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CHANGES IN PHOSPHOLIPID AND GANGLIOSIDE DURING DIFFERENTIATION OF MOUSE MYELOID LEUKEMIA CELLS

Masaki Saito[1*], Hisao Nojiri[1] and Michiyuki Yamada[2]

[1]Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173 and
[2]Biochemistry Division, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan

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SUMMARY

When mouse myeloid leukemia M1 cells were induced to differentiate into macrophages by bacterial lipopolysaccharide (LPS), phospholipids and gangliosides of the cells changed markedly. The amounts of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol per mg protein increased 30%, 20% and 30%, respectively, during differentiation, but the others, phosphatidylserine and sphingomyelin, did not increase significantly. Three species of gangliosides constituted of major portions of gangliosides in M1 cells. Several-fold increase in monosialoganglioside GM1 was observed in the LPS-treated cells with a concomitant decrease in disialogangliosides. Based upon the treatment with sialidase, this GM1 was identified to be GM1b, which was recently found in rat ascites hepatoma cells and human erythrocyte membranes.

It has been reported that mouse myeloid leukemia M1 cells can be induced to differentiate into macrophages by various reagents, such as proteins in the conditioned medium of mouse lung fibroblasts, steroids, polyanions including poly(ADP-Rib) and lipopolysaccharide (1-6). During this induction, differentiation-associated properties appeared; cell adhesion to culture flask, locomo-

*To whom all correspondence should be addressed. Mailing address: Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo-173, Japan.

The symbols for gangliosides are essentially based on the nomenclature system of Svennerholm (26): GA1, gangliotetraosylceramide, Gal-GalNAc-Gal-Glc-ceramide; GM1a, Gal-GalNAc-(NeuAc)Gal-Glc-ceramide; GM1b, NeuAc-Gal-GalNAc-Gal-Glc-ceramide; GM2, GalNAc-(NeuAc)Gal-Glc-ceramide; GM3, NeuAc-Gal-Glc-ceramide; GD1a, NeuAc-Gal-GalNAc-(NeuAc)Gal-Glc-ceramide; GD1b, Gal-GalNAc-(NeuAc-NeuAc)-Gal-Glc-ceramide; GD2, GalNAc-(NeuAc-NeuAc)Gal-Glc-ceramide; GD3, NeuAc-NeuAc-Gal-Glc-ceramide; GT1a, NeuAc-NeuAc-Gal-GalNAc-(NeuAc)Gal-Glc-ceramide; GT1b, NeuAc-Gal-GalNAc-(NeuAc-NeuAc)Gal-Glc-ceramide; Sialylparagloboside, NeuAc-Gal-GlcNAc-Gal-Glc-ceramide. The abbreviations used are: Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid; DEAE-, diethylaminoethyl-; TLC, thin-layer chromatography.

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tion, phagocytosis, Fc- and C3-receptors, lysozyme and cathepsin D and changes in cell morphology. Recently Nagata and Ichikawa reported that Fc-receptor appeared without protein synthesis at the early stage of the induction of differentiation (7). Changes in glycoprotein of cell membrane in Ml cells were also observed during the cell differentiation (8).

Phospholipids are major constituents of the cell membrane (9) and changes in their composition might cause changes of the cell morphology as well as various cellular functions. Gangliosides constitute a small portion of the glycoconjugates of cell surfaces, but might exhibit an important function as special receptors or surface markers (10-12), providing negative charges to the cell surface.

This work shows that changes in phospholipids and gangliosides of mouse myeloid leukemia Ml cells were associated with the differentiation of the cells into mature cells. In addition, evidence is presented that a monosialoganglioside GMlb, which has been uncommon in biological materials, was identified in Ml cells.

MATERIALS AND METHODS

Chemicals: Phospholipids were purchased from Sigma Chemical Co., St. Louis, and purified in this laboratory. Gangliosides GM3, GM2, GMla, GDla and GTlb were isolated and purified from bovine and human brains according to the method of Momoi et al.(13). Neutral glycosphingolipids were products of Supelco Inc., Bellefonte. Asialo-GMl (GAl) was prepared by the treatment of GMla with Arthrobacter ureafaciens sialidase in the presence of detergent as described previously (14). LPS from Salmonella typhosa was from DIFCO Laboratories. Ganglioside GMlb isolated from rat ascites hepatoma, AH7974F, was kindly supplied by Dr. T. Taki, Shizuoka Pharmaceutical College, Shizuoka.

