Characterization of Tumor-associated Ganglio-N-triaosyglyceramide in Mouse Lymphoma and the Dependency of Its Exposure and Antigenicity on the Sialyl Residues of a Second Glycoconjugate*

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Ganglio-N-triaosyglyceramide (GalNAc1→4Gal1→4Glc1→3Cer), a tumor-associated marker for L5178 cells, was previously reported to separate on thin layer chromatography into three distinct bands (bands a, b, and c). The present paper describes the characterization of these bands and the factor that determines the degree of glycolipid exposure at the cell surface and its antigenicity. 1) The resolution of ganglio-N-triaosyglyceramide into three bands was found to be due to molecules having different fatty acid compositions. Band a contained nervonic (C24:1) and lignoceric (C24:0) acids, band b contained palmitic acid (C16:0), and band c contained α-hydroxypalmitic acid. 2) Surface labeling of L5178 cells with galactose oxidase, followed by fluorography of the isolated glycolipids, revealed that all three bands were exposed on the surface of the cell. However, treatment of cells with sialidase before treatment with galactose oxidase resulted in a 10-fold increase of label incorporated into ganglio-N-triaosyglyceramide. Since no sialylated form of ganglio-N-triaosyglyceramide was detected on these cells, and no change in the chemical amount of this glycolipid could be detected, the increase of label into this molecule was due to the exposure by sialidase of a normally cryptic glycolipid. The exposure of ganglio-N-triaosyglyceramide after sialidase treatment was also reflected by the increased sensitivity of these cells to monoclonal antibodies to the glycolipid and complement after enzyme treatment. Thus, the results provide clear evidence that crypticity, as well as antigenicity, of a membrane glycolipid is determined by the degree of sialylation in a second membrane glycoconjugate.

Among various functional notions assigned to glycolipids (1, 2), their role as cell type-specific markers and antigens is the most conclusive (3, 4). A great deal of current interest has been aroused by tumor-associated glycolipid antigens (5-17), as determined by cell surface labeling or immunochromatographic activity, is affected by factors (lectins and proteases) that cause changes in the protein structure and distribution of other membrane components (18, 19, 44). Ganglio-N-triaosyglyceramide accumulates in Kirsten virus-transformed 3T3 cells and tumors derived therefrom in BALB/c mice (11). Similarly, this glycolipid is found in mouse lymphoma L5178c127, a tumor of D2A/2 origin (20). Since this glycolipid was virtually absent or present in small quantities in various tissues and organs of these mice, the glycolipid can be regarded as an excellent model for a tumor-associated glycolipid marker. OgOse3Cer3 has been successfully utilized as a target for the antibody-dependent, avidin-mediated killing of cells by biotinyl neocarzinostatin (21) and a monoclonal IgG3 antibody directed to this marker was used successfully for the serotherapy of the L5178c127 tumor in D2A/2 mice (12). This paper describes the complete chemical characterization of this glycolipid and the factors affecting its exposure and antigenic reactivity on the cell surface.

MATERIALS AND METHODS

**Culture Cells**

Isolation of L5178c127 cells from L5178, a methylcholanthrene-induced tumor of D2A/2 origin, has been described (22). Cells were cultured in RPMI-1640, supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Ganglio lipid isolation

Ganglio lipids were isolated from cultured cells by extraction with chloroform:methanol 2:1 and chloroform:methanol:0.2 M NaCl, followed by precipitation (23). Cells were washed with PBS, resuspended in 1% Triton X-100, and long chain neutral gangliosides were extracted with chloroform:methanol:0.2 M NaCl. The lower phase was deproteinized with 10% trichloroacetic acid, followed by chloroform: methanol:water 2:1:0.8, and then deproteinized with chloroform:methanol:0.2 M NaCl. The deproteinized sample was evaporated to dryness and acetylated with acetic anhydride at 80 °C for 1 h, followed by reduction and acetylation, as previously described (24).

