

Characterization of the Acid Stability of Glycosidically Linked Neuraminic Acid

USE IN DETECTING DE-*N*-ACETYL-GANGLIOSIDES IN HUMAN MELANOMA*

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The glycosidic linkage of sialic acids is much more sensitive to acid hydrolysis than those of other monosaccharides in vertebrates. The commonest sialic acids in nature are neuraminic acid (Neu)-based and are typically *N*-acylated at the C5 position. Unsubstituted Neu is thought to occur on native gangliosides of certain tumors and cell lines, and synthetic de-*N*-acetyl-gangliosides have potent biological properties *in vitro*. However, claims for their natural existence are based upon monoclonal antibodies and pulse-chase experiments, and there have been no reports of their chemical detection. Here we report that one of these antibodies shows nonspecific cross-reactivity with a polypeptide epitope, further emphasizing the need for definitive chemical proof of unsubstituted Neu on naturally occurring gangliosides. While pursuing this, we found that α 2–3-linked Neu on chemically de-*N*-acetylated G_{M3} ganglioside resists acid hydrolysis under conditions where the *N*-acetylated form is completely labile. To ascertain the generality of this finding, we investigated the stability of glycosidically linked α - and β -methyl glycosides of Neu. Using NMR spectroscopy to monitor glycosidic linkage hydrolysis, we find that only 47% of Neu α 2Me is hydrolyzed after 3 h in 10 mM HCl at 80 °C, whereas Neu5Ac α 2Me is 95% hydrolyzed after 20 min under the same conditions. Notably, Neu β 2Me is hydrolyzed even slower than Neu α 2Me, indicating that acid resistance is a general property of glycosidically linked Neu. Taking advantage of this, we modified classical purification techniques for de-*N*-acetyl-ganglioside isolation using acid to first eliminate conventional gangliosides. We also introduce a phospholipase-based approach to remove contaminating phospholipids that previously hindered efforts to study de-*N*-acetyl-gangliosides. The partially purified sample can then be *N*-propionylated, allowing acid release and mass spectrometric detection of any originally existing Neu as Neu5Pr. These advances allowed us to detect covalently bound Neu in lipid extracts of a human melanoma tumor, providing the first chemical proof for naturally occurring de-*N*-acetyl-gangliosides.

Gangliosides are amphipathic glycosphingolipids that are mostly found in the outer leaflet of the plasma membrane (1–4). They are typically characterized by the presence of at least one sialic acid residue and a lactosyl ceramide core. Other features of a ganglioside oligosaccharide moiety, such as the number and branching pattern of monosaccharides and modifications such as *O*-acetylation, are regulated in a tissue-specific and temporal manner. The role of gangliosides in cell signaling (5–9) and cell-cell and cell-matrix interactions (10–13) and host-pathogen interactions (14–16) has been extensively studied and is largely dictated by the structure of the glycan component.

Most sialic acids on gangliosides share a core neuraminic acid (Neu)¹ structure and are *N*-acylated at the C-5 position with either an *N*-acetyl or an *N*-glycolyl group (giving Neu5Ac or Neu5Gc, respectively). It was originally thought that unsubstituted glycosidically linked Neu did not occur in nature (17). However, there have been several reports suggesting its presence in gangliosides (5, 7, 18–21) and more recently in mucin-type glycoproteins (22, 23). Hakomori and colleagues (5) first defined de-*N*-acetyl-gangliosides by suggestive evidence for a Neu residue that was presumed to have arisen from de-*N*-acetylation of Neu5Ac. A possible role of such gangliosides in signaling was also suggested based on the finding that synthetic de-*N*-acetyl-G_{M3}² specifically enhanced epidermal growth factor receptor signaling when added to cells in culture, whereas the conventional *N*-acetylated G_{M3} had the opposite effect (5). However, proof for the natural occurrence of this monosialoganglioside was based upon the reactivity of a monoclonal antibody (mAb) DH5 that could recognize the synthetic molecule.

