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Structure of a Novel Sialylated Fucosyl Lacto-*N*-norhexaosylceramide Isolated from Chronic Myelogenous Leukemia Cells*

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A novel sialylated fucosyl glycolipid, which is present at an elevated level in chronic myelogenous leukemia cells, was isolated. The structure of this fucoganglioside was elucidated by methylation analysis, fast atom bombardment-mass spectrometry, and enzymatic degradation, followed by reaction with anti-Le^x, Galβ1→4(Fucα1→3)GlcNAcβ1→, monoclonal antibody. The structure of this ganglioside was found to be:

NeuNAcα2→3Galβ1→4GlcNAcβ1→3

Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
3
↑α
1
Fuc

This structure is unique in that a fucose is attached to the internal *N*-acetylglucosamine but not to the subterminal *N*-acetylglucosamine. Since this glycolipid is apparently absent in normal granulocytes or acute myelogenous leukemia cells, it can be a specific marker for chronic myelogenous leukemia cells. Based on the structures of this fucoganglioside and normal granulocyte glycolipids, a biosynthetic pathway of extension, sialylation, followed by fucosylation is proposed.

Chronic myelogenous leukemia (CML[1]) is a clonal myeloproliferative disorder and characterized by a marked overproduction of granulocytes in peripheral blood. Although malignant cells arise from a stem cell common to granulocytes and erythrocytes, these malignant cells achieve significant maturation and granulocytes produced in the chronic phase of CML patients are very similar to normal mature granulocytes in morphology and functions (1). In fact, Dacremont and Hildebrand (2), Klock *et al.* (3), and Westrick *et al.* (4) reported that granulocytes produced in the chronic phase of CML contain glycolipids very similar to those present in mature granulocytes.

Recently, we have analyzed the structures of polylactosaminoglycans isolated from CML granulocytes (5) and found that CML polylactosaminoglycans are distinctly different from normal granulocyte polylactosaminoglycans (6, 7), *i.e.* CML polylactosaminoglycans, are 1) highly sialylated, 2) shorter, and 3) characterized by a great abundance of the sialyl Le^x, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→ terminal structure and the presence of the NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)-GlcNAc structure. Our recent studies on normal granulocyte glycolipids indicate that polylactosaminoglycans and glycolipids share the same structures; in the majority of neutral glycolipids from granulocytes, fucose is attached to linear poly-*N*-acetyllactosaminyl backbones to form Galβ1→4(Fucα1→3)GlcNAcβ1→3 terminal structure, whereas sialic acid is joined to the same backbones by either α2→3 or α2→6 linkage in gangliosides (8). However, we have not detected the structures unique to CML polylactosaminoglycans in normal granulocyte gangliosides (8).

These results led us to investigate CML glycolipids in detail to see whether cells from CML patients contain any unique glycolipids. We describe here the structural characterization of novel fucosyl gangliosides uniquely present in CML cells.

EXPERIMENTAL PROCEDURES

Isolation of Glycosphingolipids from Granulocytic Cells of CML Patients—Patients were admitted to the hospital of the University of California at San Diego or the Medical Research Institute at San Francisco. All patients were in the chronic phase of CML and had blast counts of less than 10%. Leukocytes were enriched by therapeutic leukophoresis and were further purified by hypotonic lysis to remove contaminating erythrocytes as described (5, 9). The preparation obtained was found by its morphology to be more than 90% granulocytic cells with various degrees of maturation (segmented and banded neutrophils, metamyelocytes, and myelocytes).

Isolation and purification of glycolipids were carried out as described previously (8), except for the following. The granulocytic cells were extracted sequentially with 5 times the volume of hot ethanol and chloroform:methanol (2:1, v/v). The volume of chloroform:methanol was 10 times that of the residue obtained after ethanol extraction. The crude glycolipid extract was then fractionated by Folch's phase partition (10). The lower-phase glycolipids were freed from cholesterol and phospholipids by acetylation procedures (11). Deacetylated glycolipids were applied to QAE-Sephadex A-25 column chromatography and neutral and acidic glycolipids were separated by using the solvent systems described by Ando and Yu (12). Upper-phase glycolipids were also separated into neutral and acidic fractions

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[1] The abbreviations used are: CML, chronic myelogenous leukemia; FAB-MS, fast atom bombardment-mass spectrometry; HPTLC, high-performance thin layer chromatography; HPLC, high performance liquid chromatography; Cer, ceramide; CTH, ceramide trihexoside.

in the same manner and acidic glycolipids of the upper and the lower layer were pooled.

Purification of Acidic Glycolipids—Acidic glycolipids were purified by high-performance liquid chromatography with a Varian HPLC apparatus (model 5000, Varian Associates, CA). The glycolipids dissolved in a minimum amount of chloroform:methanol (2:1, v/v) were applied to a column (0.4 × 50 cm) of Iatrobeads (IRS 8010, 10 μm diameter, Iatron, Tokyo). The column was equilibrated with isopropyl alcohol:hexane:water (55:40:5), eluted over 20 min by a gradient to isopropyl alcohol:hexane:water (55:35:10), and followed by a shallower gradient to isopropyl alcohol:hexane:water (55:29:16) over an additional 180 min (8, 13). The flow rate was constant at 0.5 ml/min and the eluate was collected every 2 min. Glycolipids in each tube were analyzed by HPTLC (Si-HPF, J. T. Baker Chemical Co.) using a solvent system chloroform:methanol:3.5 M NH_4OH (60:35:8, v/v). The elution positions of glycolipids were as follows: G_1, 70–72 min; G_2, 72–74 min; G_3, 77–86 min; G_4, 82–88 min. Each glycolipid fraction recovered was then acetylated in pyridine:acetic anhydride (1:1, v/v) (11). Acetylated glycolipids were then separated on HPTLC (Si-HPF, J. T. Baker) using a solvent of dichloroethane:acetone:water (50:50:1, v/v). Acetylated glycolipids detected by iodine vapor were eluted from thin layer plates and deacetylated with sodium in methanol (11). Glycolipids obtained were further purified by HPLC in the same manner as described above, with the solvent system as follows. The solvent gradient was programmed as isopropyl alcohol:hexane:water (55:40:5 to 55:37:8) over 10 min, followed by the gradient to isopropyl alcohol:hexane:water (55:29:16) over 70 min. The flow rate was constant at 0.5 ml/min and the eluate was collected every 1 min. Glycolipids in each tube were analyzed by HPTLC as described above. The elution positions of glycolipids were as follows: G_1, 33 min; G_2, 34–37 min; G_3, 37–39 min; G_4, 40–42 min.