Fatty acids and sphingoid bases were isolated from the hydrolysate of gangliosides 90% acetic acid containing 0.5% sulfuric acid under nitrogen at 80 °C for 1 h, followed by reduction and acetylation, as previously described (24). Fatty acids and sphingoid bases were isolated from the hydrolysate of gangliosides 90% acetic acid containing 0.5% sulfuric acid under nitrogen at 80 °C for 1 h, followed by reduction and acetylation, as previously described (24).

**Analytical procedures**

The ganglioside composition was determined by gas chromatography (25) on a 10-100 mesh Supelcoport of sugar alditol acetates obtained from the hydrolysis of gangliosides with 0.5% sulfuric acid under nitrogen at 80 °C for 1 h, followed by reduction and acetylation, as previously described (24).

**Materials and Methods**

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Characterization of Ganglio-N-triaosylceramide—The major neutral glycolipids present in L5178c127 cells, separated in bands a, b, and c (Fig. 1), were purified to homogeneity by preparative thin layer chromatography. As previously reported (21), the carbohydrate composition and the same sugar sequence as evidenced by sequential degradation with beta-N-acetylhexosaminidase and beta-galactosidase. Moreover, each glycolipid showed a similar reactivity with antibodies to GalNAc1+3Gal1+4Glc1+1Cer. Thus, each band was identified as GalNAc1+3Gal1+4Glc1+1Cer. The difference between bands a, b, and c became evident upon examination of the "ceramide window" of the mass spectrum and analysis of the fatty acid composition. All three direct probe mass spectra had an ion at m/e 253 suggesting the presence of C:18:1 sphingenine as the long chain base. The differences between the mass spectra of bands a, b, and c (Fig. 2) were thus interpreted as being due to differences in the fatty acid composition of GalNAc1+3Gal1+4Glc1+1Cer from each band. Band a representing GalNAc1+3Gal1+4Glc1+1Cer containing nervonic acid (C24:1), band b representing GalNAc1+3Gal1+4Glc1+1Cer containing palmitic acid (C16:0), and band c representing GalNAc1+3Gal1+4Glc1+1Cer containing alpha-hydroxypalmitic acid (hC16:0) would be consistent with the ions detected. Gas chromatography-mass spectrometry of fatty acid methyl esters before and after trimethylsilylation (30) conclusively demonstrated that bands a and b contained normal fatty acids whereas band c had alpha-hydroxypalmitic acid. These results confirmed the predictions made from the direct probe mass spectral analysis and firmly established the difference in TLC migration of bands a, b, and c as due to differences in the fatty acid content of the GalNAc1+3Gal1+4Glc1+1Cer molecules. The relative amount of each fatty acid present in each band is summarized in Table I.

Other Glycolipids Present in L5178c127 Cells—Bands d and e (Fig. 1) were earlier identified as ganglio-N-tetraosylceramide (GalNAc1+3Gal1+4Glc1+1Cer). Small quantities of gangliosides were present on these cells. Three resorcinol staining bands that migrated close to each other were detected. Gas-liquid chromatography-reversed phase mass spectrometry revealed an additional peak on the chromatogram for band c that was not seen with bands a and b. This peak eluted just after N-acetylgalactosamine. The mass spectrum of this peak was identical with that of hydroxypalmitate. Moreover, co-injection of band c fatty acid methyl esters and methyl glycosides intensified this peak, confirming this observation. This observation emphasized one advantage of direct probe mass spectrometry over degradative methods of structural analysis: the entire molecule is examined intact, precluding the loss of components following hydrolysis.
FIG. 2. The "ceramide window" (m/e 500–700) from the direct probe mass spectra of permethylated L5178c127 glycolipid bands a (A), b (B), and c (C). The origin of the major fragment seen in each panel is depicted in the figure above each spectrum. \( \text{Gg}_3 \) represents the carbohydrate portion of the glycolipid. Mass spectrometry conditions: electron energy, 35 V; ion energy, +8.6 V; extractor, +6.0 V; lens, 16 V; emission, 0.5 mA; electron multiplier, 2000 V; sensitivity, \( 10^{-10} \) A/V. Ordinate, relative ion intensity (%).