Cells and Cell Cultures: Ml cells were grown in tissue culture flasks (Nunc, Denmark) in Eagle's minimal essential medium with a 2-fold concentration of amino acids and vitamins (Grand Island Biol. Co., Grand Island) and 10% inactivated (56°C for 30 min) calf serum. For induction of cell differentiation, the leukemic cells were seeded at 7.1 x 10^5 cells/ml and grown in the presence or absence of LPS (2 μg/ml) for 65 h.

Assays for Fc-receptors, Lysozyme and Phagocytosis: These assays were carried out as described previously (5) except that lysozyme activity was assayed with human lysozyme as a standard.

Quantitation of Phospholipids: For determination of phospholipids and gangliosides, the cells were collected, washed twice with phosphate-buffered saline (pH 7.4), and with saline, and then were lyophilized and kept at -80°C until use. Phospholipids were extracted from the lyophilized materials first with chloroform-methanol (C-M) (2:1, v/v) containing 5% (v/v) water, and then with

C-M (1:1, v/v) and C-M (1:2, v/v), successively. The extracts were combined, washed with 0.1M KCl, and twice with C-M-0.1M KCl (3:48:47, v/v/v) and dried up under a nitrogen stream as described previously (15). The phospholipids were placed onto the thin-layer plates of Silica gel 60 (10 x 10 cm, Merck), and developed two-dimensionally as described by Yavin and Zutra (16). In order to distinguish plasmalogens from the diacyl-phosphatides, a hydrolysis procedure with HCl fumes was included after the first development. The individual components were scraped and measured by determining phospholipid-phosphorus by the method of Chen et al.(17).

Determination of Gangliosides: The combined C-M extracts which also contained gangliosides were further fractionated by DEAE-Sephadex column chromatography and high-performance thin-layer chromatography (HPTLC) essentially as described by Ando et al.(18). The C-M extracts were brought to the solvent mixture of C-M-water (30:60:8, v/v/v) and loaded to a column (0.6 x 6 cm) of DEAE-Sephadex (A-25, acetate form) which was equilibrated with the same solvent. The column was washed with the same solvent and the gangliosides were eluted with C-M-0.2M sodium acetate (30:60:8, v/v/v). The ganglioside fraction was dried up and dissolved in 0.1N NaOH, and incubated at 37°C for 1 h to hydrolyze phospholipids. The hydrolysate was desalted by gel filtration on Sephadex G-50 (1.0 x 12.7 cm). The recovered gangliosides were dissolved in C-M (1:1), and developed on HPTLC plates with the solvent of C-M-0.5% $CaCl_2$ (45:45:10, v/v/v). The gangliosides were visualized by spraying with resorcinol-HCl reagent and determined in a dual-wavelength TLC scanner, Shimadzu CS-910.

In order to discriminate GM1b from GM1a, gangliosides (up to 2.1 μg of lipid-bound sialic acid) were treated with A. ureafaciens sialidase in the absence of sodium cholate for 3 h at 37°C under the condition in which the sialyl residue of GM1b was released, but that of GM1a was not, as described previously (14). Asialo-gangliosides were located by spraying with anthrone-H_2SO_4 reagent (14).

Other Analytical Methods: Lipid-bound sialic acid was estimated by the method of Suzuki (19). Protein was assayed by the method of Lowry et al.(20) with bovine serum albumin as a standard, and DNA was determined by the method of Ceriotti (21).

RESULTS

When M1 cells were cultured with LPS for 65 h, certain differentiation-associated properties appeared with their growth suppressed by about 50%. About 42% of the total LPS-treated cells became phagocytic, and Fc-receptors became detectable in 22% of the treated cells whereas only 1.7% of the cells were phagocytic and 3% of the cells had Fc-receptors in the non-treated control cultures. Lysozyme activity produced by the LPS-treated cells was 0.27 μg equivalent per 5 x 10^6 cells, but no lysozyme activity was detected in the control cultures. The cell growth with and without LPS reached 2.3 x 10^6 and 4.5 x 10^6 cells/ml, respectively.