Methylation Analysis—The glycolipids were methylated by the method of Hakomori (14). The permethylated samples were purified by LH-20 column chromatography and further purified by partition with chloroform:water (1:1) and the chloroform layer was washed four times with water as described (8, 15).

The permethylated samples thus obtained were then subjected to FAB-MS for sequence analysis as described below. In order to obtain the information on linkages between monosaccharides, the permethylated samples were subjected to acid hydrolysis with 0.5 N H_2SO_4 in 90% acetic acid at 80 °C for 4 h. The hydrolysates were then neutralized and acetylated as described (15). The alditol acetates of partially methylated sugars were analyzed by gas liquid chromatography-mass spectrometry with a modification in column temperature as described (5, 15).

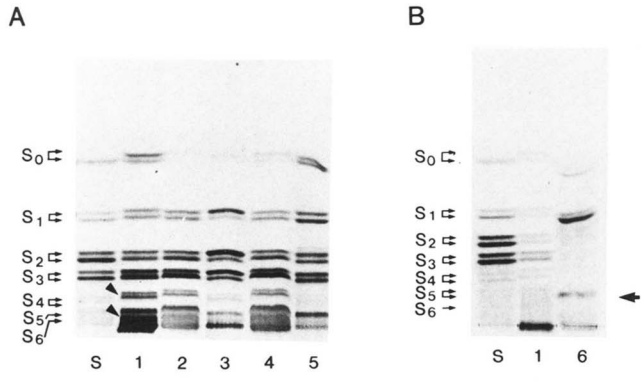


FIG. 1. **Thin layer chromatogram of gangliosides isolated from normal mature granulocytes, chronic myelogenous leukemia cells, and acute myelogenous leukemia cells.** The glycolipids were developed on HPTLC three times using the solvent system of chloroform:methanol:3.5 M NH_4OH in water (60:35:8, v/v), and detected by orcinol-H_2-SO_4. *A*, gangliosides from normal granulocytes (S) and from chronic myelogenous leukemia patients. *Lanes 1–5* represent patients 1 through 5. The bands indicated by the *arrows* are CML specific glycolipids. S_0, NeuNAcα2→3Galβ1→4Glcβ1→1Cer; S_1, NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; S_2, NeuNAcα2→6Galβ1→4GlcNAcβ1→4Galβ1→4Glcβ1→1Cer; S_3, NeuNAcα2→3(Galβ1→4GlcNAcβ1→3)$_2$→Galβ1→4Glcβ1→1Cer S_4, NeuNAcα2→6(Galβ1→4GlcNAcβ1→3)$_2$→Galβ1→4Glcβ1→1Cer (from Ref. 8). *B*, gangliosides from CML patient 1 (*lane 1*) and from acute myelogenous leukemia patient (*lane 6*). The band indicated by the *arrow* on the right side was due to contaminants.

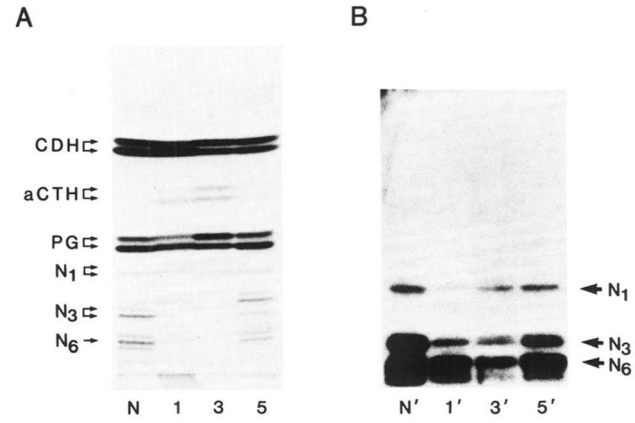


FIG. 2. **Immunostaining of Folch's upper neutral glycolipids with monoclonal anti-Le^x, Galβ1→4(Fucα1→3)GlcNAcβ1→3, antibody.** *A*, the plate was developed and visualized by orcinol-H_2SO_4. *Lanes N, 1, 3,* and *5* correspond to glycolipids from normal granulocytes (*N*), CML patients 1, 3, and 5, respectively. *B*, The same samples were subjected to immunostaining by anti-Le^x (PM-81) antibody. *Lanes N′, 1′, 3′,* and *5′* correspond to *lanes N, 1, 3,* and *5* in *A*. *CDH*, Galβ1→4Glcβ1→1Cer; *aCTH*, GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; *PG*, Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; N_1, Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → 4Glcβ1 → 1Cer; N_3, Galβ1 → 4(Fucα1→3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; N_6, Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer (from Ref. 8). PM-81 antibody reacts with N_1-glycolipid as well as Le^x-glycolipids with longer carbohydrate chains (17).

