Altered Expression of Gangliosides in Erythrocytes of Paroxysmal Nocturnal Hemoglobinuria

Hideki Nakakuma, Tatsuya Kawaguchi, Kentaro Horikawa, Michihiro Hidaka, Yuji Yonemura, Makoto Kawakita, Tadashi Kagimoto,* Masao Iwamori,‡ Yoshitaka Nagai,‡ and Kiyoshi Takatsuki

*Second Department of Internal Medicine, Kumamoto University Medical School, Kumamoto 860, Japan; *College of Medical Science, Kumamoto University, Kumamoto 862, Japan; and ‡Department of Biochemistry, Faculty of Medicine, The University of Tokyo, Tokyo 113, Japan*

Abstract

In paroxysmal nocturnal hemoglobinuria (PNH), impaired glycosyl-phosphatidylinositol (PI)-anchoring of membrane proteins such as decay-accelerating factor has been known to lead to increased susceptibility to complement. Moreover, abnormal expression of non–PI-anchoring glycoproteins such as C3b/C4b receptor (CR1) or glycophorin-α also has been shown in PNH. Therefore, we biochemically analyzed glycosphingolipids (GSL) as one of the membrane glycoconjugates of PNH erythrocytes. Erythrocytes of all seven PNH patients showed altered expression of sialosyl GSL (gangliosides) as compared with the control erythrocytes of healthy donors. Both a sialosylparagloboside (IV^{6}NeuAc-nLc$_4$Cer) among four major gangliosides and some minor gangliosides in normal erythrocytes variably disappeared in erythrocytes from the peripheral blood of PNH patients. As one of the possible mechanisms of altered expression of gangliosides in PNH erythrocytes, structural analysis suggested impaired sialylation of GSL. These results suggest not only the altered metabolism of gangliosides in PNH erythrocytes, but also a metabolic disorder of membrane glycoconjugates as a new feature of PNH. (*J. Clin. Invest.* 1990. 85:1456–1461.) glycolipid • sialosylparagloboside • sialylation • PI-glycan • membrane glycoconjugate

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH)[1] is an acquired hemolytic disease with an increased sensitivity of erythrocytes to the lytic action of complement (1, 2). This abnormal susceptibility to complement has been partly explained by a deficiency in decay-accelerating factor (DAF) (3, 4) or C8-binding protein (5), which are glycosyl-phosphatidylinositol (PI)-anchored proteins in the erythrocyte membrane (6, 7). Therefore, many studies are now being conducted on the abnormal process involving PI-anchoring of the membrane proteins (8). But the exact mechanism of the process is still unknown. On the other hand, membrane disorders other than the PI-anchoring of proteins have already been pointed out, i.e., the abnormality of C3b/C4b receptor (CR1) glycoprotein (9) or glycophorin-α (10) in PNH erythrocytes. The altered expression of membrane glycoconjugates such as glycosyl-PI (glyceroglycolipids) or glycoproteins raises the possibility of abnormal expression of other glycoconjugates like glycosphingolipids (GSL).[2]

GSL are well known to participate in various membrane-related biological functions (11, 12). Since the expression of GSL is regulated indirectly by genes through the expression of metabolic enzymes, GSL change in association with cell growth, ontogenesis, cell differentiation, and transformation. GSL in human erythrocytes are also well characterized (13, 14) and have important functions, such as blood group antigens (11, 12). Therefore, we have focused on the expression of membrane GSL in PNH erythrocytes to determine new features of membrane disorders in PNH.

Methods

Patient profile. The clinical features of seven PNH patients are summarized in Table I. Patient 1 had an inherited deficiency of the ninth component of complement (C9). Because she had never experienced spontaneous massive hemolysis nor required any blood transfusions, PNH erythrocytes were abundant in her peripheral blood. Patient 2 had moderate hemolytic attacks, but never required any blood transfusions because his anemia was mild. Patients 3 and 4 had experienced massive hemolytic attacks and required frequent blood transfusions. Patients 5, 6, and 7 had mild hemolysis but did not require any blood transfusions.