FIG. 3. \( V. \) cholera sialidase digestion of L5178c127 gangliosides. L5178, L5178c127 gangliosides (10 µg) incubated with (+) and without (−) sialidase (2.5 units in 100 µl of 50 mM acetate buffer, pH 6, 2 mM CaCl₂); SPg, sialylparagloboside (NeuAcα₂⁺₃Galβ₁→₄GlcNAcβ₁→₃Galβ₁→₄Glc→R) with and without sialidase; GM₃, bovine GM₃ (Galβ₁→₃GalNAcβ₁→₄Glcβ₁→₄Glc→R) with and without sialidase. The plate was developed in chloroform/methanol/water (60:35:8). Gangliosides were visualized with resorcinol and neutral glycolipids were then detected with orcinol. Bands d and e were revealed after orcinol staining.

TABLE I

Fatty acid composition of L5178c127 glycolipids

| Glycolipid | 16:0 | 18:0 | 18:1 | 20:0 | 22:0 | 23:0 | 24:0 | 24:1 | 25:0 |
|------------|------|------|------|------|------|------|------|------|------|
| GgOse₂Cer (reported in Ref. 48) | 1.2  | 2.6  | Trace | 2.8  | 20.4 | 12.9 | 46.4 | 9.5  | 1.2  |
| GgOse₂Cer (guinea pig red blood cells)* | − 5  | −    | −    | −    | 26.2 | 3.1  | 53.0 | 16.0 | −    |
| L5178c127 |      |      |      |      |      |      |      |      |      |
| Band a    | 78   | 16   | 6    | −    | −    | −    | −    | −    | −    |
| Band b    | 8.9  | 86.9 | Trace | 4.2  | −    | −    | −    | −    | −    |
| Band c    | 8.9  | 86.9 | Trace | 4.2  | −    | −    | −    | −    | −    |

* Results obtained in this study.

−, less than 1% of total fatty acid.
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resulted in the appearance of two neutral glycolipids that migrated on TLC with spots d and e (GgOse/Cer) (Fig. 3). Weak acid hydrolysis of L5178 gangliosides yielded neutral glycolipids d and e as well (results not shown). These results suggested that all the gangliosides present on L5178c127 cells had the gangliotetrasaccharide core and that among these were some that bore terminal sialic acids (labile to sialidase). Such a ganglioside, GM_{1b}, has been obtained in trace amounts from human erythrocyte stroma (37), and the presence of sialidase-susceptible gangliosides in murine lymphocytes has been reported (38, 39). L5178c127 cells seem to contain this ganglioside also. Thus, all glycolipids present on L5178c127 cells were derived from the ganglio family of glycolipids and were represented by glucosylceramide, lactosylceramide, GgOse/Cer, GgOse4/Cer, and gangliosides, including GM_{1b}. The alternate pathway for ganglioside biosynthesis (35, 40-42) that would result in the formation of these compounds is in contrast to the established pathway for GM_{1b} synthesis (for review, see Ref. 2).

Surface Exposure of L5178c127 Glycolipid and the Degree of Exposure and Antigenicity are Defined by the Sialosyl Residue at a Second Glycoconjugate—Glycolipid bands a, b, c, d, and e were readily labeled by treatment of cells with galactose oxidase (Fig. 4, lane 2). No label was associated with these bands by treatment of cells with sodium borotritide alone (Fig. 4, lane 1). Clearly, all three gangliotriosylceramide bands were exposed on the surface of the cell.

Treatment of cells with sialidase prior to galactose oxidase resulted in a substantial increase in label that was chloroform/methanol-soluble. As demonstrated in the experiment summarized in Table II, galactose oxidase treatment of cells followed by reduction with NaB_{3}H_{4} resulted in the extraction of 198 cpm/µg of protein by the lipid solvent. If the cells were first treated with sialidase, this value was increased to 1283 cpm/µg of protein.