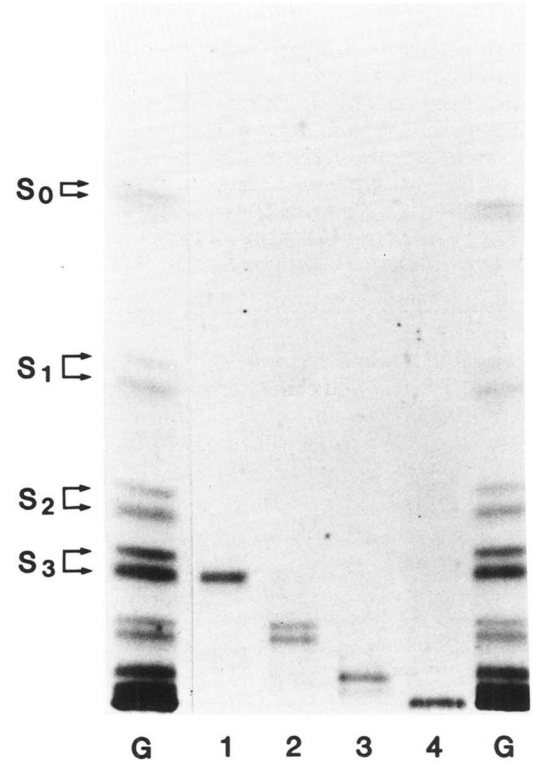


FIG. 3. **Thin-layer chromatogram of gangliosides purified from CML cells.** CML cells from patient 1 were used for starting materials. *G*, gangliosides from patient 1; *lane 1*, G_1; *lane 2*, G_2; *lane 3*, G_3; *lane 4*, G_4. The mobility of S_0, S_1, S_2, and S_3 (see legend to Fig. 1 for the structures) are shown on the left side.

Fast Atom Bombardment-Mass Spectrometry—FAB-MS was carried out on a VG analytical ZAB HF mass spectrometer (Imperial College, London) as previously described (8, 15). About 100–200 μg of glycolipids were permethylated and about 10–20 μg of each derivative were loaded onto the glycerol/thioglycerol matrix for each FAB-MS run.

Immunostaining of Glycolipid on TLC—Glycolipids were chromatographed on a HPTLC plate (alminium sheets Silica Gel 60, E. Merck) and were reacted with monoclonal anti-Le^x antibodies according to the procedure described (16). Monoclonal antibody, PM-81, which reacts efficiently with Le^x structure (17), was kindly donated by Dr. E. D. Ball of Dartmouth Medical School.

RESULTS

Glycolipids from CML Cells—Fig. 1*A* shows the thin-layer chromatogram of the acidic glycolipids (gangliosides) prepared from CML cells. CML gangliosides provide a similar profile to that of normal granulocyte gangliosides except for the following. Doublets which migrate slightly faster than S_4 were significantly increased in some cases of CMLs. This glycolipid, apparently unique to CML, was termed G_2. In addition, gangliosides migrating to the same position of S_5 and those migrating more slowly than S_5 were also increased in CML cells from most of the patients examined. In contrast, gangliosides from acute myelogenous leukemia patients were mainly composed of short-chain glycolipids (Fig. 1*B*), which is consistent with the previous report (18).

Among the patients examined, patient 5 barely had any detectable amount of G_2 (Fig. 1*A*). Apparently, higher gangliosides from patient 5 are not enriched. In order to verify these observations on gangliosides and determine whether there are differences in chain elongation, fucosylation, sialylation, neutral glycolipids with long chain carbohydrates present in Folch's upper layer were examined. As shown in Fig. 2*A*, the majority of higher neutral glycolipids are decreased in patients 1 and 3, whereas in patient 5 a comparable amount of higher neutral glycolipids to normal granulocytes was detected. Glycolipids with the Le^x structure, Galβ1→4(Fucα1→3)GlcNAc, are also reduced in patients 1 and 3, whereas the granulocytes of patient 5 still contain a comparable amount of Le^x active glycolipids to those in normal granulocytes (Fig. 2*B*). These results indicate that the glycolipids with long carbohydrate chains are more heavily sialylated in the granulocytes of the majority of CML patients including patients 1 and 3 than those present in normal granulocytes.

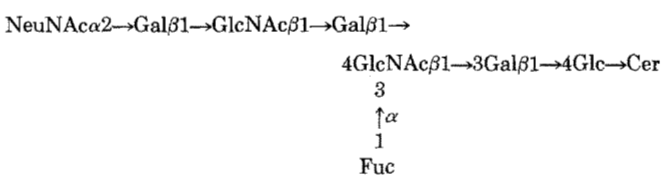
Purification of CML Gangliosides—Since the level of unique gangliosides is apparently increased in some CML cells, gangliosides from the CML cells of patient 1 were chosen for detailed structural analysis. After HPLC fractionation, the glycolipids were further purified through the procedures of acetylation, preparative HPTLC, and deacetylation followed by HPLC as described under "Experimental Procedures." Fig. 3 presents the HPTLC of CML ganglioside preparations. In the following section, the structural analysis of G_2 is described in detail, whereas the analysis of other glycolipids is described briefly.

Structure of G_2—G_2 migrated slightly faster than S_4, NeuNAcα2→6Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer (8) (Fig. 1*A*).

FAB-MS analysis of permethylated G_2 provided the molecular ions for $NeuNAc_1 \cdot Fuc_1 \cdot HexNAc_2 \cdot Hex_4 \cdot Cer_{16:0}$ (*m/z* 2421) and $NeuNAc_1 \cdot Fuc_1 \cdot HexNAc_2 \cdot Hex_4 \cdot Cer_{18:0}$ (*m/z* 2449), as shown in Fig. 4. The same analysis provided fragment ions corresponding to $NeuNAc \cdot Fuc \cdot HexNAc_2 \cdot Hex_3^+$ (*m/z* 1652), $NeuNAc \cdot Fuc \cdot Hex_2 \cdot HexNAc_2^+$ (*m/z* 1448), $NeuNAc \cdot Hex \cdot HexNAc \cdot Hex^+$ (*m/z* 1029), and $NeuNAc \cdot Hex \cdot HexNAc^+$ (*m/z* 825) (Fig. 4). If G_2 has a fucose residue at the subterminal *N*-acetylglucosamine residue, the fragment ion of *m/z* 999 should be prominent (Fig. 5*B*) (see also Ref. 5). As shown in Fig. 4, the fragment ion of *m/z* 999 was barely detected. These results indicate that G_2 has a sequence of NeuNAc→Hex→HexNAc→Hex→(Fuc→)HexNAc→Hex→Hex→Cer (Fig. 5*A*). In addition, the fragment ion of *m/z* 2183 corresponds to the $M - C_{16:0}$ acyl chain (2421–238) or $M - C_{18:0}$ (2449–266), confirming that the ceramide is composed of the sphingosine with $d_{18:1}$ as the long-chain base and the $C_{16:0}$ and $C_{18:0}$ as the major fatty acids.