Chemicals. All organic solvents were of analytical grade. DEAE-Sephadex A-25 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and was converted to its acetate form as described previously (15). Iatrobeads (activated silicic acid, 6RS-8060) were purchased from Iatron Laboratories Inc., Tokyo, Japan (16). Precoated high-performance thin-layer plates (Silica Gel 60, 0.25 mm thick) and Polygram sil G plates were purchased from E. Merck, Darmstadt, FRG, and Macherey-Nagel, Duren, FRG, respectively. The following standard glycolipids were prepared in our laboratory: II^{3}NeuAc-LacCer (G$_{M3}$), II^{3}NeuAc-Gg$_3$Cer (G$_{M2}$), II^{3}NeuAc-Gg$_4$Cer (G$_{M1}$), IV^{3}NeuAc,II^{3}NeuAc-Gg$_4$Cer (G$_{D1a}$), II^3(NeuAc)$_2$-Gg$_4$Cer (G$_{D1b}$), and IV^{3}NeuAc,II^3(NeuAc)$_2$-Gg$_4$Cer (G$_{T1b}$) from bovine brain; II^3(NeuAc)$_2$-LacCer (G$_{D3}$) from human milk; IV^{3}NeuAc-nLc$_4$Cer from human erythrocytes; and IV^{3}NeuAc-Lc$_4$Cer and IV^{6}NeuAc-nLc$_4$Cer from human meconium. nLc$_4$Cer and Lc$_4$Cer were prepared from IV^{3}NeuAc-nLc$_4$Cer and IV^{3}NeuAc-Lc$_4$Cer by treatment with neuraminidase, respectively. Gg$_4$Cer (G$_{A1}$) was prepared from G$_{M1}$ using formic acid (17).

Address correspondence to Dr. Hideki Nakakuma, Second Department of Internal Medicine, Kumamoto University Medical School, 1-1-1, Honjo, Kumamoto 860, Japan.

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1. *Abbreviations used in this paper:* DAF, decay-accelerating factor; FAB-MS, fast atom bombardment mass spectrometry; GSL, glycosphingolipids; PI, phosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria.

2. Abbreviations for GSL were based on the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) (41) or the nomenclature system of Svennerholm (42).

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Table I. Clinical Profile of PNH Patients

Patient	Sex/age	Blood group	Erythrocytes in peripheral blood	A.S. test*	S.W. test‡	Hemolytic feature	Other complications	DAF-negative erythrocytes
			$\times 10^4/\mu l$					%
1	F/55	A	250	+	+	Mild	C9-deficiency	95
2	M/77	O	250	+	+	Moderate		33
3	M/32	O	228	+	+	Massive	Thrombosis, DIC	17
4	M/53	O	188	+	+	Massive		17
5	M/60	A	344	+	+	Mild		ND
6	F/52	A	371	+	+	Mild		6
7	M/48	O	489	+	+	Mild		3

* Acidified serum test (Ham test). ‡ Sugar water test.

Extraction and purification of GSL from erythrocytes. 50 ml peripheral blood was drawn with heparin from seven PNH patients and healthy donors. Erythrocytes were lysed in 0.05% acetic acid and centrifuged at 100,000 *g* for 30 min. The precipitate was then washed several times with distilled water. An aliquot of the precipitate was used for the protein assay. GSL were purified by methods described previously (18, 19). Briefly, after lyophilization of the precipitate, total lipids were extracted successively with chloroform(C)/methanol(M)/water(W) (20:10:1, 10:10:1 vol/vol/vol) and C/M (1:2 vol/vol) at room temperature. Each extract was combined and an aliquot was used for the quantitation of lipid-bound phosphorus and lipid-bound sialic acid. Total lipids were then separated into neutral and acidic fractions by DEAE-Sephadex A-25 (acetate form) column chromatography. Gangliosides were isolated from the acidic fraction by mild alkaline hydrolysis and subsequent desalting with a Sep-Pak C18 cartridge (Waters Associates, Milford, MA). Further purification of gangliosides was performed by Iatrobead column chromatography.

