Glycolipid Changes in Murine Myelogenous Leukemias: Neutral Glycolipids as Markers for Specific Populations of Leukemias†

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ABSTRACT: We have studied the glycolipid composition of six different murine myelogenous leukemias as well as that of T-cell leukemias and normal spleen cells. Neutral and acidic lipid fractions were isolated by column chromatography on DEAE-Sephadex and analyzed by high-performance thin-layer chromatography (HPTLC) and an HPTLC overlay method. Murine myelogenous leukemias were found to contain globo- and ganglio-series neutral glycolipids, e.g., glucosylceramide (Glc-cer), lactosylceramide (Lac-cer), globotriaosylceramide (Gb3), globoside (Gb4), Forssman glycolipid (Gb5), and asialo-GM1 (GA1). Monoblastic leukemia cells contained increased proportions of Gb3, Gb4, Gb5, and GA1. Monocytic and myelomonocytic leukemia cells contained increased proportions of Glc-cer and Lac-cer. Especially, Glc-cer accounted for approximately 60% of the total neutral glycolipids in monocytic leukemia cells. Gb3 was the major neutral glycolipid in reticulum cell neoplasm type A, and it accounted for approximately 75% of the neutral glycolipids. GA1 was the major neutral glycolipid in myeloblastic and granulocytic leukemia cells as well as T-cell leukemias. Especially, granulocytic leukemia cells contained predominantly GA1, and it accounted for approximately 80% of the total neutral glycolipids. The pattern of gangliosides in myelogenous leukemias was more complex when compared with that of the neutral glycolipids; murine myelogenous leukemias contained at least 13 gangliosides, including such major gangliosides as GM1, GM1b containing *N*-acetyl neuraminic acid and *N*-glycolyl neuraminic acid, and GalNAc–GM1b. Alterations of glycolipid composition in murine myeloid leukemias may be associated with cellular differentiation and maturation, and therefore these characteristic glycolipid species may be regarded as markers for specific populations of leukemia cells.

Glycolipids are important constituents of the plasma membranes of virtually all vertebrate tissues and are known to possess specific immunological properties (Yamakawa & Nagai, 1978; Ledeen & Yu, 1982; Hakomori, 1984). The expression of individual glycolipid species is cell-specific and regulated during differentiation and neoplastic transformation (Hakomori, 1983). During the past decade considerable evidence has been accumulated indicating that glycolipid composition can undergo remarkable changes during cellular growth, differentiation, and oncogenic transformation (Mora et al., 1969; Brady & Fishman, 1974; Siddigui et al., 1978; Hattori et al., 1981; Pukel et al., 1982; Lee et al., 1982; Hakomori, 1983; Ladisch & Wu, 1985; Matyas et al., 1987; Tatsumura et al., 1988; Ariga et al., 1988). Alterations in glycolipid composition as well as glycosyltransferase activities have been found in the metastatic process of experimental tumor systems (Hakomori & Kannagi, 1983; Holm & Hakomori, 1983). These changes can be exploited diagnostically and therapeutically for malignant tumors (Hakomori, 1983).

We previously reported that the ganglioside composition in human leukemic leukocytes undergoes dramatic changes during differentiation (Tsuboyama et al., 1980). Thereafter, several reports have also shown that alterations in glycolipid patterns occur in leukocytes undergoing differentiation (Rosenfelder et al., 1980, 1982; Saito et al., 1980; Lee et al., 1982; Kannagi & Hakomori, 1983; Kniep et al., 1983; Buehler et al., 1985; Nojiri et al., 1985; Taki et al., 1988; Rokukawa et al., 1988). However, the underlying mechanism(s) of the glycolipid changes is still poorly understood (Hakomori, 1983). Recently, we reported that most of the murine myelogenous leukemias, experimentally induced by irradiation, showed aberrations in chromosome 2, which seemed to be related to this disorder (Hayata et al., 1979, 1983). In this paper we describe that changes in glycolipids of radiation-induced murine myelogenous leukemias are associated with the differentiation of the cell into more mature types.

MATERIALS AND METHODS

Materials. Neutral glycolipids and gangliosides used as references and standards were isolated from erythrocyte membranes and human brain in our laboratories (Ando & Yu, 1979; Ariga et al., 1980; Kasai et al., 1982). All reagents used were of analytical grades or higher. Solvents were usually redistilled before use.

Preparation of Leukemia Cells. Leukemia was induced in our laboratory by whole body irradiation of C3H/He mice, 8–10 weeks-old. Mice were exposed to X-rays ranging from 50 to 500 R (Hayata et al., 1983). The different leukemia types were diagnosed by hematological and histological examination and marker chromosome tests as described previously (Hayata et al., 1979, 1983). The numbers and types of leukemias in this study were 3 myeloblastic (MYB), 2 monoblastic (MOB), 3 monocytic (MC), 3 myelomonocytic (MM), 5 granulocytic leukemia cells, and 2 reticulum cell neoplasm type A (RS). T-cell leukemias, which constitutively produced interleukin 3, were prepared from C3H/He mouse spleen as previously described (Yoshida et al., 1988). These leukemia cells were maintained by serial transplantation with spleen cells into syngeneic female mice and were that of five or more in vivo passages. Leukemic spleens were removed

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from the mice immediately before death from leukemia and prepared to single-cell suspensions with McCoy 5A medium (Flow Lab, Virginia). The normal spleen cell preparation obtained from C3H/He, 8–10 weeks old, in this study consisted of approximately 60% lymphocytes (T and B cells) and erythroblastic cells. It also contained myeloblast, monoblast, granulocyte, monocyte, and macrophage as the minor components and extremely small amounts of CFU-S (colony forming unit in spleen cells; 0.004%) and GM-CFU (granulocyte/macrophage colony forming unit; 0.03%). These cells were washed with medium and PBS and then stored at −20 °C until used for glycolipid analysis. Small aliquots of cells were set aside for determination of total protein (Lowry et al., 1951).

Isolation of Neutral Glycolipids and Gangliosides. The isolation procedures for the total lipids and neutral and acidic glycolipids (gangliosides) were described previously (Sekine et al., 1984; Ariga et al., 1988). Briefly, total lipids were extracted from the leukemia cells, ca. 10^8 cells, with chloroform–methanol (2:1, 1:1, v/v) and chloroform–methanol–water (30:60:8 v/v), successively. The combined extracts were then applied to a DEAE-Sephadex A-25 column (acetate form, 3-mL bed volume), which was further eluted with 20 mL of chloroform–methanol–water (30:60:8 v/v). The neutral lipids were eluted in this fraction. Acidic lipids were then eluted with 30 mL of chloroform–methanol–0.8 M sodium acetate in water (30:60:8 v/v). Portions of the neutral and acidic lipid fractions were set aside for neutral glycolipid and ganglioside analysis, respectively.