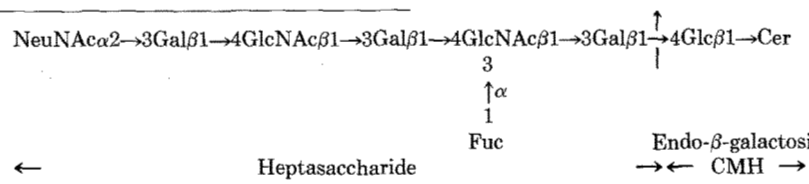
Methylation analysis of G_2 produced 1 mol of 2,3,4-tri-*O*-methylfucose, 3 mol of 2,4,6-tri-*O*-methylgalactose, 1 mol of 6-*O*-methyl-*N*-acetylglucosamine, 1 mol of 3,6-di-*O*-methyl-*N*-acetylglucosamine, and 1 mol of 2,3,6-tri-*O*-methylglucose (Table I).

G_2 was sequentially digested with clostridial neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase. The product was reacted with anti-Le^x monoclonal antibody, PM-81, and gave a positive spot at the same position of the N_1-glycolipid, Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glcβ1→1Cer (8), whereas the starting material did not react with the same antibody (Fig. 6*A*). These results indicate the following structure for G_2.



Endo-β-galactosidase from *Escherichia freundii* hydrolyzed G_2 glycolipid to produce CMH and a large oligosaccharide (Fig. 6*B*).

It has previously been shown that endo-β-galactosidase cannot hydrolyze the β-galactosidic linkage adjacent to the fucosylated *N*-acetylglucosamine (6, 19). Thus, the result can be interpreted as follows (where CMH represents Glcβ1→1Cer):



These results obtained by FAB-MS, methylation analysis, immunostaining and endo-β-galactosidase digestion indicate that the structure of G_2 glycolipid is $VI^3NeuNAcIII^3Fuc\text{-}LcnOse_6Cer$ as shown in Table II.

Structure of G_4—G_4 migrated slower than G_3 or S_5 (Figs. 1 and 3), which has a composition of $NeuNAc \cdot Hex_5 \cdot HexNAc_3 \cdot Cer_{16:0\ (or\ 18:0)}$ (Fig. 7*B*).[2]

FAB-MS analysis (Fig. 7*C*) of permethylated G_4 provided the fragment ions corresponding to $NeuNAc \cdot Hex \cdot HexNAc^+$

[2] Fig. 7 is presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2676, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