Phospholipids and gangliosides were analysed on the differentiated cultures, and compared with the controls. Phospholipids of the LPS-treated cells increased by 26% compared with those of the control cultures, and gangliosides of the

Table I

Comparison of Phospholipid-Phosphorus and Ganglioside-Sialic Acid Contents between Myeloid Leukemic Cells (Ml) and Cells Differentiated by Lipopolysaccharide[a)]

	Phospholipid-phosphorus		Ganglioside-sialic acid	
	(μg/mg-prot.)	(μg/mg-DNA)	(μg/mg-prot.)	(μg/mg-DNA)
Control	4.73 ± 0.22	37.0 ± 1.4	0.194 ± 0.007	1.54 ± 0.08
Lipopolysaccharide	5.96 ± 0.24	63.4 ± 3.1	0.217 ± 0.010	2.31 ± 0.09

[a)]Determinations of content of phospholipid-phosphorus, ganglioside-sialic acid, protein and DNA were performed using lyophilized samples as described in MATERIALS AND METHODS. Each value represents the mean value ± S.E.M. in two observations.

former cells increased by 12% on the basis of milligram of protein. The amounts of phospholipids and gangliosides per milligram DNA were increased by 71% and 50%, respectively (Table I).

The relative percentage of each phospholipid species did not significantly change when Ml cells were induced to differentiate by LPS. However, the amount of each phospholipid species were generally increased when expressed on the basis of milligram of protein and DNA (Table II). It was found that the two major phospholipids, phosphatidylcholine and phosphatidylethanolamine, increased by 32% and 24%, respectively, in the LPS-treated cells while choline- and ethanolamine-plasmalogens were also increased by 45% and 18%, respectively. Phosphatidylinositol also increased by about 30% in the LPS-differentiated culture. In contrast, both sphingomyelin and phosphatidylserine were almost unchanged. The ratio of ethanolamine phosphoglycerides to choline phosphoglycerides was calculated to be 0.32 in the LPS-treated Ml cells and 0.35 in the corresponding control culture. The differences of each phospholipid species between the LPS-treated Ml cells and the control culture were much more evident when expressed on the basis of mg DNA.

The ganglioside composition in Ml cells and those differentiated with LPS were analysed by HPTLC. Three major ganglioside species were present in both

Table II

Phospholipid Compositions of Ml Cells and of Those Differentiated with Lipopolysaccharide[a)]

Phospholipid	Culture: Control (A) µg Phosphorus per mg protein	Culture: Control (B) µg Phosphorus per mg DNA	Culture: LPS-induced (A) µg Phosphorus per mg protein	Culture: LPS-induced (B) µg Phosphorus per mg DNA	Ratio LPS (A) / Control (A)
Phosphatidylcholine	2.50 ± 0.17	19.6 ± 1.27	3.31 ± 0.05	35.3 ± 0.6	1.32
Choline-plasmalogen	0.080 ± 0.017	0.63 ± 0.13	0.116 ± 0.003	1.23 ± 0.03	1.45
Lysophosphatidylcholine	0.024 ± 0.004	0.19 ± 0.03	0.039 ± 0.004	0.40 ± 0.04	1.62
Sphingomyelin	0.230 ± 0.051	1.80 ± 0.41	0.236 ± 0.054	2.51 ± 0.58	1.03
Phosphatidylethanolamine	0.639 ± 0.106	4.99 ± 0.80	0.791 ± 0.073	8.43 ± 0.77	1.24
Ethanolamine-plasmalogen	0.267 ± 0.029	2.09 ± 0.22	0.316 ± 0.012	3.36 ± 0.12	1.18
Phosphatidylserine	0.271 ± 0.031	2.11 ± 0.24	0.296 ± 0.014	3.14 ± 0.16	1.09
Phosphatidylinositol	0.383 ± 0.041	3.01 ± 0.32	0.497 ± 0.024	5.29 ± 0.26	1.30

[a)] The total phospholipid fractions were extracted from LPS-treated and untreated Ml cells, and individual phospholipids were quantitated after separation by two-dimensional TLC as described in MATERIALS AND METHODS. Each value represents the mean value of two determinations, each in duplicate, plus/minus the standard error of means (SEM).