TLC. TLC of GSL was performed with the following solvent systems: C/M/W (60:40:10 vol/vol/vol) for neutral GSL; and C/M/0.5% aqueous $CaCl_2$ (50:50:12 vol/vol/vol) for gangliosides. Neutral GSL and gangliosides were located with orcinol-H_2SO_4 and resorcinol-HCl reagents, respectively. The distribution of lipid-bound sialic acid in the individual gangliosides on TLC plates was quantitated densitometrically with a dual wave-length TLC densitometer (CS-9000; Shimadzu Co., Kyoto, Japan) as described previously (18, 19).

Structural elucidation of gangliosides from human erythrocytes. β-Galactosidase, with a specificity reacting to β1-4 linkage but not to β1-3 linkage, was prepared from the culture medium of *Diplococcus pneumoniae* as described previously (20). Neuraminidase (*Vibrio cholerae;* Behring, Marburg, FRG) and the β-galactosidase were used for structural determination as described before (18, 20). In brief, gangliosides were incubated in 100 μl of distilled water at 37°C for 2 h with 20 mU of the neuraminidase. Products were then treated with the β-galactosidase in 50 μl of 200 mM phosphate buffer (pH 6.5) containing 5 μg of sodium deoxycholate. The reaction products were examined by TLC.

Gangliosides and their degradates were further analyzed by TLC-immunostaining (21) with a mouse MAb (Y916 MAb) that recognized the terminal NeuAcα2-6Gal structure of gangliosides (21), a human MAb (HMST-1) which reacted with Lc_4Cer (21), a mouse anti-G_{M3} MAb donated from MECT Co., Tokyo, Japan, or a rabbit anti-G_{A1} antibody (22). Fast atom bombardment mass spectrometry (FAB-MS) was also used for structural determination (23).

Compositions of fatty acid and sphingosine (long chain base) of purified gangliosides were determined by gas-liquid chromatography as described previously (18, 24).

Quantitative determination of membrane constituents. Protein in the starting material was determined by the method of Bradford (25). Lipid-bound sialic acid and lipid-bound phosphorus in the total lipid extracts were determined by the resorcinol-HCl (26) and Bartlett's methods, respectively (27).

Immunofluorescence staining of erythrocytes with anti-DAF MAb. Peripheral blood was diluted 10 times with PBS. 10 μl of the diluted blood ($\sim 5 \times 10^6$ erythrocytes) was incubated with 10 μl of mouse anti-DAF MAb (Wako Pure Chemical Industries, Osaka, Japan) at 4°C for 30 min and labeled with fluorescein-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). Cells were then analyzed by laser cytofluorometry (FACStar; Becton Dickinson Laboratories, Mountain View, CA) (28).

Results

Population of DAF-negative erythrocytes. To estimate the population of abnormal erythrocyte clones in peripheral blood of PNH patients, we took DAF-negative erythrocyte population analyzed by FACS with anti-DAF MAb. The results of immunofluorescence staining of erythrocytes are summarized in Table I. Patient 1 showed an extremely high population of DAF-negative erythrocytes because her C9 deficiency prevented massive hemolytic attacks. Patient 2 had a moderately high population of DAF-negative cells. Neither of them received any blood transfusions. On the other hand, patients 3 and 4 had massive hemolytic exclusion of PNH erythrocytes and received frequent transfusions of erythrocytes from healthy donors. However, both of them showed moderately low populations of DAF-negative erythrocytes. Patients 5, 6, and 7 were clinically mild. The latter two showed very low populations of DAF-negative erythrocytes. Thus, all of the seven patients were classified into three groups on the basis of clinical features and DAF-negative erythrocyte populations: group I (patients 1 and 2); group II (patients 3 and 4); group III (patients 5, 6, and 7).