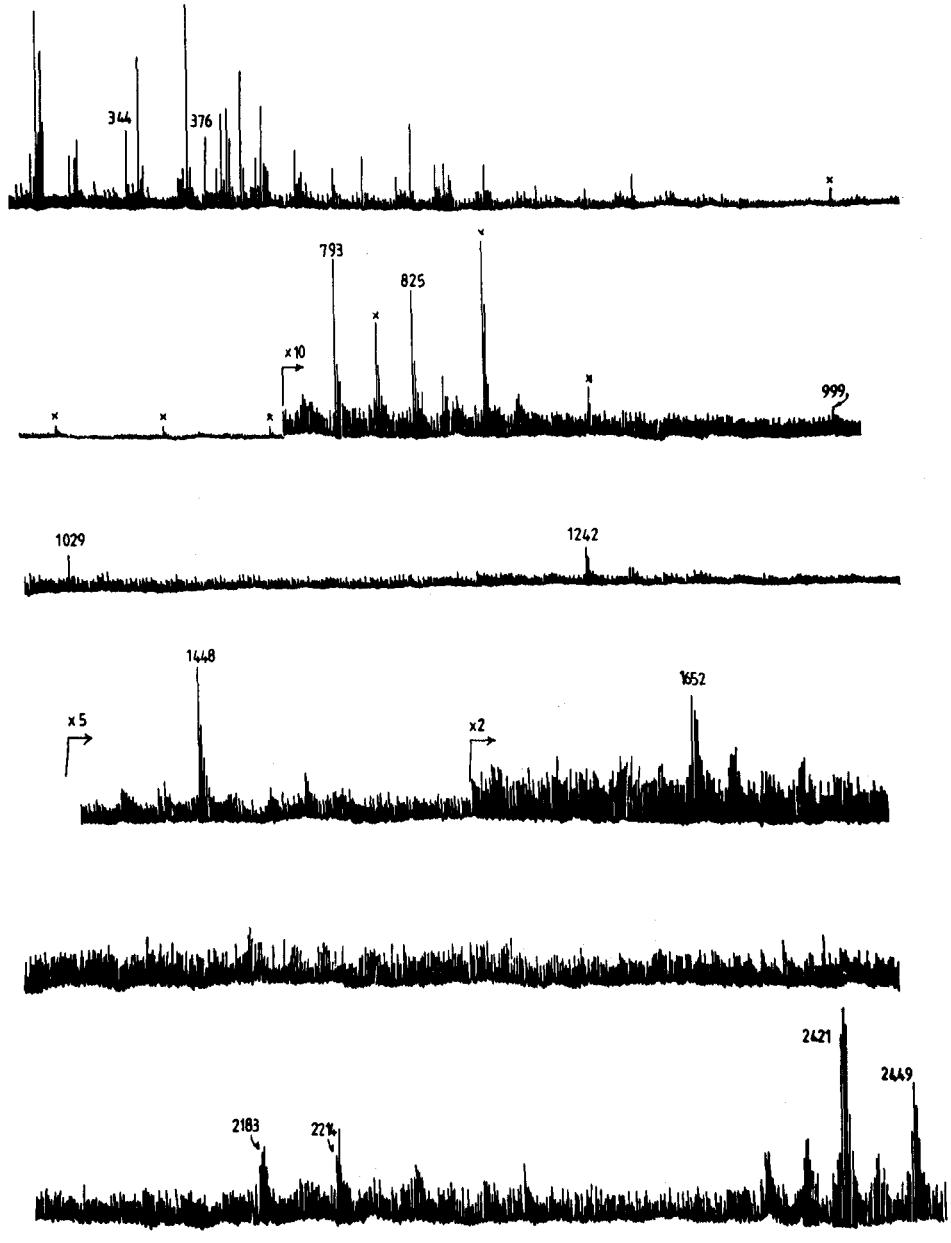


FIG. 4. **Fast atom bombardment-mass spectrum of permethylated G_2.** The positive spectrum was recorded. The molecular ions for the fully methylated NeuNAc·Fuc·$HexNAc_2$·Hex_4·$Cer_{16:0}$ and for the fully methylated NeuNAc·Fuc·$HexNAc_2$·Hex_4·$Cer_{18:0}$ are at the signals of 2421 and 2449, respectively. These signals are accompanied by the fragment ions for NeuNAc·Hex·HexNAc·Hex·Fuc·HexNAc·Hex^+ (*m/z* 1652), NeuNAc·Hex·HexNAc·Hex·Fuc·$HexNAc^+$ (*m/z* 1448), NeuNAc·Hex·HexNAc·Hex^+ (*m/z* 1029), NeuNAc·Hex·$HexNAc^+$ (*m/z* 825), and $NeuNAc^+$ (*m/z* 376). The signal at 2183 (2421–238 and 2449–266) corresponds to loss of acyl chain. The signal at 1242 corresponds to the fragment produced from NeuNAc·Hex_2·Fuc·$HexNAc_2^+$ by β-elimination of FucOH. Note that the fragment ion for NeuNAc·Gal·Fuc·$GlcNAc^+$ (*m/z* 999) is barely seen.

(*m/z* 825), NeuNAc·Fuc·Hex_2·$HexNAc_2^+$ (*m/z* 1448), and NeuNAc·Fuc·Hex_3·$HexNAc_3^+$ (*m/z* 1897). These results suggest the following sequence for one of the major components of G_4 glycolipids (see also Fig. 5*A* and Table I).

NeuNAc→Hex→HexNAc→Hex→

HexNAc→Hex→HexNAc→Hex→Hex→Cer

↑

Fuc

Further analysis could not be made due to the limited amount of G_4 available. It is, however, significant that fucose is attached to the internal *N*-acetylglucosamine.

Structures of G_1 and G_3 Glycolipids—G_1 migrated at the same position as the lower component of S_3 (Fig. 3). FAB-MS of permethylated G_1 provided molecular ions of *m/z* 2247 for NeuNAc·$HexNAc_2$·Hex_4·$Cer_{16:0}$ and 2275 for NeuNAc·$HexNAc_2$·Hex_4·$Cer_{18:0}$ (Fig. 7*A*). The same analysis showed that G_1 has a sequence of NeuNAc→$(Hex→HexNAc)_2$→Hex→Hex→Cer (see the legend for Fig. 7*A*). Endo-β-galac-

FIG. 5. **Possible structures revealed by fast atom bombardment-mass spectrometry of permethylated G_2 (see Fig. 4).** The fragment ions obtained by the loss of acyl chain or β-elimination of FucOH were omitted.

TABLE I

Relative proportions of methylated sugars obtained from gangliosides of chronic myelogenous leukemia cells

Methylated sugars	G_1	G_2	G_3	G_4
Fucitol				
2,3,4-Tri-*O*-methyl	0	1.0	0	0.8
Glucitol				
2,3,6-Tri-*O*-methyl	1.0	1.0	1.0	1.0
Galactitol				
2,3,4,6-Tetra-*O*-methyl	0	0	0	0.2
2,4,6-Tri-*O*-methyl	3.0	3.0	3.8	3.7
2-*N*-Methylacetamido-2-deoxyglucitol				
3,6-Di-*O*-methyl	2.0	1.0	3.0	2.1
6-Mono-*O*-methyl	0	1.0	0	1.0

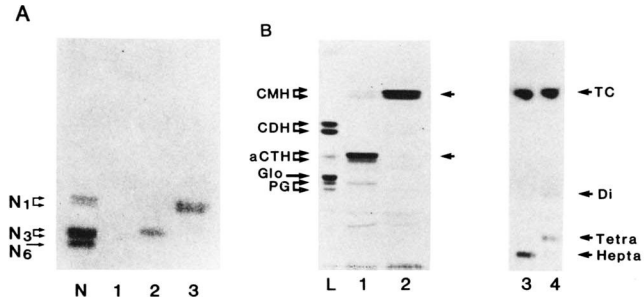
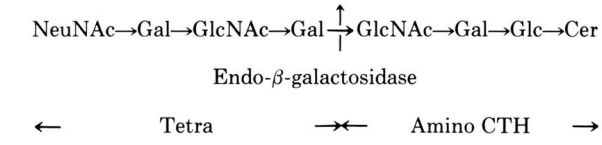


FIG. 6. **HPTLC of hydrolysis products of G_2 by exo- and endo-β-galactosidase.** *A*, immunostaining of glycolipids by anti-PM-81 monoclonal antibody. *N* and *lane 2*, Folch's upper phase neutral glycolipids (*N*) and N_3-glycolipid, Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer (*lane 2*). These are from normal granulocytes (8). *Lane 1*, untreated G_2; *lane 3*, G_2 after clostridial neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase. The monoclonal antibody reacts with Galβ1→4(Fucα1→3)GlcNAcβ1→R terminal structure. The solvent system used was chloroform:methanol:water (60:35:8, v/v). *B*, HPTLC of hydrolysis products of G_1 (*lanes 1* and *4*) and G_2 (*lanes 2* and *3*) gangliosides by endo-β-galactosidase. Glycolipids were digested under condition 1 as described (29, 30). Endo-β-galactosidase hydrolysis products were partitioned by chloroform:methanol:water (4:2:1, v/v) and each organic phase was applied to *lanes 1* and *2* for analysis of shorter glycolipids. Water phases were applied to *lanes 3* and *4* for analysis of oligosaccharides. The solvent systems used were chloroform:methanol:water (60:35:8, v/v) for glycolipids and 1-butanol:acetic acid:water (3:3:2, v/v) for oligosaccharides. *L*, Folch's lower phase glycolipid mixture from total blood cells as a Ref. 8. *TC*, sodium taurodeoxycholate; *Di*, GlcNAcβ1→3Gal; *Tetra*, NeuNAcα2→3Galβ1→4GlcNAcβ1→3Gal. *CMH*, Glcβ1→1Cer; *Glo*, globoside, GalNAcβ1→3Galα1→4Galβ1→4Glc→Cer. Structures for other standard glycolipids are shown in Fig. 2.