The neutral lipid fraction was dissolved in 1 mL of 0.3 M NaOH in methanol and incubated at 40 °C for 2 h in order to remove the alkali-labile phospholipids. After neutralization with 4 M acetic acid in methanol, the sample was desalted on a Sephadex LH-20 column (26-mL bed volume; 0.7 cm i.d. × 48 cm) with the elution of methanol (Ariga et al., 1982). This glycolipid fraction was evaporated to dryness. An aliquot of this glycolipid fraction was examined by high-performance thin-layer chromatography (HPTLC)[1] with two different solvent systems: (A) chloroform–methanol–water (65:35:8 v/v) and (B) chloroform–methanol–2.5 M ammonium hydroxide (60:40:10 v/v). The neutral glycolipids were visualized by spraying with the orcinol–sulfuric acid reagent and heating at 120 °C. Quantitation of the neutral glycolipids was carried out by gas–liquid chromatography (GLC) of the trimethylsilyl derivatives of the sugars with myoinositol as an internal standard according to the procedure of Vance and Sweeley (1967).

The acidic lipid fraction was evaporated to dryness, and the sample was desalted by a Sephadex LH-20 column as described above. An aliquot of this acidic glycolipid fraction was examined by HPTLC with two different solvent systems: (C) chloroform–methanol–0.2% $CaCl_2{\cdot}2H_2O$ (55:45:10 v/v) (Ando et al., 1978) and (D) chloroform–methanol–0.4 M ammonium hydroxide–0.4% $CaCl_2{\cdot}2H_2O$ (50:40:5:4 v/v) (Ariga et al., 1982). In a separate experiment, gangliosides were examined by two-dimensional (2D) HPTLC with two different solvent systems according to the procedure of Nakamura et al. (1983): (E) 1-propanol–0.1% $CaCl_2{\cdot}2H_2O$ (80:20 v/v) and (F) 1-butanol–pyridine–0.1% $CaCl_2{\cdot}2H_2O$ (60:40:20 v/v). The gangliosides were visualized by spraying with the resorcinol–hydrochloric acid reagent and heating at 95 °C. The liquid-bound sialic acid of gangliosides was determined by GLC according to the procedure of Yu and Ledeen (1970).

Fast-Atom Bombardment (FAB) Mass Spectrometry. In order to characterize the structures of the neutral glycolipids, an aliquot of the neutral glycolipid fraction of leukemia cell lines (myelomonocytic leukemia cells) was applied to preparative TLC, and the individual glycolipids were separated by use of the solvent system A as described above. Each glycolipid band was scrapped from the TLC plate and extracted from the silica gel with chloroform–methanol (1:1 and 1:3 v/v). Further purification was carried out on a small Iatrobeads column. This final purification method is necessary for the subsequent FAB mass spectrometry analysis (Ariga et al., 1988). FAB mass spectrometry of the isolated glycolipids were performed as follows. The sample, ~50 μg, was dissolved in 10 μL of chloroform–methanol (2:1 v/v), and then 2 μL of triethanolamine–tetramethyl urea (1:1) was added. The solvent mixture (about 1 μL) was applied to a stainless steel sample holder (1 × 4 mm) and analyzed by a mass spectrometer (JMS HX-110; JEOL, Tokyo, Japan) equipped with a negative-ion FAB ion source and JMA-3500 computer system (JEOL, Tokyo, Japan). Xenon gas was used at a 6-kV neutral beam (Ariga & Yu, 1988).

High-Performance Thin-Layer Chromatography (HPTLC) Overlay Method of Glycolipids. The HPTLC overlay method of glycolipids using specific anti-glycolipid antibodies was carried out according to the procedure described by Saito et al. (1985). Briefly, the glycolipids were chromatographed on an HPTLC plate and the plate was air-dried and then dipped in a solution of 0.2% poly(isobutyl methacrylate) in *n*-hexane. After being air-dried, the plate was overlaid with an anti-glycolipid antibody at a dilution of 1:300 in PBS (pH 7.3) containing 1% bovine serum albumin and incubated for 2 h at room temperature. After being washed with PBS, the plate was overlaid with a second antibody, peroxidase-conjugated anti-rabbit IgG, at a dilution of 1:500 in the dilution buffer for 2 h, and washed as described above. The peroxidase activities were revealed by the addition of in a 0.02% solution of hydrogen peroxide containing 0.5% 4-chloronaphthol. Rabbit polyclonal antibodies against asialo-GM1 (GA1) were prepared by immunization with GA1 in complete Freund adjuvant (Suzuki & Yamakawa, 1981; Jacobson et al., 1982). Polyclonal antibodies against GM2 and Forssman glycolipid were purchased from Seikagaku Kogyo, Ltd. (Japan). In order to detect GM1, was also used biotinylated cholera toxin B subunit (choleragenoid) (List Biological Laboratories Inc., Campbel, CA), which specifically reacted with GM1. In a separate experiment, gangliosides were developed by HPTLC and the bends were treated in situ with neuraminidase (*Vibrio cholerae*, EC 3.2.1.18; 0.064 units/mL in sodium acetate buffer, pH 4.8) in the absence of detergent as described previously (Saito et al., 1985; Hirabayashi et al., 1986); the neuraminidase-sensitive gangliosides were detected with a polyclonal antibody against GA1 as described above.

RESULTS

Glycolipid Contents. Table I shows the glycolipid contents in murine myelogenous leukemias. Values for neutral glycolipids or gangliosides are expressed as nanograms of glucose or lipid-bound sialic acid per milligram of protein, respectively. The contents of neutral glycolipids in various cells changed dramatically. The values for myeloblastic and monoblastic leukemias and reticulum cell neoplasm type A were increased 4–5 times as compared to those of normal spleen cells. On the other hand, the ganglioside contents in monocytic leukemia cells did not show significant changes when compared with

[1] Abbreviations: HPTLC, high-performance thin-layer chromatography; GLC, gas–liquid chromatography; FAB, fast-atom bombardment. The nomenclature used for glycolipids is based on that recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1976).