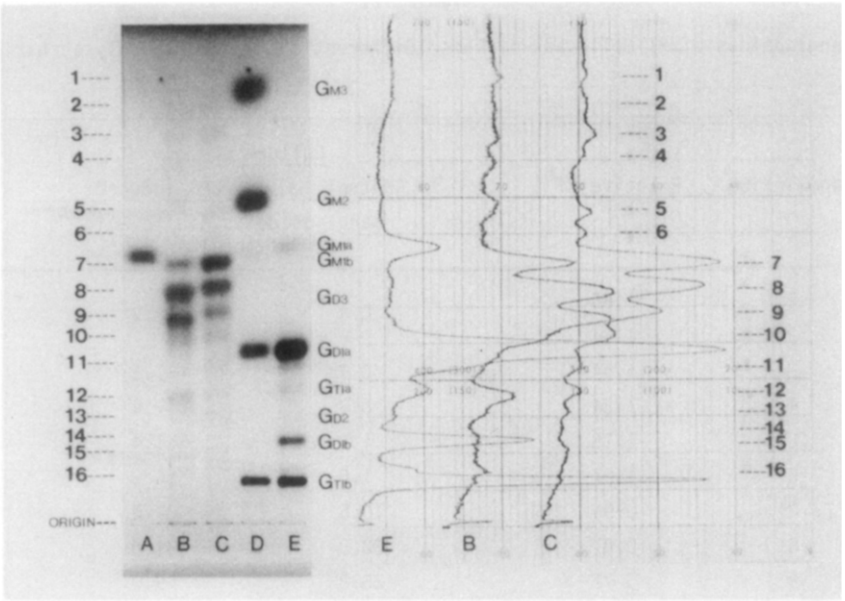

Fig. 1. The high-performance thin-layer chromatogram and its densitometric scanning pattern of gangliosides isolated from M1 cells and those differentiated with LPS. The total ganglioside fractions were isolated from M1 cells untreated and treated with LPS, and HPTLC was carried out as described in MATERIALS AND METHODS. Each lane represents as follows: A, ganglioside GM1a purified from bovine brain (equivalent to 0.48 μg lipid-bound sialic acid); B, gangliosides isolated from untreated M1 cells (equivalent to 2.5 μg lipid-bound sialic acid); C, gangliosides isolated from LPS-treated M1 cells (equivalent to 2.5 μg lipid-bound sialic acid); D, a mixture of gangliosides GM3, GM2, GD1a and GT1b standards (each, equivalent to 1.10, 1.26, 0.72 and 0.48 μg lipid-bound sialic acid, respectively); E, the total bovine brain gangliosides (equivalent to 3.16 μg lipid-bound sialic acid). Numbers at both sides represent ganglioside spots distinguishable by both the naked eye and the densitometry. The band 7 was tentatively identified as GM1b as shown in Fig. 2.

cell cultures and in addition, more than 10 species of minor gangliosides could be observed as shown in Fig. 1. One of the major gangliosides, corresponding to band 7 in Fig. 1, chromatographed closely to brain ganglioside GM1a. This ganglioside was increased about 3-fold in the LPS-treated M1 cells as compared to the control culture (Fig. 1 and Table III). This material co-chromatographed with the monosialoganglioside GM1b which has been recently identified as a naturally occurring monosialotetraosylganglioside in rat ascites hepatoma AH-7974F (22) and human erythrocyte membranes (23). Furthermore, the treatment of gangliosides with A. ureafaciens sialidase confirmed that the ganglioside of

Table III

Gangliosides of Ml Cells and of Those Differentiated with Lipopolysaccharide[a)]

Ganglioside	Relative Rf[b)]	Culture	
		Control	LPS-induced
		Ganglioside as sialic acid[d)] (% of total)	
1	1.66	0.8	0.7
2	1.54		
3	1.44	0.7	1.5
4	1.34	0.7	1.4
5	1.16	1.8	1.4
6	1.07	1.5	0.9
7[c)]	0.96	10.5	31.2
8	0.87	32.8	31.0
9	0.77	26.8	15.0
10	0.68	9.1	9.2
11	0.59	3.2	3.0
12	0.47	6.6	2.2
13	0.40	2.8	1.4
14	0.32	1.2	1.0
15	0.26		
16	0.18	1.3	N.D.[e)]

a)Ganglioside-bound sialic acid was determined by the densitometric procedure on thin-layer chromatograms as described in MATERIALS AND METHODS.
b)The Rf value of ganglioside GMla was taken as 1.00.
c)The band 7 was tentatively identified to be ganglioside GMlb as described in the text.
d)Each value represents the mean of two determinations.
e)not detectable.

band 7 is GMlb (Fig. 2). As shown previously (14), the sialidase could hydrolyze the terminal sialyl residue of GMlb in both the absence and presence of sodium cholate, but could hydrolyze the internal sialyl residue of GMla only in the presence of the detergent. Sialylparagloboside is also a sialidase-labile monosialotetraosylganglioside and has an Rf value similar to that of GMl with neutral solvents. It could be easily discriminated from GMlb after sialidase treatment since paragloboside, the product of the former sialidase-treated sialoglycolipid, had an Rf of about 0.61 and that of GAl around 0.50 on HPTLC