tosidase treatment of G_1 yielded lacto-*N*-triaosylceramide (amino CTH) and tetrasaccharide (Fig. 6*B*), as follows:



It was also apparent that a portion of amino CTH was further digested to produce Glcβ1→1Cer and disaccharide (*lanes 1* and *4* in Fig. 6*B*). Combining these results with methylation analysis (Table I), the structure of G_1 was proposed to be NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer with a $C_{16:0}$ or $C_{18:0}$ fatty acid. Thus, the G_1 glycolipid has the same carbohydrate sequence as the S_3 glycolipid which is present in normal granulocytes (8).

G_3 migrated approximately at the same position as S_5 (Figs. 1*A* and 3). FAB-MS (Fig. 7*B*) of permethylated G_3 provided molecular ions for NeuNAc·Hex_5·$HexNAc_3$·$Cer_{16:0}$ (*m/z* 2696) and NeuNAc·Hex_5·$HexNAc_3$·$Cer_{18:0}$ (*m/z* 2724). The same analysis indicates that G_3 has a sequence of NeuNAc→(Hex→HexNAc)$_3$→Hex→Hex→Cer. The methylation analysis indicates that sialic acid is linked to galactose through a 2→3 linkage and that no branched galactose is present (Table I). Combining these results, the structure of G_3 was suggested to be NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer with a $C_{16:0}$ or $C_{18:0}$ fatty acid.

DISCUSSION

The present study revealed the presence of a unique fucosyl ganglioside (G_2) in chronic myelogenous leukemia cells and elucidated its structure as NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glcβ1→1Cer. This is the first report of the isolation of this glycolipid although related fucogangliosides have been isolated from colonic tumor tissues (20, 21) (see Table II). Previously, we have established the structure of glycolipids present in normal granulocytes (8). Although a fucosyl ganglioside, which has the same composition as G_2, has been detected in the S_5 fraction from normal granulocytes, the amount was significantly low (8). In addition, the S_5 glycolipid migrated much slower than the G_2 glycolipid on TLC, suggesting that G_2 and S_5 glycolipids are different in carbohydrate structure. Since the G_2 glycolipid is not accumulated in acute myelogenous leukemia cells (Fig. 1*B*), the accumulation of G_2

TABLE II

Structures of sialylated fucosylnorhexaosylceramides

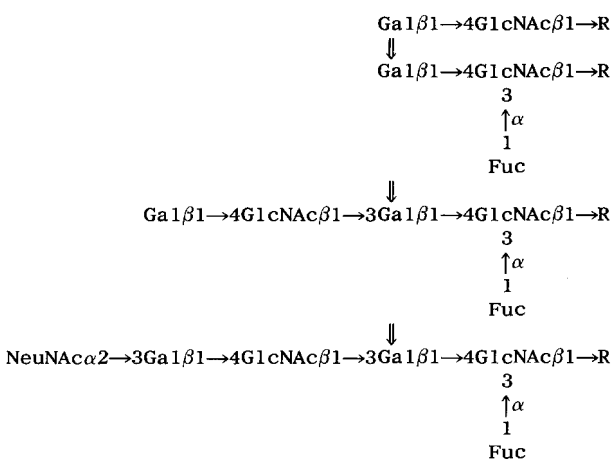
Systematic name[a]	Structure	Reference
$VI^3NeuNAcIII^3FucnorLcnOse_6Cer$	NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 ↑α 1 Fuc	Present study
$VI^6NeuNAcIII^3FucnorLcnOse_6Cer$	NeuNAcα2→6Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 ↑α 1 Fuc	20
$VI^3NeuNAcV^3III^3Fuc_2norLcnOse_6Cer$	NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑α ↑α 1 1 Fuc Fuc	21

[a] According to the recommendations of the Nomenclature Committee of the International Union of Pure and Applied Chemistry (27).

glycolipid may be a characteristic marker for CML cells.

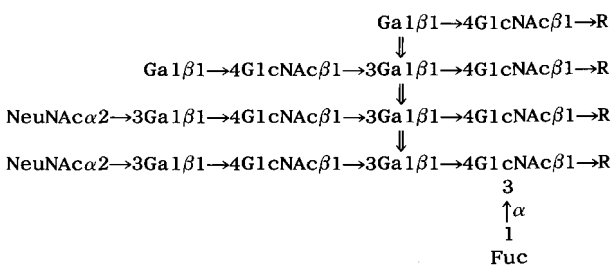
It is significant that fucose is linked to the *N*-acetylglucosamine which is distal from the nonreducing end, but not to the subterminal *N*-acetylglucosamine. This was somewhat surprising to us since we have observed fucosylation at the subterminal *N*-acetylglucosamine which forms the sialyl Le^x, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→ structure in CML polylactosaminoglycans (5). However, preferential fucosylation at internal *N*-acetylglucosamine residues in CML polylactosaminoglycans has also been noticed by FAB-MS; the signal at *m/z* 1448 for NeuNAc→Gal→GlcNAc→Gal→(Fuc→)GlcNAc$^+$ or NeuNAc→Gal→(Fuc→)GlcNAc→Gal→GlcNAc$^+$ was stronger than that for NeuNAc→Gal→(Fuc→)GlcNAc→Gal→(Fuc→)GlcNAc$^+$ (*m/z* 1622) or that for NeuNAc→Gal→(Fuc→)GlcNAc$^+$ (*m/z* 999) (see Fig. 5*A* in Ref. 5). The signal at *m/z* 1448, therefore, mostly represents the NeuNAc→Gal→GlcNAc→Gal→(Fuc→)GlcNAc→ structure. Thus, internal monofucosylation appears to take place more frequently than external monofucosylation or difucosylation at both internal and external sites.