In related studies, our group used radiolabeling and pulse-chase techniques to indicate that ganglioside sialic acids were undergoing a de-*re*-*N*-acetylation cycle in cultured human melanoma cells (18). In collaboration with Tai's group (19), we also described monoclonal antibody SGR37 raised against synthetic

¹ The abbreviations used are: Neu, neuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac α 2Me, Neu5Ac α -methyl glycoside; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Pr, *N*-propionylneuraminic acid; Neu α 2Me, neuraminic acid α -methyl glycoside; Neu β 2Me, neuraminic acid β -methyl glycoside; CHO, Chinese hamster ovary; DMB, 1,2-diamino-4,5-methylene dioxybenzene; ELISA, enzyme-linked immunosorbent assay; HPTLC, high performance thin layer chromatography; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PLC, phospholipase C; Sia, sialic acid, type unspecified; SiaQ, sialic acid quinoxalinone (DMB adduct); ESI, electrospray ionization.

² Ganglioside nomenclature is based on the system of Svennerholm (41). G_{M3}, Neu5Ac α 2,3Gal β 1,4Glc β 1,1-ceramide; G_{D3}, Neu5Ac α 2,8Neu5Ac α 2,3Gal β 1,4Glc β 1,1-ceramide; G_{M1}, Gal β 1,3GalNAc β 1,4(Neu5Ac α 2,3)Gal β 1,4Glc β 1,1-ceramide.

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de-*N*-acetyl-G_{D3}, which was used to suggest that this disialo-ganglioside is specifically expressed in some human melanomas and lymphomas (20).

Despite all of these suggestive reports, no one has provided definitive structural proof for the natural existence of de-*N*-acetyl-gangliosides. Although Hidari *et al.* (21) provided ¹H NMR data defining traces of de-*N*-acetyl-G_{M1} in bovine brain gangliosides, these preparations had been subjected to alkaline saponification for phospholipid degradation under conditions that would have caused some chemical de-*N*-acetylation. Previous attempts to purify de-*N*-acetyl-gangliosides from natural sources have also been unsuccessful. Contaminating molecules in total lipid extracts, such as phospholipids and more abundant *N*-acylated gangliosides, can interfere with purification and detection by mAbs. Additionally, cell lines and cell line-based tumors have proven to be unreliable sources of de-*N*-acetyl-gangliosides because the expression level based on monoclonal antibody detection is low and variable. Furthermore, as reported here, at least one of these mAbs shows nonspecific cross-reactivity to a peptide.

In unrelated studies we have reported an acid-stable, mono-carboxylated modification of as yet unknown structure on the *N*-linked glycans of bovine lung (24, 25). In seeking to understand the nature of this carboxylated moiety, we considered the possibility that it might be a modified type of sialic acid. It is known that the glycosidic linkage of amino sugars like glucosamine is more stable to acid hydrolysis when the amino group is unsubstituted (26). Although sialic acids are generally among the most acid labile of glycosidically linked sugars, we considered the possibility that the glycosidic linkage of Neu with its unsubstituted amino group might be acid-resistant. As it turned out, the carboxylate in question was not part of a sialic acid, and further studies are currently under way to define its true nature. However, in the course of exploring this issue, we discovered that the glycosidic linkage of Neu is indeed quite resistant to acid. Employing this knowledge, we report here a new approach for detection of naturally occurring Neu in de-*N*-acetyl-gangliosides.

EXPERIMENTAL PROCEDURES

Materials—Hybridoma cells secreting mAbs SGR37 or SMR36 were prepared in collaboration with Dr. Tadashi Tai as previously described (19). Expired clinical grade mAb R24 (Celltech Ltd.) (27) was obtained from the National Cancer Institute; mAb DH2 (28) was kindly provided by Dr. Sen-itiroh Hakomori. All hybridomas were cultured in RPMI 1640, 10% heat-inactivated fetal calf serum, 1 ng/ml IL-6. B16 mouse melanoma cells were maintained in Dulbecco's modified Eagle's medium (regular glucose), 10% heat-inactivated fetal calf serum, and human U937 cells in RPMI 1640, 10% heat-inactivated fetal calf serum. Chinese hamster ovary (CHO) cells (K1 strain) were cultured in α -minimum essential medium, with 10% heat-inactivated fetal calf serum. Human tissue samples were kindly provided by Dr. Nissi Varki through the University of California, San Diego Cancer Center Histology Core Service. Neu5Ac α 2Me was purchased from Sigma, and Neu β 2Me was purchased from ICN Biomedicals. Unless otherwise stated, all other chemicals were of reagent grade or higher and purchased from commercial sources.