Table I: Glycolipid Content of Murine Myelogenous Leukemias[a]

	neutral glycolipids (glucose)	gangliosides (lipid-bound sialic acid)
N (2)	1.4	1.7
MYB (3)	5.1 ± 0.3	3.8 ± 0.2
MOB (2)	5.1	2.5
MM (3)	2.2 ± 0.1	3.1 ± 0.1
MC (3)	3.0 ± 0.3	1.6 ± 0.1
RS (2)	6.8	3.3
G (5)	3.8 ± 0.1	3.9 ± 0.2
T (3)	4.1 ± 0.2	3.6 ± 0.2

[a] N, normal spleen cells; MYB, myeloblastic leukemia cells; MOB, monoblastic leukemia cells; MM, myelomonocytic leukemia cells; MC, monocytic leukemia cells; RS, reticulum cell neoplasm type A; G, granulocytic leukemia cells; T, T-cell leukemias. Values are expressed as nanomoles of glucose or sialic acid per milligram of protein (average or average ± SD). The number in parentheses indicates the number of experiments.

those of normal spleen cells. However, the ganglioside contents were elevated 1.5–2 times in myelomonocytic, monoblastic, and myeloblastic leukemia cells as well as in reticulum cell neoplasm type A and granulocytic leukemia cells. In T-cell leukemias, the ganglioside content was 2 times higher than that in normal spleen cells.

Neutral Glycolipid Composition of Murine Myelogenous Leukemias. Qualitative and quantitative analyses of the neutral glycolipids were carried out by HPTLC (Sekine et al., 1984). The neutral glycolipid patterns of murine myelogenous leukemia cells experimentally induced by irradiation are shown in Figure 1 and Table II. Murine myelogenous leukemia cells contained globo- and ganglio-series neutral glycolipids, e.g., glucosylceramide (Glc-cer), lactosylceramide (Lac-cer), globotriaosylceramide (Gb3), globoside (Gb4), Forssman glycolipid (globopentaosylceramide), (Gb5) and asialo-GM1 ganglioside (GA1), which comigrated with standard samples of these neutral glycolipids on the basis of two different solvent systems as shown in Figure 1. Further structural identification of each purified neutral glycolipid is described later. The neutral glycolipid composition of murine myelogenous leukemias was shown to differ from that of normal spleen cells in C3H/He mice. Normal spleen cells in this study contained approximately 60% lymphocytes (T and B cells) and erythroblastic cells and expressed three major glycolipids, being GA1, Gb5, and Gb4. GA1 was the major neutral glycolipid in myeloblastic leukemia cells, and it accounted for about 55% of the total neutral glycolipids. Monoblastic leukemia cells contained increased proportions of Gb3, Gb4, Gb5, and GA1; notably, Gb3 was increased to about 30% of the total neutral glycolipids. In monocytic leukemia cells Glc-cer was the major glycolipid, which accounted for slightly less than 60% of the total neutral glycolipids. This cell line also expressed Gb5 and GA1 as the major neutral glycolipids. As compared with monoblastic leukemia cells, the levels of Lac-cer and Gb3 in

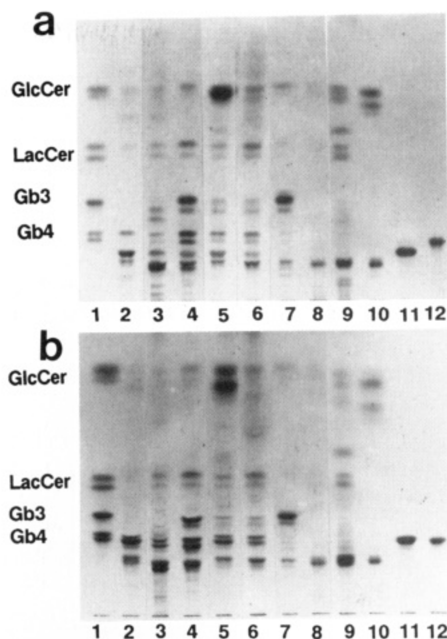


FIGURE 1: Thin-layer chromatogram of neutral glycolipids from murine myelogenous leukemias. The lanes represent (1) authentic samples of neutral glycolipid mixtures; (2) normal spleen cells; (3) myeloblastic leukemia cells; (4) monoblastic leukemia cells; (5) monocytic leukemia cells; (6) myelomonocytic leukemia cells; (7) reticulum cell neoplasm type A; (8) granulocytic leukemia cells; (9) T-cell leukemias; (10) standard samples of Gal-cer and GA1; (11) standard sample of Gb5; and (12) standard sample of paragloboside. Plates a and b were developed with the solvent systems A and B, respectively (see Materials and Methods). The bands were visualized with the orcinol–sulfuric acid reagent.

myelomonocytic leukemia cells were increased in proportion to the decrease of Gb5. Gb3 was also the major neutral glycolipid in reticulum neoplasm cell type A, which accounted for approximately 75% of the neutral glycolipids. In both monoblastic leukemia cells and reticulum cell neoplasm type A, the level of Glc-cer was low. No significant differences were seen among the individual granulocytic leukemia cells; GA1, the major neutral glycolipid, accounted for approximately 80% of the total neutral glycolipids. In T-cell leukemias, GA1 was the major neutral glycolipid, which accounted for approximately 50% of the total neutral glycolipids. Lac-cer and Glc-cer accounted for approximately 12 and 20% of the total neutral glycolipids, respectively.

Negative-Ion Fast-Atom Bombardment (FAB) Mass Spectrometry of Neutral Glycolipids. In order to further characterize the neutral glycolipids in murine myelogenous leukemias, each neutral glycolipid was isolated from large batches of myelomonocytic leukemia cells as shown in Figure 2. This cell line contained Glc-cer, Lac-cer, Gb3, Gb4, Gb5, and GA1, which comigrated with their respective neutral glycolipid standards. Negative-ion FAB mass spectra of each

Table II: Neutral Glycolipid Composition of Murine Myelogenous Leukemias

	GA1	Gb5	Gb4	Gb3	Lac-cer	Glc-cer
N (2)	23.1	50.7	12.0	1.5	3.7	9.0
MYB (3)	54.8 ± 1.7	12.1 ± 0.7	4.8 ± 0.3	16.4 ± 1.8	7.5 ± 0.6	3.2 ± 0.2
MOB (2)	19.6	12.9	23.3	32.0	6.8	4.8
MM (3)	26.2 ± 2.0	11.4 ± 1.7	12.4 ± 0.7	11.0 ± 0.3	24.8 ± 0.8	14.3 ± 0.6
MC (3)	11.5 ± 1.0	13.5 ± 0.7	4.3 ± 0.6	6.7 ± 0.3	4.5 ± 0.2	59.6 ± 2.2
RS (2)	12.2	3.3	1.7	75.0	0.9	7.0
G (5)	79.2 ± 0.9	4.4 ± 0.3	3.5 ± 0.1	4.3 ± 0.2	5.9 ± 0.3	4.1 ± 0.1
T (3)	53.6 ± 2.4	7.5 ± 0.5	3.1 ± 0.8	3.4 ± 0.3	11.9 ± 0.5	20.4 ± 0.5

[a] N, normal spleen cells; MYB, myeloblastic leukemia cells; MOB, monoblastic leukemia cells; MM, myelomonocytic leukemia cells; MC, monocytic leukemia cells; RS, reticulum cell neoplasm type A; G, granulocytic leukemia cells; T, T-cell leukemias. Values are expressed as percentage of the total neutral glycolipids (average or average ± SD). The number in parentheses indicates the number of experiments.