The structure of G_2 and the results of previous structural studies on granulocyte glycolipids provide significant information on the biosynthetic steps of poly-*N*-acetyllactosamine carbohydrates in terms of extension, fucosylation, and sialylation. Previously, we have shown that no such structure as extended N_1, Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)-GlcNAcβ1→3Galβ1→4Glcβ1→Cer, is present in granulocyte glycolipids (8). This is consistent with the recent report that fucosylation takes place after lactosaminyl backbone is formed and that fucose is preferentially added to the subterminal *N*-acetylglucosamine and then to the internal *N*-acetylglucosamine (22). These observations (8, 22) indicate that "extension precedes fucosylation" and thus the possibility that G_2 is formed by the following pathway is less likely.



SCHEME I

Therefore, G_2 must be formed by the following alternative pathway which is expressed as "extension, sialylation, then fucosylation."



SCHEME II

Sialylation preceding fucosylation may explain why the fucose residue is present at the internal *N*-acetylglucosamine. The presence of sialic acid at the nonreducing terminal may hamper the addition of fucose to the subterminal *N*-acetylglucosamine. Thus, fucosylation takes place at the internal *N*-acetylglucosamine. If fucosylation takes place before sialylation, fucose should be preferentially added at the subterminal *N*-acetylglucosamine as seen in neutral glycolipids (8, 22). Recently, α1→3 specific fucosyltransferases were detected in mutant Chinese hamster ovary cell lines or human lung cancer cell lines. These enzymes exclusively form α1→3 fucosyl linkage on *N*-acetylglucosamine (22, 23), and α1→3 specific fucosyltransferases can apparently add fucose to sialylated lactosaminyl chains (23). On the other hand, it has been shown that α1→3 and α1→4 fucosyltransferase activities cannot be separated in human milk (24, 25). In this enzyme system, fucosyltransferase could not add fucose to sialylated substrates (24). Therefore, at least two kinds of α1→3 fucosyltransferases are known (for a detailed discussion, see Ref. 26). In CML cells, an enzyme similar to the former fucosyltransferase may be responsible for the formation of G_2.

Table II lists the structures of the fucosyl gangliosides so far reported which have two *N*-acetylglucosamine residues available for fucosylation. In all of these structures, fucose residues are attached only at the internal *N*-acetylglucosamine or at both sites. This evidence is in agreement with the proposed sequence of sialylation followed by fucosylation (Scheme II). It is plausible that fucose is preferentially added to the internal *N*-acetylglucosamine and then to the subterminal *N*-acetylglucosamine, due to the steric hindrance caused by the terminal sialic acid residue. In order to test whether Scheme II is correct, it will be essential to characterize an α1→3 fucosyltransferase by using a defined substrate, such as S_1 and S_3 glycolipids.

It is noted that CML gangliosides contain $C_{18:0}$ fatty acid in addition to $C_{16:0}$ and $C_{24:1}$.[3] We have previously shown that glycolipids from normal, mature granulocytes almost exclusively contain $C_{16:0}$ and $C_{24:1}$ fatty acids (8). These results suggest that fatty acid metabolism is altered in CML cells and this is reflected in the glycolipids.

The present study also shows the heterogeneity of ganglioside profiles among patients. Although cells from the majority of CML patients express a unique fucoganglioside, G_2, some of the patients do not show this glycolipid (Fig. 1*A*). There may be two possible explanations for this. One possibility is that this glycolipid is aberrently expressed in malignant cells. Another possibility is that the glycolipid is expressed in a subpopulation of immature CML cells which are not present in normal peripheral blood. For this, it will be important to correlate the presence or absence of G_2 with maturational stages or cell types of dominant leukemic cells in a given stage of the disease. It will also be important to see whether any immature cells of normal granulocyte development also express this carbohydrate structure. These studies will provide a basis for diagnosis and prognosis of chronic myelogenous leukemia and hopefully provide a basis for immunotherapy (28).

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[3] Our analysis showed that CML glycolipids corresponding to the upper band of S_3 contain $C_{24:1}$ fatty acid.

Note Added in Proof—After submitting our manuscript, the biosynthetic pathway of sialylation followed by fucosylation has been shown in sialyl Le^a oligosaccharide structure (Hansson, G. C., and Zopf, D. (1985) *J. Biol. Chem.* **260,** 9388–9392.

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SUPPLEMENTAL MATERIAL
TO
STRUCTURE OF A NOVEL SIALYLATED FUCOSYL LACTO-N-NOR-HEXAOSYLCERAMIDE
ISOLATED FROM CHRONIC MYELOGENOUS LEUKEMIA CELLS

by

Michiko N. Fukuda, Anne Dell, Philip R. Tiller, Ajit Varki,
John C. Klock and Minoru Fukuda

