunostaining data for bands 14, 6 and 3 (Figure 7) may provide clues to the function of these gangliosides in platelet physiology. A ganglioside with an identical structure to that of band 6, known variously as sialyl Lewis Xi, sialyl dimeric Le^x, FH6 antigen, 5B or sialodifucosyl Y2 ganglioside, has been isolated from adenocarcinoma of the colon. The expression of this ganglioside in adenocarcinoma cells seemed to correlate with the potential for metastasis of the primary tumour (Fukushi *et al.*, 1984b). Oligosaccharides similar to platelet band 3 have been suggested to exist on colon carcinoma and high endothelial venules (Sawada *et al.*, 1993). In all of the above noted situations, adhesion to other cells is a prominent feature of the source cell. Branching (or multivalency) of oligosaccharides, as seen for bands 14 and 3, has recently been shown to be important in enhancing binding (DeFrees and Gaeta, 1993). Such a complex, branched, O-linked sLe^x glycan, very similar to the glycan of bands 14 and 3, has recently been reported to be a high-affinity P-selectin ligand carried on myeloid cells (Moore *et al.*, 1994). Thus, the apparent structures observed for platelet sLe^x gangliosides (Figure 7) suggest that these gangliosides may possibly play a role in platelet cell–cell adhesion.

Although the importance of selectin expression in leukocyte migration and inflammation is well recognized (McEver, 1991; Springer and Lasky, 1991; Lasky, 1992; Bevilacqua and Nelson, 1993; Rosen and Bertozzi, 1994), the possible role of P-selectin in thrombosis is a matter of conjecture. A recently proposed model (Ware and Heistad, 1993) suggests an essential role for leukocytes as bridging units. Our data show, alternatively, that it is possible that platelets may interact directly with each other through P-selectin/sLe^x interactions. In support of our proposal are several recent studies. Lyp20, an anti-GMP-140 (or P-selectin) mAb, has been shown to inhibit platelet aggregation to multiple platelet agonists including thrombin, ADP and collagen (Parmentier *et al.*, 1991). In a more recent study (Yen *et al.*, 1993), cisplatin-induced platelet aggregation was found to be an agglutination phenomenon inhibitable by both fluid-phase GMP140 and Fab fragments directed against the GMP140 molecule. Finally, electron microscopy studies of thrombin-induced platelet aggregates found that P-selectin, but not fibrinogen, GPIIb/IIIa, GP1b, von Willebrand factor, thrombospondin, or fibrinonectin, was localized along the long cell–cell contact zones of aggregated platelets, leading to the speculation that GMP140 may play a role in the stabilization of platelet aggregates (Isenberg *et al.*, 1987). All of these studies suggest that platelets possess a ligand capable of binding the P-selectin receptor.

What are now needed are studies to determine whether or not activated platelets express sLe^x epitopes, and whether anti-sLe^x agents actually block the agglutination and aggregation of purified platelets. In addition, large-scale isolation and definitive characterization of platelet sLe^x-GSLs may help in the design of potential therapeutic analogues tailored to specifically inhibit pathological adhesive events mediated by P-selectin.

Materials and methods

Tissue preparation

Platelet concentrates (PCs) were prepared from whole blood donation by volunteer donors according to the procedures of the American Association of Blood Banks (AABB; Walker, 1990). After 120 h of storage with agitation at room temperature, purified platelet pellets were obtained from PCs by differential centrifugation. PCs were spun first to remove contaminating leukocytes and red cells (350 *g*, 15 min), then to separate platelets from plasma (4500 *g*, 30 min). The resulting platelet pellets were pooled by ABO blood types, washed twice with isotonic ammonium bicarbonate buffer, frozen and lyophilized. Each unit of PC yielded ~93 mg of desiccated platelets. For leukodepletion experiments, pooled platelet concentrates were prepared as described from 100 pooled PCs (4800 ml). Two-thirds of the pooled platelet concentrates were passed over polyester leukodepletion filters (500 ml/filter; Pall Filter, PL-100 HF), then divided in half, with one-half leukodepleted by filtration a second time. The remaining original pooled PCs (1600 ml) and leukofiltered pooled platelet concentrates (1600 ml each) were then centrifuged, washed, frozen and lyophilized as before. Leukocyte contamination of the original platelet pool and leukofiltered fractions was microscopically determined (Brandwein and Dickstein, 1991; Masse *et al.*, 1992) using a Naegotte chamber (Polylabo, Strasbourg, France) and Türk's solution (Rebulla *et al.*, 1993). The percentage of leukocyte and granulocyte contamination was based on the total number of leukocytes/total cells.