Since we had shown that there were gangliosides present that yielded GgOse/Cer after treatment with sialidase (Fig. 3), we initially predicted that the increased label would appear in this neutral glycolipid. As expected, label associated with GgOse/Cer was increased 24-fold (Table II) after sialidase treatment of cells, and the amount of chemically detectable GgOse/Cer was increased (Table II).

Sialidase treatment of cells, however, yielded an unexpected

![Thin layer chromatogram and fluorogram of surface-labeled L5178c127 glycolipids. Lane 1, cells treated with sodium borotritide alone; lane 2, cells treated with galactose oxidase followed by sodium borotritide; lane 3, cells treated with sialidase and galactose oxidase followed by sodium borotritide. Glycolipids were extracted from sodium dodecyl sulfate-dissolved cells. The Folch lower phase was then applied to TLC plates. Each lane received the glycolipids from 10 million cells. The radiochromatograph scans to the right of the figure reflect the actual number of counts applied to each lane. TLC plates were developed in chloroform/methanol/0.5% sulfuric acid reaction according to the method as previously described (45). Both activity and chemical quantity are the values from 2×10^{6} cells. The chemical quantity of GgOse/Cer is the summation of three spots, a, b, and c. Spots a and b were estimated to be 0.4 µg; spot c was 0.1 µg.

CaCl_{2} (60:35:8) and then sprayed with 0.4% 2,5-diphenyloxazole in 2-methylnaphthaline (32). Film was developed at -70 °C.

**TABLE II**

| Glycolipids in L5178c127 cells and their chemical quantity |
|-------------------------------------------------------------|
| 2×10^{7} cells were labeled with NaB_{3}H_{4} after enzyme treatment. Labeled cells were suspended in 0.54 ml of NaCl/P, and 0.01-ml aliquots were taken for determination of radioactivity for determination of protein by the fluorescamine assay (43). 0.5 ml of the cell suspension was extracted with chloroform/methanol (2:1) and extract equivalent to 2×10^{6} cells (163 µg of protein) was applied to high performance TLC plate. Regions on the plate that corresponded to GgOse/Cer and GgOse4/Cer (located by fluorography) were scraped and counted for radioactivity. The chemical quantity was analyzed by high performance TLC after reaction with the orcinol/sulfuric acid reaction according to the method as previously described (45). Both activity and chemical quantity are the values from 2×10^{6} cells. The chemical quantity of GgOse/Cer is the summation of three spots, a, b, and c. Spots a and b were estimated to be 0.4 µg; spot c was 0.1 µg. |
| | Total | Trinitium label | Glycolipids and their chemical quantity |
| | | lipid associated | |
| | cpm/µg protein | cpm/µg protein | cpm | µg | Activity | Chemical quantity |
| Galactose oxidase | 2,078 | 198 | 3,569 | 0.9 | 1,157 | ND |
| Sialidase + galactose oxidase | 3,492 | 1,283 | 38,469 | 0.9 | 26,751 | 0.1 |
| Trypsin + galactose oxidase | 2,068 | 170 | 3,652 | 0.9 | 1,136 | ND |
| Trypsin + sialidase + galactose oxidase | 3,643 | 1,349 | 39,345 | 0.9 | 28,028 | 0.1 |

* A possible error that could occur in visual comparison of spot could be 10-20%.

† ND, not detectable.
result. Label associated with GgOse3Cer (bands a, b, and c) was enhanced 11-fold (Table II and Fig. 4, lane 3), with no detectable change in the chemical amount of these bands (Table II), and since no sialylated forms of this glycolipid were found on these cells (Fig. 3), the increase in label was ascribed to the increased exposure of GgOse3Cer after sialidase treatment. This phenomenon was also revealed by the enhanced reactivity of sialidase-treated cells to monoclonal antibodies directed to GgOse3Cer (Fig. 5). Cells treated with sialidase (Fig. 5, open symbols) were 10 times more sensitive to complement-mediated lysis than untreated cells (Fig. 5, closed symbols). It is interesting to note that treatment of cells with trypsin prior to treatment with galactose oxidase had no effect on the exposure of GgOse3Cer or GgOse4Cer (Table II).