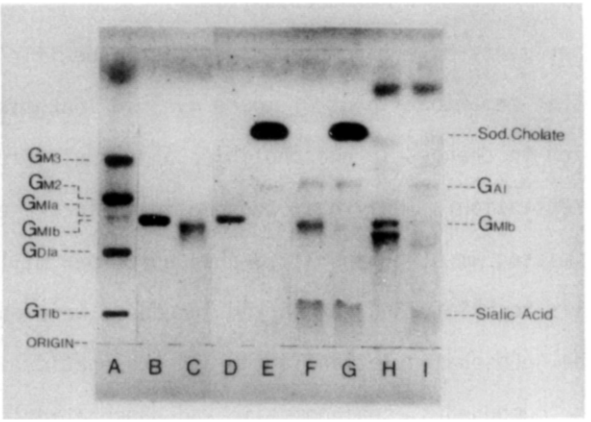

Fig. 2. HPTLC of gangliosides after treated with A. ureafaciens sialidase. Gangliosides of various origins were subjected to sialidase-treatment in the presence or absence of sodium cholate, and the HPTLC was performed with these mixtures, spots being visualized by spraying with anthrone-H_2SO_4 reagent, as described in MATERIALS AND METHODS. Each lane represents as follows: A, a mixture of gangliosides GM3, GM2, GD1a and GT1b standards (each, the same amount as shown in lane D in Fig. 1); B, ganglioside GM1a (0.95 μg lipid-bound sialic acid) purified from bovine brain; C, ganglioside GM1b (0.71 μg lipid-bound sialic acid) isolated from rat ascites hepatoma AH7974F; D, GM1a (0.51 μg lipid-bound sialic acid) was incubated with the sialidase (10 milli-units) for 3 h at 37°C in the absence of detergent; E, GM1a (0.51 μg lipid-bound sialic acid) was incubated with the same amount of the sialidase as in lane D in the presence of sodium cholate (8.6 μg); F, GM1b (0.71 μg lipid-bound sialic acid) was incubated with the sialidase (5 milli-units) in the absence of detergent for 3 h at 37°C; G, GM1b (1.78 μg lipid-bound sialic acid) was hydrolyzed with the sialidase (25 milli-units) in the presence of sodium cholate (25 μg); H, gangliosides (equivalent to 2.13 μg lipid-bound sialic acid) isolated from LPS-treated Ml cells were incubated in the absence of both the sialidase and the detergent for 3 h at 37°C; I, the same amount of gangliosides of LPS-treated Ml cells as in lane H was incubated with the sialidase (5 milli-units) in the absence of the detergent as shown in lane F.

with a solvent of C-M-0.5% $CaCl_2$ (45:45:10). The major gangliosides, bands 7, 8 and 9 could be hydrolyzed with A. ureafaciens sialidase in the absence of detergent, and the production of asialo-GM1 (GA1) was clearly demonstrated as shown in Fig. 2. Bands 8 and 9 ganglioside chromatographed close to brain ganglioside GD3. The ganglioside corresponding to band 9 markedly decreased when the Ml cells were induced to differentiate with LPS. In contrast, the ganglioside corresponding to band 8 retained a constant value of one third of the total gangliosides recovered (Table III). Among the minor gangliosides, a marked decrease was noticed with gangliosides corresponding to bands 12 and 16 in Fig. 1.

DISCUSSION

The present experiments have attempted to document specific differentiation-associated changes of membrane lipids of mouse myeloid leukemic cells with particular attention to changes in phospholipids and gangliosides of Ml cells during cell differentiation. Individual phospholipids increased in general when expressed as microgram of phospholipid-phosphorus per milligram DNA after exposure of Ml cells to LPS for 65 h. Marked increases were observed with phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol while the other two major components, sphingomyelin and phosphatidylserine were almost unchanged. These phospholipid changes might be related to the differentiation-associated appearance of specific receptors, such as the Fc and C3 receptors, as well as with the appearance of morphological characteristics of cell differentiation, such as the general decrease in the mass ratio of nucleus to cytoplasm. Recently, Honma et al.(24) reported changes in the phospholipid composition of Ml cells during differentiation with steroids, and argued that the ratio of phosphatidylethanolamine to phosphatidylcholine increased as the process of differentiation proceeded for longer than 3 days. However, the values of lysophosphatidylcholine of the differentiated cells (24) seems to be abnormally high, which could suggest some problems in their quantitative analyses of phospholipids, or some artifacts of degradative processes during the processing of lipids in the steroid-treated cells.