Altered expression of gangliosides in PNH erythrocytes. Gangliosides from control erythrocytes showed four major bands (*a, b, c,* and *g* in Fig. 1, lane *C* > 70% of the total lipid-bound sialic acid) and five minor bands (*d, e, f, h,* and *i* in Fig. 1, lane *C*). Variable expression of gangliosides in PNH erythrocytes was observed. Patient group I (Fig. 1, lanes *1* and *2*) showed dramatic changes in ganglioside expression: deficiency in band *g*, which was located between the reference gangliosides G_{D1a} and G_{D1b}; and disappearance of gangliosides more polar than band *g* on TLC plates. These changes were also demonstrated by TLC densitometry (*I* in Fig. 2). On the

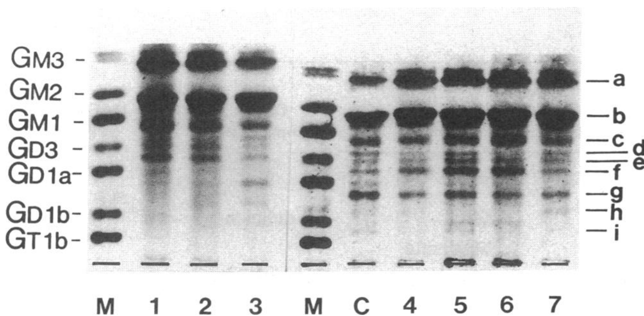

Figure 1. Thin-layer chromatograms of gangliosides in erythrocytes from PNH patients and a healthy donor. The ganglioside fractions corresponding to ~ 10 μg of lipid-bound sialic acid were separated on TLC plates and located as described in the text. Clear resorcinol-positive bands are denoted in alphabetical order from top to bottom. Lanes *1–7*, gangliosides of PNH erythrocytes from patients 1–7, respectively; lane *C*, representative of six healthy donors as normal control; lane *M*, mixture of reference gangliosides. Lanes *M, 1, 2, 3* (*left*) and *M, C, 4, 5, 6,* and *7* (*right*) were developed on different TLC plates.

other hand, the erythrocytes in group III (Fig. 1, lanes *5–7*) had both band *g* and polar gangliosides, which were not detected in group I. However, the highly polar gangliosides showed variable expression among the three patients of group III. In group II (Fig. 1, lanes *3* and *4*), both gangliosides *g* and *h* of the polar gangliosides clearly appeared in patient 3, and *g* in patient 4. The other highly polar gangliosides, which variably appeared in group III, were not detected in group II. The ganglioside pattern was confirmed using a TLC densitometer (Fig. 2) that detected only resorcinol-positive bands on TLC plates. These results suggest that polar gangliosides variably disappeared in the erythrocytes from PNH patients. There were no significant changes in the neutral fractions of GSL from PNH erythrocytes (data not shown).

Chemical structure of gangliosides purified from human erythrocytes. For structural analysis of band *g*, which variably disappeared from PNH erythrocytes, we isolated the glycolipid from the total ganglioside fraction of erythrocytes in 23.2 liters of peripheral blood from 116 healthy donors by Iatrobead column chromatography with a linear gradient solvent system (24) or preparative TLC (14). Homogeneity and mobility of isolated *g* were confirmed by TLC (Fig. 3, lane *3*). After neuraminidase treatment, *g* was converted to resorcinol-negative, but orcinol-positive neutral glycolipid (Fig. 3, lane *4*) that had the same mobility as nLc_4Cer (Fig. 3, lane *2*). Sequential treatment with the diplococcal β-galactosidase, which cleaved Galβ1-4 but not Galβ1-3 linkage, yielded a neutral glycolipid (indicated by top arrow in Fig. 3, lane *6*) that migrated on TLC plate in a manner similar to Lc_3Cer derived from $IV^3NeuAc\text{-}nLc_4Cer$ of human erythrocytes (Fig. 3, lane *5*), indicating that the structure is nLc_4Cer with sialic acid at the nonreducing terminal. Moreover, both ganglioside *g* (Fig. 3, lane *6*) and reference $IV^3NeuAc\text{-}nLc_4Cer$ from human erythrocytes (Fig. 3, lane *5*) gave the same products when analyzed by TLC after sequential enzymatic hydrolysis (Fig. 3 *B*).