Ganglioside De-*N*-acetylation—Synthetic de-*N*-acetyl-gangliosides were prepared as described (19, 29, 30). Briefly, 1 mg of G_{M3} or G_{D3} (Matreya) was dissolved in 14.3 ml of 1 M tetramethylammonium hydroxide in butanol and heated to 100 °C in an oil bath for 3 h with constant stirring. After adding 175 ml of water, the solution was neutralized with 14.3 ml of 1 M acetic acid and dried on a rotary evaporator. Small volumes of water were added throughout to ensure complete evaporation of butanol. The de-*N*-acetyl-gangliosides were recovered after dialysis and lyophilization, dissolved in methanol, and stored at -20 °C. The extent of de-*N*-acetylation was determined by high performance thin layer chromatography (HPTLC) to be ~50%.

Immunohistochemistry of CHO Cells—CHO cells were plated on tissue culture slide chambers (LabTek) and grown to 70–90% confluence. The media was removed, and cells were washed once in PBS and

fixed in 2% paraformaldehyde in PBS for 30 min. Blocking was performed by incubating the slides in PBS containing 1% bovine serum albumin, and 3% goat serum for 30 min. Slides were incubated with primary antibody solutions for 30 min. Primary antibodies included R24 (5 μ g/ml final) and SGR37 (50% solution of hybridoma supernatant) and were prepared in the same solution that had been used for blocking. Slides were washed three times with PBS, and the horseradish-conjugated secondary antibody was added (goat-anti-mouse IgG-horseradish peroxidase; 1:50) in PBS. Slides were washed again in PBS 3 times and developed using the AEC (3-amino-9-ethylcarbazole) substrate system (Vector Labs). The substrate solution was removed, slides were washed twice, and nuclei were counterstained with hematoxylin. Images were captured using the 40 \times objective on a Zeiss Axioptot microscope fitted with a Sony DKC-5000 using NIH Image software.

Western Blot Analysis of CHO and Melur Cell Polypeptides—CHO and Melur cells were grown to 80% confluence in large culture dishes (15-cm diameter) and harvested using a cell scraper. 30 million cells were washed in PBS twice, and 300 μ l of reducing SDS-PAGE sample buffer was added. DNA was sheared by repeated pipetting using a 20-gauge needle. The samples were boiled at 100 °C for 5 min followed by a 5-min spin at 15,000 \times *g*. 10 μ l of the samples were used per lane on a 7.5% SDS-PAGE that was subsequently run at 100 V for approximately 1 h. The proteins in the gel were transferred to a polyvinylidene difluoride membrane at 50 V for 3 h. Scissors were used to cut the membrane for separation of each lane. Strips of polyvinylidene difluoride were rocked gently in either 2 mM sodium periodate in PBS for 30 min at 4 °C (mild periodate) or 25 mM sodium periodate in 0.1 M sodium acetate buffer, pH 5.0, for 30 min at room temperature (strong periodate) or left untreated. After washing the periodate-treated lanes in PBS, all strips were soaked in blocking buffer (PBS, 1% powdered milk) to block nonspecific binding sites. Primary antibodies R24 (10 μ g/ml) and SGR37 (50% hybridoma supernatant) were diluted in blocking buffer and incubated with the polyvinylidene difluoride strips for 1 h. The polyvinylidene difluoride strips were washed 3 times for 5 min and incubated with the alkaline phosphatase-conjugated secondary antibody (Goat-anti-mouse IgG-AP; 1:4000) in blocking buffer. After three washes, the blots were developed using the alkaline phosphatase-conjugate substrate system (Bio-Rad).