Monoblasts (AML M5b) from a therapeutic leukopheresis (6.4×10^{11} cells), 24-h-old granulocytes (Type B positive, 3.0×10^{11} cells) from a donor cytapheresis and 35-day-old packed red cells (Type AB positive, 1 U) were collected according to the procedures of the AABB (Walker, 1990). Differential counting of the leukocytes present in the granulocyte cytapheresis unit showed that it contained 83% polymorphonuclear granulocytes and 17% mononuclear leukocytes. Erythrocytes were washed twice with normal saline (350 *g*, 15 min), frozen and lyophilized. The monoblasts and granulocytes were diluted with 2 vols of isotonic ammonium bicarbonate, centrifuged (4500 *g*, 30 min), washed twice with isotonic ammonium bicarbonate, frozen and lyophilized. Human kidney (Type A positive, 30 g) was obtained from the autopsy service (Department of Pathology, University of Iowa). Endothelium was obtained from 15 human thoracic aortas by scrapping the luminal surface with a glass microscope slide. Both kidney and aorta specimens were obtained within 12 h of death. All tissue procurement was carried out with the supervision and approval of the institutional human investigation review committee.

Ganglioside extractions

The total ganglioside fraction of tissues was obtained using the general procedures of Ledeen and Yu (1982) as we have adapted them to blood cells (Koerner *et al.*, 1989b). Lyophilized blood cell powders (5 g) were extracted with at least 100 ml of chloroform–methanol (1:1 v/v). The resulting total lipid extract was dissolved in chloroform–methanol–water (30:60:8 v/v, 1.24 l), applied to a DEAE–Sephadex column (A-25, Pharmacia; 62 ml bed volume) and washed with 1.24 l of the same solvent. The bound acidic lipid was eluted with chloroform–methanol–aqueous 0.8 M sodium acetate (30:60:8 v/v, 930 ml). The resulting eluate was evaporated *in vacuo*, saponified (0.1 N methanolic sodium hydroxide, overnight) and dialysed against 20 or more volumes of distilled water (Spectra/Por tubing, mol. wt cut-off 1400, Houston, TX) until the pH of the dialysate was neutral. The lyophilized dialysis retentate was taken up in chloroform–methanol (85:15 v/v; 150 ml) and applied to a silicic acid column (Bio-sil A, Bio-Rad Labs; bed volume 45 ml). After washing the column with the same solvent (750 ml) to remove sulphatides and free fatty acids, the total ganglioside fraction was eluted with chloroform–methanol (1:2 v/v, 600 ml). Depending on the cell type and particular batch, the yield of total ganglioside varied between 1 and 12 mg. Kidney gangliosides were isolated following the Ledeen and Yu (1982) procedure exactly.

Monoclonal antibodies (Table I)

mAbs LM112-161, LM119-181 and ES4 were purchased from Gamma Biologicals (Houston, TX). mAb P001 was purchased from Accurate (San Diego, CA). mAb PM81 (CD15) was supplied by Medarex (West Lebanon, NH). mAb MH04 against the A blood group antigen was kindly provided by Donald Davies of Ortho Diagnostic Systems (Raritan, NJ). mAbs MC631, MC813-70, MC480 (SSEA-1) and A5 were purchased as hybridoma supernatants from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences at Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology at the University of Iowa, Iowa City, IA, under contract no. NO1-HD-2-3144. mAbs CSLEX1 (HB 8580), FH4 (HB 8775), FH5 (HB 8770), BR55.252a (HB 9347) and BE2 (TIB 182) were purchased as hybridoma cell lines from the American Type Culture Collection (ATCC, Rockville, MD) and grown in the University of Iowa Hybridoma Facility. mAb 1B2 was originally a gift of Dr William Young (University of Louisville), then purchased as a hybridoma (1B2-1B7, TIB 189) from ATCC. The hybridoma producing mAb SH34 was a gift from Dr T.J.Higgins (University of Pennsylvania, Philadelphia, PA). All purchased antibodies or hybridoma supernatants were used as provided after appropriate dilution into Buffer A, as determined by preliminary titration studies.