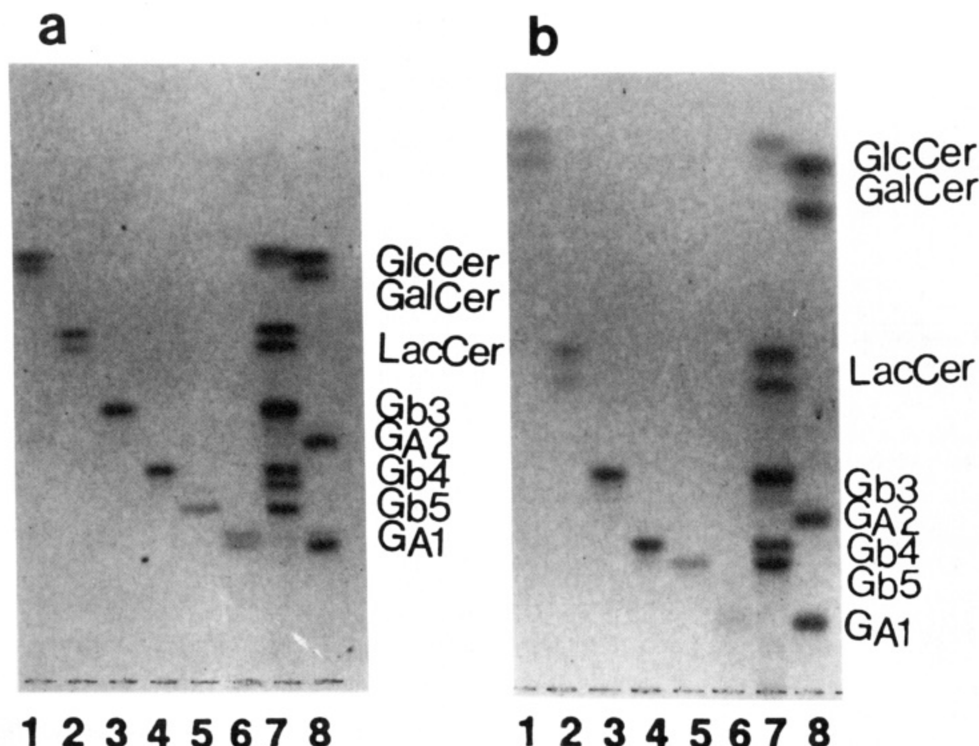


FIGURE 2: Thin-layer chromatogram of isolated neutral glycolipids from myelomonocytic leukemia cells. The lanes represent (1–6) isolated neutral glycolipids; (7) standard samples of Glc-cer, Lac-cer, Gb3, Gb4, Gb5, and GA1; and (8) standard samples of Gal-cer, GA2 (asialo GM2), and GA1. The plates a and b were developed with the solvent systems A and B, respectively. The bands were visualized with the orcinol–sulfuric acid reagent.

neutral glycolipid revealed the prominent quasimolecular ions m/z $[M - H]^-$ and fragment ions having C18 sphingosine and various fatty acids with chain lengths ranging from C16:0 to C24:0 (Figure 3).

High-Performance Thin-Layer Chromatography (HPTLC) Overlay Method of Neutral Glycolipids. Figure 4 shows the HPTLC overlay method of neutral glycolipids with the use of polyclonal antibodies against Gb5 and GA1. All neutral glycolipid fractions in murine myelogenous leukemias contained bands reacting with these polyclonal antibodies, indicating the presence of Gb5 and GA1.

Gangliosides in Murine Myelogenous Leukemias. Figure 5 shows the ganglioside patterns of murine myelogenous leukemia cells. The ganglioside patterns in myelogenous leukemia cells were extremely complex as compared with those of the neutral glycolipids. In myeloblastic and myelomonocytic leukemia cells, the ganglioside patterns resembled each other, being simple gangliosides as the predominating species. No significant differences were observed for granulocytic leukemia cells or T-cell leukemias.

In order to further characterize myelogenous leukemia gangliosides, we employed an HPTLC overlay method using specific anti-glycolipid antibodies (Figure 6). Myelogenous leukemia cells contained many gangliotetraosyl series gangliosides, including GM1 species having *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc), which were characterized by comigration with standard gangliosides (Figure 6a, lanes 10 and 11) as well as by binding with choleraginoid (Figure 6b) and anti-GM1 antibody (data not shown). In addition, two gangliosides that migrated between GD1a and GD1b reacted with choleraginoid, suggesting the presence of additional choleraginoid-binding gangliosides (Gal–GalNAc–GM1b having NeuAc and NeuGc, which are marked by asterisks in Figure 6b, lanes 6) as described by Nakamura et al. (1987). After neuraminidase treatment in the absence of detergent, two gangliosides reacted with anti-GA1 antibody, suggesting the presence of GM1b gangliosides having NeuAc and NeuGc (Figure 6d, lane 7). In addition, there were two gangliosides that reacted with anti-GM2 antibody (Figure 6e). The fast-migrating band comigrated with GM2, which was observed predominantly in myelomonocytic leukemias (Figure 5, lane 6). The major band that migrated between GD1a and GD1b also reacted with anti-GM2 antibody. This ganglioside may have a terminal GalNAc–Gal structure, suggesting the presence of GalNAc–GM1b, which was reported in murine T lymphocytes (Muthing et al., 1987), murine spleen (Nakamura et al., 1987), and Tay-Sachs brain (Itoh et al., 1981).

Figure 7 shows the two-dimensional HPTLC of gangliosides from murine myelogenous leukemias. Myeloblastic leukemia cells contained at least 13 gangliosides, including three or four major gangliosides as shown in Figure 7b. HPTLC overlay method using choleragenoid revealed the presence of many gangliotetraosyl series gangliosides, two of which were tentatively identified as GM1 gangliosides having NeuAc and NeuGc (Figure 7c). The presence of GM1b ganglioside was confirmed by using anti-GA1 antibody after treatment with neuraminidase without detergent (Figure 7d).