Enzyme-linked Immunosorbent Assay (ELISA)—Aliquots of gangliosides (600 ng/well) in methanol were added to wells of 96-well plates (NUNC) and allowed to dry overnight at room temperature. All washes and incubations were performed at room temperature in ELISA buffer (PBS, 1% bovine serum albumin). Nonspecific binding sites were blocked with ELISA buffer for 2 h followed by a 2-h primary antibody incubation. Primary antibodies used include DH2 (hybridoma supernatant) and SMR36 (ammonium sulfate precipitate in 50% saturated salt solution). mAbs were titrated to determine the optimal dilution. Wells were washed 3 times for 5 min each in ELISA buffer before a 1-h incubation with secondary antibody conjugated to horseradish peroxidase (1:4000) (goat-anti-mouse IgG DH2; goat-anti-mouse IgM for SMR36) (Bio-Rad). Wells were washed again 3 times for 5 min each and developed in citrate phosphate buffer, pH 5.0, containing 400 μ g/ml *o*-phenylenediamine and 0.12% hydrogen peroxide. Reactions were allowed to proceed for several minutes until a yellow color was visible and then quenched with the addition of 1/3 volume 9 M sulfuric acid. Absorbance was measured at 490 nm on a SpectraMax-250 96-well plate reader (Molecular Devices).

HPTLC—Samples in methanol were applied 1 cm above the bottom of an activated silica gel 60 glass-backed HPTLC plate (Merck) using a 1- μ l Hamilton syringe (4 μ g of ganglioside/lane; phospholipid extract originating from 2 \times 10⁶ B16 cells/lane). Plates were developed in glass TLC tanks pre-equilibrated with chloroform:methanol:water (65:25:4) for phospholipid separation or chloroform, methanol, 0.02% CaCl₂ (60:40:9) for separation of gangliosides. Phospholipids were visualized using phosphomolybdate spray reagent (Sigma) according to the manufacturer's directions. Individual gangliosides were visualized using the appropriate monoclonal antibody in an immuno-overlay assay.

Immuno-overlay—This procedure was performed as described elsewhere (19, 31). Briefly, HPTLC plates were allowed to air dry after development and then plasticized by immersing the plate for 1 min in hexane, 2% polyisobutylmethacrylate in chloroform (84:16). The plate was allowed to air dry, placed horizontally in a humidified chamber, covered with primary antibody in overlay buffer (PBS, 1% bovine serum albumin), and incubated at 4 °C overnight. Antibodies were used as described for ELISA. After primary antibody incubation, the surface of the plate was washed with overlay buffer 3 times for 5 min each and then covered with a 1:1000 dilution of secondary antibody. Secondary antibodies were identical to those used for ELISAs. The secondary

incubation was allowed to proceed at room temperature for 1 h. After three 5-min washes, the gangliosides were visualized using 400 $\mu\text{g/ml}$ *o*-phenylenediamine in citrate phosphate buffer, pH 5.0, with 0.12% hydrogen peroxide. Once bands were visible, plates were rinsed with water and dried with a blow drier.

Lipid Extraction—Tissue samples were sliced into small pieces with a scalpel and homogenized in 10 mM HEPES, pH 7.4, using a Polytron homogenizer (Brinkmann Instruments). Ten volumes of chloroform:methanol (1:1) were added, samples were sealed under nitrogen gas, and the extraction was allowed to proceed at room temperature for 12 h with gentle agitation. Protein precipitates were pelleted by centrifugation ($10,000 \times g$ for 15 min), supernatants were collected and stored at 4 °C under nitrogen, and the extraction was repeated using the same volume of chloroform:methanol (1:1). The organic extracts were pooled and dried under a stream of nitrogen.

Phospholipase Treatment—Dried lipids were resuspended in 50 mM potassium phosphate buffer, pH 7.4, using probe sonication and/or bath sonication. Phospholipase C (PLC) (Sigma; P-9439) was added at 5 milliunits/mg of tissue extracted and incubated with vigorous agitation at 37 °C in 3-h increments until degradation was complete (as determined by HPTLC, showing that excess PLC failed to further degrade a test aliquot of a given sample).