TLC immunostaining with Y916 MAb showed positive staining of ganglioside *g* (Fig. 4, lane *2*) as well as the control $IV^6NeuAc\text{-}nLc_4Cer$ from human meconium (Fig. 4, lane *1*) (21). Differences in mobility on TLC plates may be due to the structural difference in their ceramide moieties. Neuraminidase-treated band *g* (Fig. 4, lane *6*) migrated to the position identical to that of nLc_4Cer (Fig. 4, lane *5*) among reference glycolipids with four carbohydrates on TLC plates (Fig. 4 *B*). HMST-1 MAb, which recognized Lc_4Cer, did not react with the neutral glycolipid derived from *g* after neuraminidase treatment (Fig. 4, lane *10*). Thus, the chemical structure of ganglioside *g* was concluded to be $IV^6NeuAc\text{-}nLc_4Cer$, designated as G4 by Watanabe, K., et al. (14). Expression of band *g* in normal erythrocytes and altered concentrations in PNH erythrocytes were clearly shown by TLC immunostaining of total gangliosides with Y916 MAb (data not shown), and were consistent with those obtained by TLC with orcinol staining in Fig. 1.

In addition to ganglioside *g*, the other major gangliosides denoted as bands *a*, *b*, and *c* of human erythrocytes in Fig. 1 were also isolated by Iatrobead column chromatography. Both ganglioside *a* and a product after neuraminidase treatment of

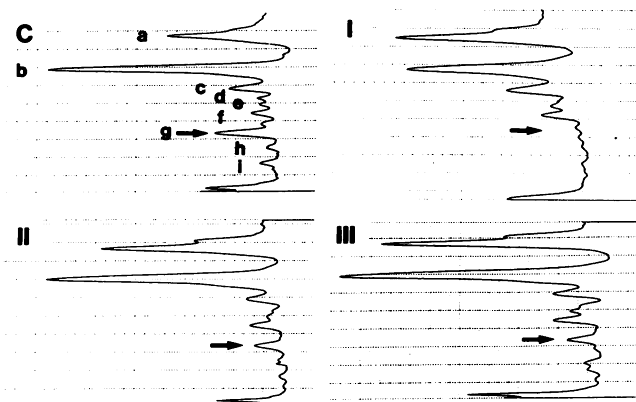

Figure 2. Densitometric scanning pattern of gangliosides in erythrocytes from PNH patients and a healthy donor. The chromatograms shown in Fig. 1 were scanned as described in the text. Panels *I, II,* and *III* are representative of patient groups I, II, and III, respectively. Arrows indicate the characteristic ganglioside, band *g*, which variably disappeared in PNH erythrocytes. *C, I, II,* and *III* correspond to lanes *C, 1, 4,* and *5* in Fig. 1, respectively.