Discussion

The classification of myelogenous leukemia cells is primarily based on morphologic and cytochemical features. The extensive studies by Rosenfelder et al. (1980, 1982), Saito et al. (1980), and Rokukawa et al. (1988) have indicated that glycolipids are excellent markers for diagnosis of leukemic cells and appear to be suitable for classification purposes. Most leukemic cells used in these studies are obtained by in vitro passage. However, leukemias used in the present study are induced by whole body irradiation of C3H/He male mice and these cells are maintained by serial transplantation in spleen cells of syngeneic mice (Hayate et al., 1983). It would be important to determine whether glycolipid changes might be

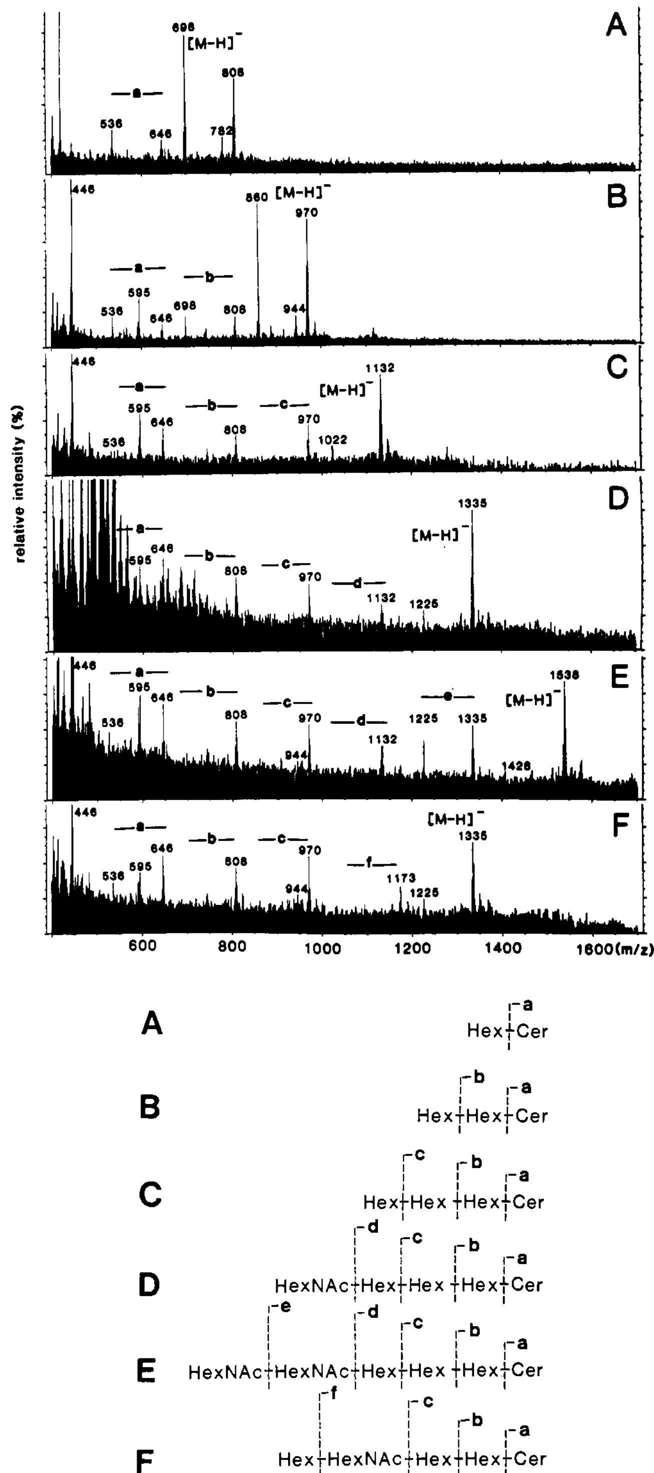


FIGURE 3: Negative-ion FAB mass spectra of isolated neutral glycolipids: (a) mass spectra and (b) fragmentation diagram. The mass spectra of A–F corresponded to the lanes 1–6 of Figure 2.

related to the cellular differentiation in in vivo passages. In this study, we have shown that the glycolipid patterns change dramatically, and this finding may be useful as an aid in defining the phenotypic characteristics of these cells. In the case of neutral glycolipids, we have shown that their concentrations and complexity increase in mature cells (Tables I and II; Figure 1). These glycolipid changes might be related to the expression of differentiation-associated glycolipid antigens (Hakomori, 1983). Buehler et al. (1985) reported that well-differentiated forms of human lymphoid cells contained only globo-type neutral glycolipids, whereas well-differentiated forms of human myeloid cells contained neolacto- and gala-type neutral glycolipids. However, this type of generalization may not be entirely true since we have shown that murine myelogenous leukemias express both globo-series neutral glycolipids, e.g., Lac-cer, Gb3, Gb4, and Gb5, and a ganglio-series neutral glycolipid, GA1. Monocytic leukemia cells, which are differentiated, contain predominantly Glc-cer. Buehler et al. (1985) also reported that human myeloid leukemia cells, K562 (undifferentiated blast cell line), KG1 (myeloblastic cell line), and HL 60 (promyelocytic cell line) contained predominantly Glc-cer. In a more recent study, HL 60 cells were shown to contain both Gal-cer and Glc-cer (Xia et al., 1989).

In contrast, the major neutral glycolipid in reticulum cell neoplasm type A, which is differentiated, appears to be Gb3. Gb3 is known to be a marker glycolipid of Burkitt lymphoma (Wiels et al., 1984) and murine myeloid leukemia M1 cells (Kannagi et al., 1983a). Recently, Kannagi et al. (1983b) reported that the glycolipid composition and metabolism change dramatically during the differentiation of M1 cells into macrophages. Taki et al. (1988) recently confirmed that Gb3 is expressed during differentiation of M1 cells into macrophage-type cells, M1R1 (differentiation-resistant clone of murine myeloid leukemia cells), and they further reported that Gb3–α-galactosyltransferase activities are increased more than 10 times during differentiation. Similarly, Kniep et al. (1983) reported the presence of Gb3 in murine myeloid cells and B blasts, whereas Gb4 (globoside) was shown to be a marker for helper T cells. In our present study, Gb4 is expressed only in small amounts in T-cell leukemias, whereas it is a major glycolipid in myelomonocytic and monoblastic leukemia cells. In this regard, it is interesting to note that Muhlradt et al. (1984) found Gb4 to be a differentiation marker for murine T lymphocytes, which consisted primarily of splenic T cells. However, in the T cells from blood, this glycolipid was present only in small amounts. The difference therefore may reflect the origin of the T cells.