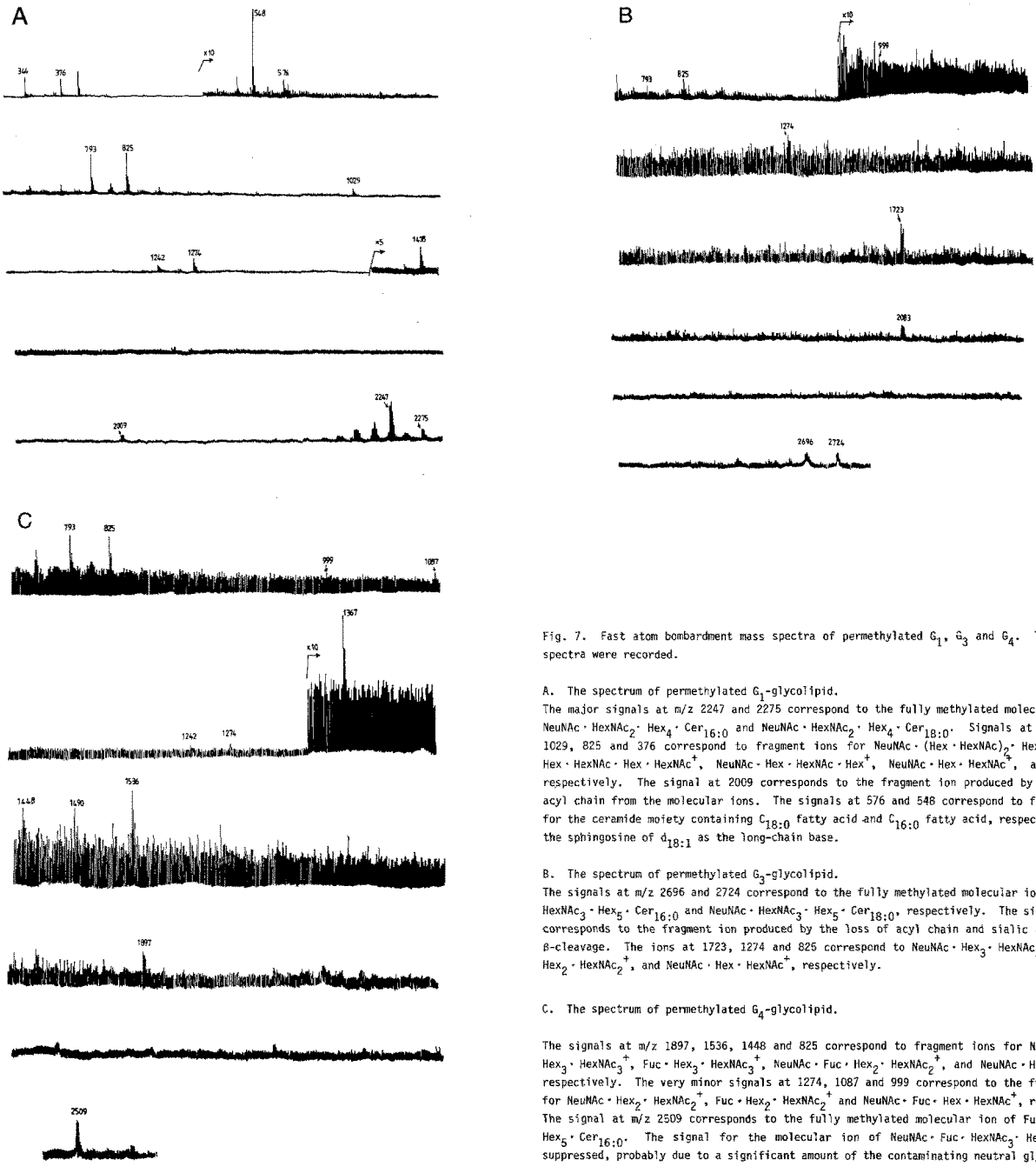

Fig. 7. Fast atom bombardment mass spectra of permethylated G_1, G_3 and G_4. The positive spectra were recorded.

A. The spectrum of permethylated G_1-glycolipid.
The major signals at m/z 2247 and 2275 correspond to the fully methylated molecular ions of NeuNAc · $HexNAc_2$ · Hex_4 · $Cer_{16:0}$ and NeuNAc · $HexNAc_2$ · Hex_4 · $Cer_{18:0}$. Signals at 1478, 1274, 1029, 825 and 376 correspond to fragment ions for NeuNAc · $(Hex \cdot HexNAc)_2$ · Hex^+, NeuNAc · Hex · HexNAc · Hex · $HexNAc^+$, NeuNAc · Hex · HexNAc · Hex^+, NeuNAc · Hex · $HexNAc^+$, and $NeuNAc^+$, respectively. The signal at 2009 corresponds to the fragment ion produced by the loss of acyl chain from the molecular ions. The signals at 576 and 548 correspond to fragment ions for the ceramide moiety containing $C_{18:0}$ fatty acid and $C_{16:0}$ fatty acid, respectively, with the sphingosine of $d_{18:1}$ as the long-chain base.

B. The spectrum of permethylated G_3-glycolipid.
The signals at m/z 2696 and 2724 correspond to the fully methylated molecular ions of NeuNAc · $HexNAc_3$ · Hex_5 · $Cer_{16:0}$ and NeuNAc · $HexNAc_3$ · Hex_5 · $Cer_{18:0}$, respectively. The signal at 2083 corresponds to the fragment ion produced by the loss of acyl chain and sialic acid through β-cleavage. The ions at 1723, 1274 and 825 correspond to NeuNAc · Hex_3 · $HexNAc_3^+$, NeuNAc · Hex_2 · $HexNAc_2^+$, and NeuNAc · Hex · $HexNAc^+$, respectively.

C. The spectrum of permethylated G_4-glycolipid.

The signals at m/z 1897, 1536, 1448 and 825 correspond to fragment ions for NeuNAc · Fuc · Hex_3 · $HexNAc_3^+$, Fuc · Hex_3 · $HexNAc_3^+$, NeuNAc · Fuc · Hex_2 · $HexNAc_2^+$, and NeuNAc · Hex · $HexNAc^+$, respectively. The very minor signals at 1274, 1087 and 999 correspond to the fragment ions for NeuNAc · Hex_2 · $HexNAc_2^+$, Fuc · Hex_2 · $HexNAc_2^+$ and NeuNAc · Fuc · Hex · $HexNAc^+$, respectively. The signal at m/z 2509 corresponds to the fully methylated molecular ion of Fuc · $HexNAc_3$ · Hex_5 · $Cer_{16:0}$. The signal for the molecular ion of NeuNAc · Fuc · $HexNAc_3$ · Hex_5 · Cer was suppressed, probably due to a significant amount of the contaminating neutral glycolipid.