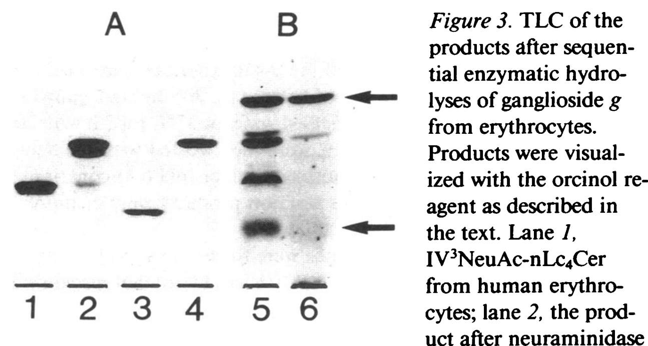

Figure 3. TLC of the products after sequential enzymatic hydrolyses of ganglioside *g* from erythrocytes. Products were visualized with the orcinol reagent as described in the text. Lane *1*, $IV^3NeuAc\text{-}nLc_4Cer$ from human erythrocytes; lane *2*, the product after neuraminidase treatment of the $IV^3NeuAc\text{-}nLc_4Cer$ of lane *1;* lane *3*, ganglioside *g;* lane *4*, the product after neuraminidase treatment of ganglioside *g;* lanes *5* and *6*, the products after treatment of $IV^3NeuAc\text{-}nLc_4Cer$ and ganglioside *g* with both neuraminidase and β-galactosidase, respectively. The top arrow indicates the position of Lc_3Cer and the bottom arrow indicates an orcinol-negative spot that was derived from the sodium deoxycholate used in the reaction with β-galactosidase. *A* (lanes *1–4*) and *B* (lanes *5* and *6*) were developed separately.

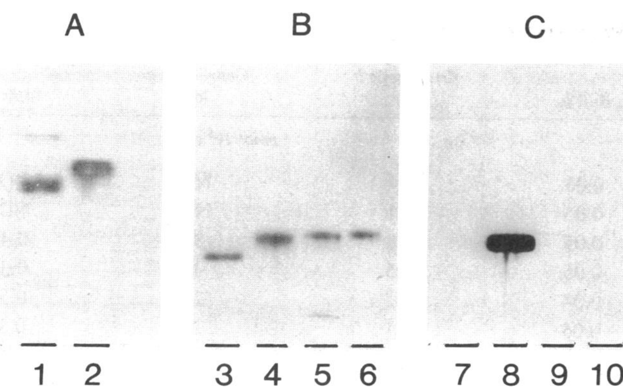

Figure 4. TLC and TLC immunostaining of ganglioside *g* and its products after neuraminidase treatment. TLC immunostaining with Y916 MAb (*A*) of both ganglioside *g* (lane *2*) and IV^6NeuAc-nLc_4Cer from human meconium for positive staining (lane *1*); TLC (*B*, orcinol staining) and TLC immunostaining with HMST-1 MAb (*C*) of nLc_4Cer analogues such as Gg_4Cer (G_{A1}, lanes *3* and *7*), Lc_4Cer (lanes *4* and *8*), nLc_4Cer (lanes *5* and *9*), and the products after neuraminidase treatment of ganglioside *g* (lanes *6* and *10*).

ganglioside *c* were analyzed by TLC immunostaining with a mouse MAb against G_{M3} or anti-G_{A1} antibody, respectively. Ganglioside *b* was analyzed by FAB-MS with reference IV^3NeuAc-nLc_4Cer. The chemical analyses suggested that bands *a, b,* and *c* were G_{M3}, IV^3NeuAc-nLc_4Cer, and IV^3NeuAc-Gg_4Cer (G_{M1b}), respectively (data not shown). They were also previously designated as G1, G2, and G5, respectively (14).

Interestingly, the structural difference in sugar moieties between gangliosides *b* and *g* was a sialosyl linkage alone. Hence, to examine whether the ceramide moiety defines the carbohydrate determinants (29), we analyzed the ceramide structures of both bands *b* and *g* from normal erythrocytes. As a result, band *b* had a longer chain of fatty acids as compared with that of band *g,* while there was no difference in the composition of long chain bases between them (Table II).

Contents of membrane constituents. We also determined the quantities of protein, lipid-bound phosphorus, lipid-bound