Murine myelogenous leukemia cells and granulocytic leukemia cells as well as T-cell leukemias also display Gb5 and GA1. Bethke et al. (1987) reported that Gb5 is not found on T cells. However, using a highly sensitive HPTLC overlay method, we could show that all T-cell leukemias and granulocytic leukemia cells expressed a small amount of Gb5. Gb5 has been shown to be an antigenic marker for a major subpopulation of macrophages from murine spleen and peripheral lymph nodes (Bethke et al., 1987). In addition, all T-cell leukemias and granulocytic leukemia cells contained GA1 as a major neutral glycolipid. GA1 is known to be a marker glycolipid for T lymphocytes (Stein et al., 1978; Schwarting & Summers, 1980; Kniep et al., 1983) and natural killer (NK) cells (Kasai et al., 1980; Young et al., 1980). The increased levels of GA1 in T-cell leukemias might be related to the previous finding that granulocytic leukemia cells are increased in the host cells due to constitutive production of interleukin 3 (Yoshida et al. 1988).

Myelomonocytic leukemia cells expressed Glc-cer and Lac-cer as well as GA1 as the major neutral glycolipids, in which both types of granulocytic and monocytic leukemia cells were contained.

Scheme I shows the relationship between myelogenous leukemia cell differentiation and the expression of major neutral glycolipids in various cell types diagnosed by histological examination. On the basis of our results, we propose that neutral glycolipid patterns in murine myelogenous leukemias are simplified during cellular differentiation, e.g., GA1 in granulocytic leukemia cells, LacCer and GlcCer in mono-

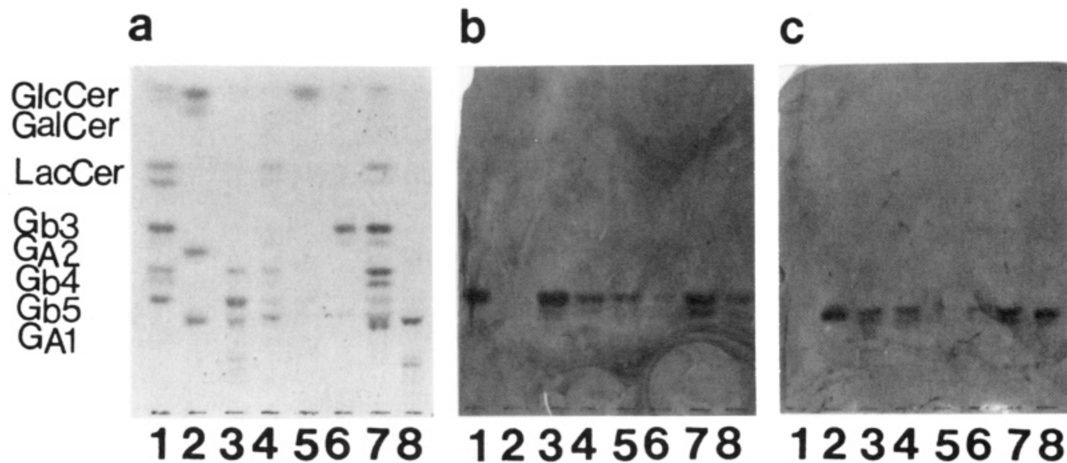


FIGURE 4: HPTLC overlay method of neutral glycolipids from myelogenous leukemias. The lanes represent (1) standard samples of Glc-cer, Lac-cer, Gb3, Gb4, and Gb5; (2) standard samples of Gal-cer, GA2, and GA1; (3) normal spleen cells; (4) myelomonocytic leukemia cells; (5) monocytic leukemia cells; (6) reticulum cell neoplasm type A; (7) monoblastic leukemia cells; and (8) granulocytic leukemia cells. The plates were developed with the solvent system A. The bands of plate a were visualized with the orcinol–sulfuric acid reagent, and plates b and c were immunostained with anti-Gb5 antibodies and anti-GA1 antibodies, respectively.

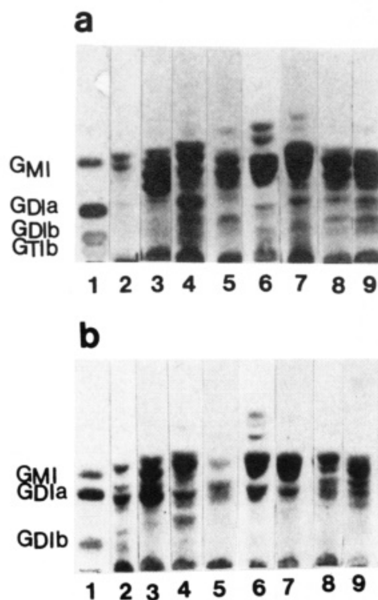
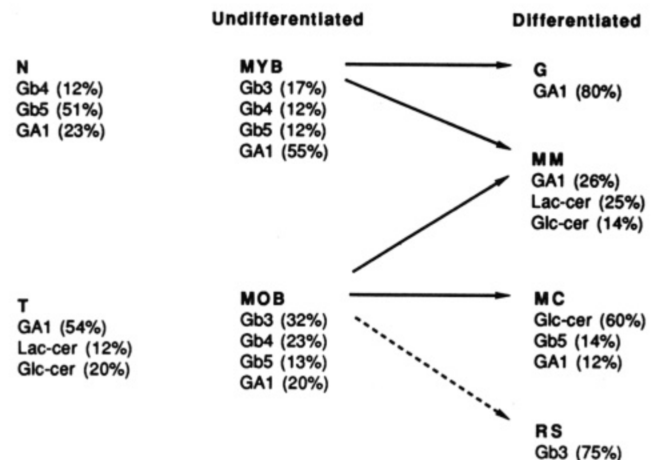


FIGURE 5: Thin-layer chromatogram of gangliosides from murine myelogenous leukemias. The lanes represent (1) standard samples of human brain gangliosides; (2) normal spleen cells; (3) myeloblastic leukemia cells; (4) monoblastic leukemia cells; (5) monocytic leukemia cells; (6) myelomonocytic leukemia cells; (7) reticulum cell neoplasm type A; (8) granulocytic leukemia cells; and (9) T-cell leukemias. Plates a and b were developed with solvent systems C and D, respectively. The bands were visualized with the resorcinol–hydrochloric acid reagent.

cytic leukemia and myelomonocytic leukemia cells, and Gb3 in reticulum cell neoplasm type A.