High-performance thin-layer chromatography

HPTLC was performed according to published procedures (Ando *et al.*, 1978; Koerner *et al.*, 1989b) using aluminium-backed HPTLC plates (E.Merck, Darmstadt, FRG). Plates spotted with neutral GSLs were developed in a solvent of chloroform–methanol–water 65:25:4 (v/v). Acidic GSLs were developed in one of three solvents of chloroform–methanol–water with 0.2% $CaCl_2$: Solvent A, 55:45:10; Solvent B, 45:55:10; or Solvent C, 40:60:10 (v/v). GSLs were detected by spraying with diphenylamine reagent (Harris and McWilliam, 1954), resorcinol reagent (Ledeen and Yu, 1982) or charring reagent (Macala *et al.*, 1983), or by immunostaining as described below. GSL bands were characterized by intensity (% total staining density) and relative mobility (R_f) by scanning densitometry at 560 nm (Shimadzu Instruments, Columbia, MD). Error in mobility measurements was ± 0.01 R_f or less unless otherwise specified.

HPTLC–immunostaining

HPTLC–immunostaining was performed as described by Magnani *et al.* (1980) and modified by Buehler and Macher (1986). Briefly, air-dried solvent-developed plates were dipped in a hexane solution of 0.04% poly (iso-butyl) methacrylate for 60 s and air dried. Plates were blocked with Buffer A [50 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.1% azide, 1% bovine serum abumin (BSA), (pH 7.8)] for 45 min, and then overlaid with primary mAb diluted in buffer A for 1 h at room temperature. The plates were washed with phosphate-buffered saline (PBS; pH 7.4) and incubated with a biotinylated secondary antibody diluted in PBS–1% BSA (pH 7.4) for 1 h, washed with PBS and reacted with an avidin-linked alkaline phosphatase (Vector Laboratories, Burlingame, CA) for 30 min.

Enzymic digestions of gangliosides

Poly (iso-butyl) methacrylate-coated HPTLC plates were treated *in situ* with 2.0 ml of sialidase (*Clostridium perfringens* Type V, EC 3.2.1.18; Sigma, St Louis, MO, 0.15 U/ml) in PBS (pH 7.44) containing 1% BSA for 2 h at 37°C. Plates were washed repeatedly with PBS prior to immunostaining.

Endoglycoceramidase digestions were carried out in glass test tubes. Total platelet gangliosides (~1.0 mg) were incubated for 48 h at 37°C with endoglycoceramidase (EC 3.2.1.45) from *Rhodococcus* (Genzyme, Boston, MA; 0.5 mU) in 50 µl of sodium acetate buffer (50 mM, pH 5.0) containing sodium taurodeoxycholate (1.0 mg/ml; Sigma, St Louis, MO). All reactions were terminated by addition of 100 µl of chloroform–methanol 1:1 (v/v), dried under nitrogen and analysed by HPTLC (diphenylamine reagent) and HPTLC–immunostaining (sialidase/MC480).

High-performance liquid chromatography

Underivatized platelet gangliosides were separated by HPLC (Shimadzu LC-610, Columbia, MD) using laser light scattering detection (Varex ELSD II, Burtonsville, MD) according to the procedures of Christie (1985) and Koerner *et al.* (1992). The sample (13.0 mg) was applied to a preparative silica gel column (1.0 × 25 cm, 83-IIII-C, Dynamax-60A, 8 μm; Ranin, Woburn, MA) and eluted with a gradient of chloroform–methanol–water from 80:20:0 to 34:66:17, applied at a rate of 5 ml/min for 2 h. Fractions were collected every 2 min and analysed by HPTLC–immunostaining with sialidase/MC480.

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Abbreviations

AABB, American Association of Blood Banks; AML, acute myelocytic leukaemia; BSA, bovine serum albumin; CD62, cluster designation 62 (i.e. GMP-140 or P-selectin); GM3, ganglioside GM3 (NeuAcα2→3Galβ1→4Glcβ1→1′ceramide); GMP-140, 140 kDa glycoprotein from membranes of platelets; GSL, glycosphingolipid; HPTLC, high-performance thin-layer chromatography; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PC, platelet concentrate; sLe^x, sialyl Lewis X epitope; SPG, sialyl paragloboside (NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1′ceramide); CSLEX, MC480, etc., monoclonal antibodies with specificities as defined in Table I.

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