Table II. Fatty Acid and Long Chain Base Compositions of Gangliosides b and g

	Ganglioside *b* (IV^3NeuAc-nLc_4Cer)	Ganglioside *g* (IV^6NeuAc-nLc_4Cer)
Fatty acid		
14:0	0	3.8
16:1	0	3.1
16:0	6.7	22.3
18:1	0	8.5
18:0	1.7	20.8
20:0	1.4	1.3
22:0	28.3	11.2
24:1	2.6	11.3
24:0	59.3	17.9
14 + 16 + 18 (a)	8.4	58.5
20 + 22 + 24 (b)	91.6	41.7
a/b	0.09	1.40
Long chain base		
18d:1	100.0	100.0

sialic acid, and major gangliosides in erythrocytes (Table III). Patients 1 and 2 showed slightly higher levels of both lipid-bound phosphorus and lipid-bound sialic acid than the other patients or the healthy donor. However, the ratio of lipid-bound sialic acid/lipid-bound phosphorus was similar among the PNH erythrocytes and those of the control (b/a ratio in Table III). The levels of IV^3NeuAc-nLc_4Cer (ganglioside *b*) in patients 1 and 2 increased in association with the disappearance of both band *g* (IV^6NeuAc-nLc_4Cer) and the highly polar gangliosides.

Discussion

In all seven PNH patients we found marked changes in the expression of gangliosides on the erythrocyte membrane. Both a characteristic ganglioside designated as *g* and highly polar gangliosides, which clearly appeared in normal erythrocytes, variably disappeared from erythrocytes in the peripheral blood of PNH patients. Although we used erythrocytes from peripheral blood without the isolation of PNH clones, biochemical analysis of erythrocytes from patient 1 with a very high population of DAF-negative erythrocytes strongly supported the ganglioside alteration in PNH erythrocytes. These results suggest a new feature of membrane disorders in PNH, i.e., metabolic disorders of membrane glycoconjugates, when taken together with the reported abnormalities of PI (glyceroglycolipid)-anchored proteins like DAF (3, 4), and non–PI-anchored glycoproteins like CR1 (9) and glycophorin-α (10).

Among the altered gangliosides, the most characteristic band *g* was structurally characterized for both clinical use and for understanding the underlying molecular mechanism of metabolic changes in PNH. Chemical analyses identified IV^6NeuAc-nLc_4Cer as the plausible structure, which had already been isolated from human erythrocytes and analyzed previously (14). Interestingly, the structural difference between both gangliosides *b* (IV^3NeuAc-nLc_4Cer) and *g* (IV^6NeuAc-nLc_4Cer) was a sialosyl linkage alone in sugar moieties: NeuAcα2-3Gal and NeuAcα2-6Gal. Ganglioside *b* was, however, contained in both PNH and normal erythrocytes, whereas ganglioside *g* was detected clearly in the normal erythrocytes but variably in the PNH erythrocytes. These results suggest an impaired α2-6 sialylation of glycolipids in PNH erythrocytes. As to the molecular basis of impaired glycosylation of glycolipids, some possible mechanisms are suggested: either quantitative or qualitative changes in the expression of the responsible glycosyltransferase or alteration in the regulatory system of the enzyme activity (30). These mechanisms are also expected to be operated in erythroid precursors in PNH. Clarification of the precise mechanisms of impaired glycosylation in PNH may lead not only to disclosure of the mechanism of altered expression of other polar gangliosides than *g* in PNH (Fig. 1), but also to a clue to the abnormal process involving PI-anchoring of membrane proteins. At present, we have not found a common structure in the precisely determined carbohydrate moieties between IV^6NeuAc-nLc_4Cer of human erythrocytes and PI glycans, such as DAF of human erythrocytes (31), Thy-1 glycoprotein of rat brain (32), or variant surface glycoprotein of *Trypanosoma brucei* (33). We showed and focused on impaired sialylation, however, and these results suggest that we cannot rule out coexistence of the abnormalities in either glycosylation other than α2-6 sialylation or degradation pathway of membrane glycoconjugates in PNH erythrocytes.