It is well known that gangliosides play a role in maintaining negative charges on the cell surface and providing specific receptors for biological active substances, such as thyrotropin (11) and choleragen (10). In Ml cells, three major gangliosides were detectable with more than 10 additional species of minor gangliosides. During the induction of differentiation of Ml cells into macrophages with LPS, a monosialoganglioside GMlb (band 7) markedly increased while two other gangliosides, the one (band 9) which chromatographed close to the location of GD3 and the other (band 12) chromatographed close to the position of GD2 or GTla, concomitantly decreased. One of the major ganglio-

sides (band 8) remained constant in its relative amount. Such changes in ganglioside compositions of the LPS-differentiated M1 cells might be related to appearances of specific surface receptors during cell differentiation. The monosialoganglioside GM1b which was markedly increased in the LPS-treated cells was only recently detected in biological materials when Hirabayashi et al.(22) identified this ganglioside in rat ascites hepatoma AH7974F and Watanabe et al. (23) also found it in human erythrocyte membranes. Stoffyn et al.(25) have recently reported that ganglioside GM1b could be synthesized in vitro using homogenates of various animal cells as the enzyme source. The biological significance of this uncommon ganglioside is currently under investigation in this laboratory, using various hematopoietic cells of murine and human origins.

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REFERENCES

1. Ichikawa, Y. (1969) J. Cell. Physiol. 74, 223-234.
2. Fibach, E., Landau, Y., and Sachs, L. (1972) Nature New Biol. 237, 276-278.
3. Lotem, J., and Sachs, L. (1974) Proc. Natl. Acad. Sci. USA 71, 3507-3511.
4. Nakayasu, M., Shimamura, T., Sato, S., and Sugimura, T. (1978) Cancer Res. 38, 103-109.
5. Yamada, M., Shimada, T., Nakayasu, M., Okada, H., and Sugimura, T. (1978) Biochem. Biophys. Res. Commun. 83, 1325-1332.
6. Weiss, B., and Sachs, L. (1978) Proc. Natl. Acad. Sci. USA 75, 1374-1378.
7. Nagata, K., and Ichikawa, Y. (1979) J. Cell. Physiol. 98, 167-176.
8. Sugiyama, K., Tomida, M., and Hozumi, M. (1979) Biochim. Biophys. Acta 587, 169-179.
9. Finean, J. B. (1973) Form and Function of Phospholipids, 2nd edition, pp. 171-203, Elsevier, Amsterdam.
10. Van Heyningen, S. E. (1974) Nature 249, 415-417.
11. Fishman, P. H., and Brady, R. O. (1976) Science 194, 906-915.
12. Marcus, D. M., and Schwarting, G. A. (1976) Adv. Immunol. 23, 203-240.
13. Momoi, T., Ando, S., and Nagai, Y. (1976) Biochim. Biophys. Acta 441, 488-497.
14. Saito, M., Sugano, K., and Nagai, Y. (1979) J. Biol. Chem. 254, 7845-7854.
15. Saito, M., Bourque, E., and Kanfer, J. (1975) Arch. Biochem. Biophys. 169, 304-317.
16. Yavin, E., and Zutra, A. (1977) Anal. Biochem. 80, 430-437.
17. Chen, P. S. Jr., Toribara, T. Y., and Warner, H. (1956) Anal. Chem. 28, 1756-1758.
18. Ando, S., Chang, N.-C., and Yu, R. K. (1978) Anal. Biochem. 89, 437-450.

19. Suzuki, K. (1964) Life Sci. 3, 1227-1233.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
21. Ceriotti, G. (1955) J. Biol. Chem. 214, 59-70.
22. Hirabayashi, Y., Taki, T., and Matsumoto, M. (1979) FEBS Lett. 100, 253-257.
23. Watanabe, K., Powell, M. E., and Hakomori, S. (1979) J. Biol. Chem. 254, 8223-8229.
24. Honma, Y., Kasukabe, T., and Hozumi, M. (1980) Biochem. Biophys. Res. Commun. 93, 927-933.
25. Stoffyn, P., and Stoffyn, A. (1980) Carbohydr. Res. 78, 327-340.
26. Svennerholm, L. (1963) J. Neurochem. 10, 613-623.