Studies from several laboratories have revealed the ganglioside differences among human leukocytes, leukemia cells, and leukemia cell lines (Tuboyama et al., 1980; Rosenfelder et al., 1980; Saito et al., 1980; Xia et al., 1989). We have shown that the ganglioside patterns can be useful for distinguishing different types of human leukemia cells (Tsuboyama et al., 1980). These findings indicate that the degree of cellular differentiation could be correlated with particular ganglioside patterns (Rosenfelder et al., 1980). With regard to the expression of gangliosides, a striking finding in the present study has been the marked heterogeneity among the different cell lines. Rosenfelder et al. (1978) reported that Con-A-treated thymoyctes contained at least 12 gangliosides that were labeled by [^{14}C]galactose incorporation. Schwarting and Gajewsky

Scheme I: Murine Myelogenous Leukemia Cell Phenotypes and the Expression of Major Neutral Glycolipids[a]



[a] N, normal spleen cells; T, T-cell leukemias; MYB, myeloblastic leukemia cells; MOB, monoblastic leukemia cells; G, granulocytic leukemia cells; MM, myelomonocytic leukemia cells; MC, monocytic leukemia cells; RS, reticulum cell neoplasm type A.

(1983) also reported the structures of 11 murine thymus gangliosides having a gangliotetraosyl backbone. Yohe et al. (1988) recently described that murine thymoyctes contained 8 major gangliosides and 22 minor species, many of which had different types of the sialic acid residue, e.g., NeuAc or NeuGc, different fatty acid chain lengths at the ceramide moiety, and different carbohydrate core structures. In the present study, we achieved satisfactory separation of murine myelogenous gangliosides by two-dimensional (2D) TLC using the combination of 1-propanol–0.1% $CaCl_2{\cdot}2H_2O$ (first dimension) and 1-butanol–pyridine–0.1% $CaCl_2{\cdot}2H_2O$ (second dimension) as previously reported (Nakamura et al., 1983). This method is very useful for comparative studies of ganglioside patterns, including the detection of minor components (Nakamua et al., 1983; Yohe et al., 1988). 2D TLC immunostaining using specific glycolipid antibodies can provide additional information on ganglioside structures. According to 1D and 2D TLC as well as by the TLC overlay technique using specific glycolipid antibodies, we could identify five major gangliosides containing the gangliotetraosyl backbone: GM1 and GM1b, having NeuAc or NeuGc and GalNAc–GM1b as well as two minor gangliosides that reacted with choleraginoid as reported

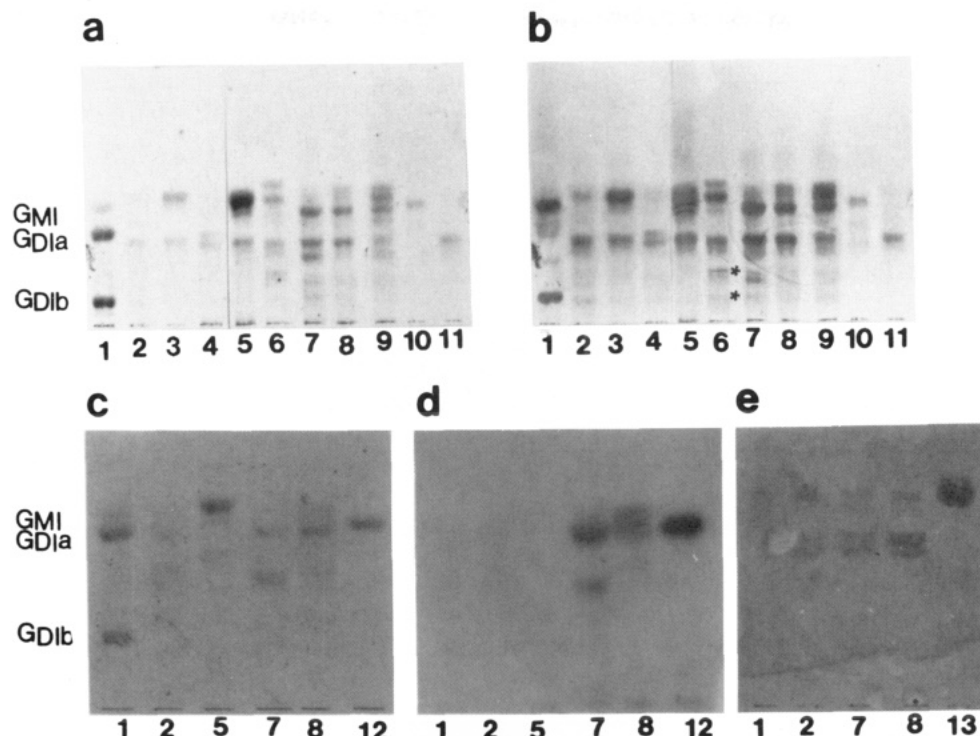


FIGURE 6: HPTLC overlay method of gangliosides from murine myelogenous leukemias. The lanes represent (1) standard samples of human brain gangliosides; (2) normal spleen cells; (3) monoblastic leukemia cells; (4) monocytic leukemia cells; (5) myelomonocytic leukemia cells; (6) myeloblastic leukemia cells; (7) reticulum cell neoplasm type A; (8) granulocytic leukemia cells; (9) T-cell leukemias; (10) standard sample of GM1 (NeuAc); (11) standard sample of GM1 (NeuGc); (12) standard sample of GM1b (NeuAc); and (13) standard sample of GM2 (NeuAc). The plates were developed with the solvent system D. The bands of plate a were visualized with the resorcinol–hydrochloric acid reagent. Plate b was treated with cholera toxin B subunits (choleraginoid). Plates c and d were immunostained with anti-GA1 antibodies after treatment by neuraminidase without detergent and anti-GM2 antibodies, respectively.