Table III. *Membrane Constituents of Erythrocytes from PNH Patients and a Healthy Donor*

Patient	Protein	Lipid-P* (a)	Lipid-NA‡ (b)	(b)/(a)	Ganglioside *b* (c)	Ganglioside *g* (d)	(d)/(c)
	μg/10⁶ cells		*nmol/10⁸ cells*			*pmol/10⁸ cells*	
1	0.4	10.0	0.6	0.06	214	ND	ND
2	0.4	13.1	0.7	0.05	251	ND	ND
3	0.4	7.8	0.4	0.05	98	39	0.4
4	0.4	7.8	0.4	0.05	105	31	0.3
5	0.6	5.2	0.2	0.04	52	26	0.5
6	0.4	5.9	0.3	0.05	81	24	0.3
7	0.3	6.6	0.3	0.05	80	30	0.4
HD§	0.3	5.9	0.3	0.05	74	35	0.5

* Lipid-bound phosphorus. ‡ Lipid-bound sialic acid. § Representative of six healthy donors.

With regard to the ceramide moieties of gangliosides *b* and *g*, there was a marked difference in the fatty acid compositions. This finding supports a previous report that the ceramide structure defines the organization of glycosyltransferases for synthesis of the carbohydrate determinants (29). Ganglioside *b* seemed to increase in association with the disappearance of ganglioside *g* (Table III), especially in the erythrocytes from patients 1 and 2. Thus, it is of further interest to investigate whether the ceramide structure accounts for the impaired glycosylation (sialylation) in PNH erythrocytes by structural analysis of the ceramides of $IV^3NeuAc\text{-}nLc_4Cer$ (ganglioside *b*) from PNH erythrocytes.

For the clinical usage of ganglioside *g*, we next tried to detect it in GSL of erythrocytes with Y916 MAb, which reacted with the terminal NeuAcα2-6Gal structure. TLC immunostaining of purified gangliosides with the MAb discriminated between PNH erythrocytes and normal erythrocytes, while FACS analysis with the MAb could not detect the ganglioside even in normal erythrocytes. Cryptic expression of the ganglioside in cell membranes may partly explain this (34).

It is important to clarify the relation between the altered expression of gangliosides and the clinical features of PNH. The effect of membrane gangliosides on complement activation is not yet clear; however, recent reports suggest the importance of sialic acid residue of gangliosides in inhibiting the alternative pathway of complement activation (35, 36). Moreover, we showed an increase in lipid-bound sialic acid in PNH patients 1 and 2, but no difference in the ratio of lipid-bound sialic acid to lipid-bound phosphorus between control and PNH patients (Table III). Thus, our results might suggest that the increase in total sialic acid would reflect a functional or structural compensation for the loss of DAF or other PI glycoproteins in PNH erythrocytes. In this respect, it is interesting to examine whether or not the loss of both ganglioside *g* and highly polar gangliosides in PNH erythrocytes accelerates the alternative pathway. DAF expression and the ganglioside abnormalities, however, did not closely correlate. Considering that the increased sensitivity to complement is simply one aspect of many membrane disorders in PNH erythrocytes (2, 9, 10), it is likely that the ganglioside alteration in PNH reflects new features of membrane disorders other than the abnormal susceptibility of erythrocytes to complement. For example, impaired sialylation in PNH erythrocytes is similar to the blocked synthesis theory of ganglioside changes in cancer cells or in association with malignant transformation (11, 34). Taken together with the unusual leukemic conversion of PNH (37, 38), PNH might share ganglioside alteration as a common phenotypic expression in cell membrane with leukemia.

Concerning the control erythrocytes, we should have analyzed the normal erythrocytes from patients before the onset of the disease. However, the biochemical analysis of erythrocytes from the six healthy donors gave the same pattern of gangliosides on TLC plates (data not shown), irrespective of their different ABO blood groups, which are mainly defined by very small but immunologically sufficient amounts of fucosylated neutral GSL. Our control was representative of the healthy donors and thus was a good reference.

If the change in gangliosides is one of the changes that result from unusual hematopoiesis in PNH patients, it is possible that PNH leukocytes and platelets also have metabolic disorders involving GSL, because PNH is a stem-cell disorder of a clonal nature (1, 39). Furthermore, it would also be interesting to examine membrane GSL of blood cells in other hemolytic disorders or in PNH-related disorders, such as PNH aplastic anemia syndrome (40) and leukemia evolved from PNH (37, 38).

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