DISCUSSION

The mouse lymphoma tumor-associated glycolipid, GgOse3Cer, was resolved by TLC into three distinct bands (a, b, and c). By direct probe mass spectrometry of the intact permethylated glycolipids and analysis of the glycolipid fatty acids of each band by gas chromatography-mass spectrometry, these differences were found to be in the fatty acid composition of the molecules. Band a was GgOse3Cer which contained nervonic (C24:1) and lignoceric (C24:0) acids, band b contained palmitic acid (C16:0), and band c contained a-hydroxypalmitic acid. Since band e showed the same mobility as an authentic GgOse3Cer isolated from guinea pig erythrocytes, band b had the same mobility as authentic globo-N-tetraosylceramide from human erythrocytes, and band c coincided with lacto-N-tetraosylceramide, identification of glycolipids by migration on TLC alone is misleading.

By cell surface labeling with galactose oxidase/sodium borohydride, we were able to show that all three bands were exposed on the surface of the cell and potentially available for interaction with antibodies directed toward the carbohydrate determinant expressed on these molecules (20). We observed, unexpectedly, that after sialidase treatment, the amount of label incorporated into GgOse3Cer by this technique increased 11-fold. No change in the chemical levels of this glycolipid could be detected. Since the only sialidase-sensitive gangliosides present on these cells had the GgOse3Cer core (GM1a), the increased label incorporated into GgOse3Cer was due to the exposure of normally cryptic glycolipid to the activity of galactose oxidase. The increase exposure of GgOse3Cer was also revealed by monoclonal antibodies directed to this glycolipid. Cells treated with sialidase were 6–10 times more sensitive to complement-mediated lysis with this antibody than were untreated L5178c127 cells. Clearly, the antigenicity of these cells was influenced by the sialylation of other membrane components.

This study revealed two aspects about the antigenicity of glycolipids that are important in understanding the potential of these molecules as tumor markers.

1) The reactivity of L5178c127 cells with antibodies directed to GgOse3Cer was the result of the expression on these cells of a complex family of ganglio-N-triaosylceramides. The heterogeneity of ceramides containing the same carbohydrate moiety raises the intriguing possibility that the specific activity of these compounds with antibody may differ. Perhaps GgOse4Cer with a-hydroxypalmitic acid is more exposed or exposed in a different orientation in the cell membrane, and is hence more reactive with antibody.

2) The organization and topography of the membrane are important parameters in defining the exposure and subsequent antigenicity of these molecules. Several examples of the importance of membrane organization to glycolipid antigenicity exist in the literature. Human fetal erythrocytes, for example, are much more reactive to anti-globo-N-tetraosylceramide, even though the chemical level of this glycolipid in fetal and adult erythrocytes is nearly identical (18). Human adult erythrocytes become reactive to anti-globo-N-tetraosylceramide only after sialidase or protease digestion (18). Globo-N-tetraosylceramide can, however, be labeled equally well by the galactose oxidase-sodium borohydride procedure, with or without treatment of protease or sialidase, suggesting that it is exposed on the surface of adult erythrocytes, but hidden from the view of the larger antibody molecules (19). The reactivity of globo-N-tetraosylceramide with galactose oxidase at the surface of hamster fibroblast NIL cell was twice enhanced when cells were pretreated with a small quantity of concanavalin A or Rictinus communis lectin (19). The present study indicates a much more drastic change of crypticity of glycolipid induced by sialidase, and so far, this is the clearest evidence that the crypticity and the antigenicity of a glycolipid are affected by a second membrane component.

This study clearly established the molecular basis for the ganglio-N-triaosylceramide antigenicity of this T-cell lymphoma. This analysis also enabled us to better understand the glycolipids present on normal murine lymphoid cells, where sufficient numbers of cells for structural analysis are difficult to obtain and information about the structure of glycophospholipids in these cells is scarce.

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