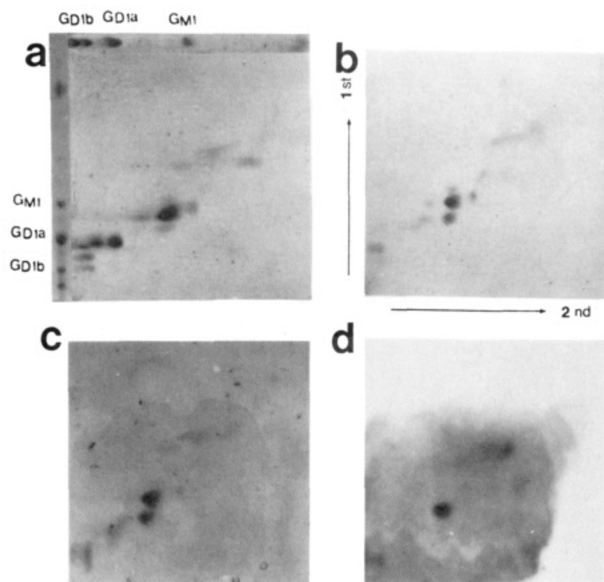


FIGURE 7: Two dimensional HPTLC overlay method of myelogenous leukemia gangliosides. The plates were developed with solvent system E and solvent system F successively. Plate a: acidic lipid fraction from normal spleen cells. Plates b, c, and d: acidic lipid fraction from monoblastic leukemia cells. The bands of plates a and b were visualized with the resorcinol–hydrochloric acid reagent. Plate c was treated with cholera toxin B subunits (choleraginoid), and plate d was immunostained with anti-GA1 antibodies after neuraminidase treatment without detergent, respectively.

by Nakamura et al. (1987) (Figure 6b, lane 6).

Although Schwarting and Gajewski (1983) reported the presence of O-acetylated gangliosides in murine thymocytes and T-lymphocytic cells, we could not detect these glycolipids. This is consistent with the observation of Yohe et al. (1988), who also failed to find these ganglioside species.

Siddiqui et al. (1984) reported that GD3 ganglioside could serve as a differentiation marker of human leukemia cells. However, in the present study, we could not detect this ganglioside using the R24 monoclonal antibody, which reacts specifically with GD3 (Dippold et al., 1980) (data not shown).

Muthing et al. (1987) reported that GalNAc–GM1b (NeuGc) could be a marker for murine T-lymphoblastic cells. More recently, the same authors reported that T cells also contained GalNAc–GM1b (NeuAc) and GM1b, and they suggested that GalNAc–GM1b might be expressed on mature peripheral T blasts (Muthing et al., 1989). The expression of this ganglioside is much decreased in thymocytes (Muthing et al., 1989). In the present study, we also detected a similar major ganglioside, which migrated closely with GD1a on one-dimensional TLC (Figure 6e) and reacted with anti-GM2 antibody, suggesting that this ganglioside might indeed be GalNAc–GM1b. GalNAc–GM1b has been shown to be a minor ganglioside in Tay-Sachs brain (Itoh et al., 1981), murine tumor MDAU-D2 (Hirabayashi et al., 1979; Schwartz et al., 1985), and mouse spleen (Nakamura et al., 1987). Our study and the studies by Muthing et al. (1987, 1989) suggest that it may be present in all T cells and murine myelogenous leukemia cells.

We have also detected GM1b containing etiher NeuAc or NeuGc using an anti-GA1 antibody following neuraminidase treatment without detergent. This ganglioside has been reported to be present on mouse thymocytes (Yohe et al., 1988), myeloid leukemia M1 cells (Saito et al., 1980), rat ascites hepatoma AH-7974 F (Hirabayashi et al., 1979), and mouse spleen cells (Nakamura et al., 1984). We have previously shown that GM1b containing NeuAc is also present as a minor ganglioside of human brain (Ariga & Yu, 1988). The presence

of this ganglioside in the nervous and immune systems suggests that it may serve as a common antigen between these two compartments. Additionally, it may play an important role in the loss of adhesiveness of tumor cells (Hirabayashi et al., 1979) and in cell maturation (Hirabayashi et al., 1979; Taki et al., 1983).

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Extensive Segregation of Acidic Phospholipids in Membranes Induced by Protein Kinase C and Related Proteins†

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ABSTRACT: Protein kinase C and two other proteins with molecular masses of 64 and 32 kDa, purified from bovine brain, constitute a type of protein that binds a large number of calcium ions in a phospholipid-dependent manner. This study suggested that these proteins also induced extensive clustering of acidic phospholipids in the membranes. Clustering of acidic phospholipids was detected by the self-quenching of a fluorescence probe that was attached to acidic phospholipids (phosphatidic acid or phosphatidylglycerol). Addition of these proteins to phospholipid vesicles containing 15% fluorescently labeled phosphatidic acid dispersed in neutral phosphatidylcholine resulted in extensive, rapid, and calcium-dependent quenching of the fluorescence signal. Fluorescence-quenching requirements coincided with protein–membrane binding characteristics. As expected, the addition of these proteins to phospholipid vesicles containing fluorescent phospholipids dispersed with large excess of acidic phospholipids produced only small fluorescence changes. In addition, association of these proteins with vesicles composed of 100% fluorescent phospholipids resulted in no fluorescence quenching. Protein binding to vesicles containing 5–50% fluorescent phospholipid showed different levels of fluorescence quenching that closely resemble the behavior expected for extensive segregation of the acidic phospholipids in the outer layer of the vesicles. Thus, the fluorescence quenching appeared to result from self-quenching of the fluorophores that become clustered upon protein–membrane binding. These results were consistent with protein–membrane binding that was maintained by calcium bridges between the proteins and acidic phospholipids in the membrane. Since each protein bound eight or more calcium ions in the presence of phospholipid, they may each induce clustering of a related number of acidic phospholipids. This property, which was very striking for this class of proteins, was barely detectable for another class of proteins that display calcium-dependent binding to membranes containing acidic phospholipids. The membrane-altering behavior of protein kinase C and other related proteins may contribute unique features to the total calcium response of the cell.

Protein kinase C (PKC)[1] is a regulatory enzyme that is believed to be involved in many cell functions (Nishizuka, 1986). With appropriate substrates, the activation of this enzyme is dependent on calcium and phospholipids (Bazzi & Nelsestuen, 1987a). Understanding the interaction of PKC with these two cofactors is essential for understanding the process of its activation and its role in the calcium response [reviewed in Nelsestuen and Bazzi (1991)]. PKC binds to membranes containing acidic phospholipids in a calcium-dependent manner. This property is shared with other diverse groups of proteins including various intracellular proteins (Klee, 1988), vitamin K dependent proteins of the plasma (Schwalbe et al., 1989), and others (Creutz et al, 1987; Khanna et al., 1990; Schwalbe, 1990). However, PKC and other recently described proteins (Bazzi & Nelsestuen, 1991a) constitute a category that appears distinguishable from the others by several unusual properties.

The interaction of PKC with calcium was strongly dependent on the presence of phospholipids (Bazzi & Nelsestuen,

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[1] Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; NBD-PA, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidic acid; NBD-PC, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-phosphatidylcholine; NBD-PG, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylglycerol; PKC, phospholipid- and calcium-dependent protein kinase C; SUV, small unilamellar vesicles of about 30-nm diameter; LUV, large unilamellar vesicles of 100-nm diameter.

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