***N*-Acylation with Acyl Anhydrides**—Dry samples containing known or putative de-*N*-acetyl sialic acids were dissolved in saturated sodium bicarbonate and treated with 3.3% acetic anhydride or propionic anhydride for 15 min at room temperature. An identical mixture of sodium bicarbonate and the acyl anhydride was added 2 more times and allowed to react for 15 min each time. Samples were neutralized by adding an appropriate volume of 1 M HCl. Control samples contained amounts of acetic or propionic acids equivalent to the amounts of the anhydride used.

Analysis of Free Sialic Acids by 1,2-Diamino-4,5-methylene Dioxymethylene (DMB) Derivatization and HPLC Analysis—Samples containing native or chemically acylated sialic acids were incubated at 80 °C for 3 h in 2 M acetic acid. In some cases, samples were then passed over a cation exchange column (Dowex AG50W-X2, H⁺ form), and the resulting volatile acids were removed by lyophilization. Free sialic acids from some samples were finally bound to an anion exchange column (Dowex AG1-X8, formate form) (Bio-Rad), eluted with 1 M formic acid, and lyophilized to remove the acid. Aliquots of free sialic acids were derivatized in 8 mM DMB, 1.5 M acetic acid, 0.8 M β -mercaptoethanol, 14 mM sodium hydrosulfite for 2.5 h at 50 °C in the dark (32). DMB-derivatized sialic acids were resolved using a reverse phase C18 Microsorb-MV column (Varian, 4.6-mm internal diameter \times 25 cm, 5 μm) on a Rainin Dynamax SD-200 HPLC. Samples were eluted at a flow rate of 0.9 ml/min using either a 50-min isocratic elution in 8% acetonitrile, 7% methanol in water or a 70-min isocratic elution in 7% acetonitrile, 7% methanol in water. A Spectrovision FD-300 on-line fluorescence detector was used to visualize the sialic acid derivatives as they eluted (excitation at 373 nm, emission at 448 nm).

Mass Spectrometric Analysis of DMB Derivatives—DMB derivatives of sialic acids were validated by mass spectrometry. Fractions eluting from the C18 column were collected based on the elution position of known standards, dried down using a speed-vac and/or shaker-evaporator and stored in the dark. The fractions were reconstituted in water and run on a Finnigan MAT HPLC with online Mass Spectrometer model LCQ-Mass Spectrometer System. A Varian C18 column was used and eluted at 0.9 ml/min in the isocratic mode with 8% acetonitrile, 7% methanol, and 0.1% formic acid in water over 50 min. The eluant was simultaneously monitored by absorbance at 373 nm and by electrospray ionization (ESI) mass spectrometry. The following ESI settings were used. Capillary temperature was set at 210 °C, the capillary voltage was set at 31 V, and the lens offset voltage set at 0 V. The spectra were acquired by scanning from m/z 150–2000 in the positive-ion mode. Tandem mass spectrometry spectra were acquired by selecting the parent mass and using a 20% normalized collision energy. Data analysis was performed using the Xcalibur data analysis program from the instrument manufacturer.

Synthesis of Neu5Ac2Me—De-*N*-acetylation of Neu5Ac2Me was accomplished using hydrazine. Two micromoles of lyophilized Neu5Ac2Me were dissolved in 0.2–0.3 ml anhydrous hydrazine, capped tightly in a nitrogen atmosphere, and incubated at 100 °C for 6 h. To remove the hydrazine, the sample was brought to room temperature, uncapped, and placed in an evacuated chamber containing a beaker of concentrated sulfuric acid and allowed to sit overnight. After hydrazine evaporation, 0.2 ml of toluene was added to the sample twice and blown off under a stream of nitrogen. The sample was resuspended in 0.5 ml of water, and Neu5Ac2Me was separated from surviving

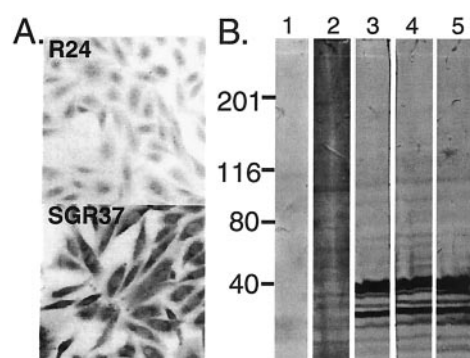


FIG. 1. Cross-reactivity of mAb SGR37 with polypeptide epitopes in CHO cells. Panel A, immunostaining of fixed CHO cells with mAbs R24 or SGR37. Note that the nuclei of all cells were visualized with the general stain hematoxylin. Panel B, Western blot of SDS-PAGE-separated polypeptides from CHO cells. Lanes were either untreated (lanes 1–3), treated with mild periodate (lane 4), or treated with strong periodate (lane 5) before incubation without primary antibody (lane 1), with R24 (lane 2), or with SGR37 (lanes 3–5).

Neu5Ac2Me by loading onto a 0.5-ml column of Dowex AG50W-X2 (H⁺ form) resin, washing with 2 ml of water, and eluting the de-*N*-acetylated material with 2 ml of 1 M HCl. The HCl was evaporated using a shaker-evaporator, and the sample was resuspended in water and stored at –20 °C.

NMR Spectroscopy—Solutions of methyl glycosides (~100 nmol) in D₂O (0.7 ml; 99.9% D; Aldrich) were transferred into 5-mm NMR tubes (Wilmad; 528PP), acidified with HCl in H₂O (a few μl) to the desired molarity (*i.e.* pH), and sealed. The pH of the stock solutions (without the glycosides) was measured using a Corning 240 pH meter. Time courses of glycoside hydrolysis were monitored discontinuously as follows. The tubes were placed in a water bath at 80 °C and removed for NMR analysis at specified time points. The time that samples spent at room temperature during NMR data collection or between 80 °C incubations was determined to be insignificant with regard to sample degradation. NMR experiments were carried out using a 500-MHz Varian Unity Inova spectrometer controlled by a SUN Microsystems Ultra-10 computer running Varian VNMR software (version 6.1B). ¹H NMR spectra were acquired at 27 °C in 512 transients each; the samples were not spun. The residual HDO signal was suppressed by low power presaturation. Data processing included line-broadening ($lb = 0.5$) and zero-filling (from 16 K to 32 K complex points) before Fourier transformation followed by base-line correction and integration. Chemical shifts (δ) are reported relative to TSP-*d*₄ (sodium 3-(trimethylsilyl)-propionate-2,2,3,3-*d*₄) (Aldrich); chemical shifts were actually measured using acetic acid (δ 2.081 ppm at pH < 4) or acetone (δ 2.217 ppm; pH-independent) as internal standards.

RESULTS AND DISCUSSION

A Monoclonal Antibody against a De-*N*-acetyl-ganglioside Shows Nonspecific Cross-reactivity with Peptide Epitopes—All prior reports of detection of de-*N*-acetylated gangliosides have used monoclonal antibodies. However, data using such antibodies cannot be considered definitive, since reactivity can be abrogated by interfering molecules (*e.g.* phospholipids).³ Additionally, antibodies directed against carbohydrates can sometimes show nonspecific cross-reactivity with other epitopes. Indeed, we have observed such cross-reactivity when staining CHO cells with mAb SGR37, which was thought to be specific for de-*N*-acetyl-G_{D3} (20). CHO cells do not express GD3, the most likely precursor of de-*N*-acetyl-G_{D3} (33). Despite this, the cytoplasm of CHO cells was highly positive when stained with mAb SGR37 (Fig. 1A). The lack of G_{D3} expression and the cytoplasmic localization of the SGR37 reactivity suggested that the antibody was cross-reacting with another epitope in the CHO cells. To further examine the nature of the CHO cell reactivity, we performed a Western blot on an SDS-PAGE of CHO cell extracts using anti-ganglioside mAbs. Although an-

³ R. Chammas and J. L. S., unpublished observations.

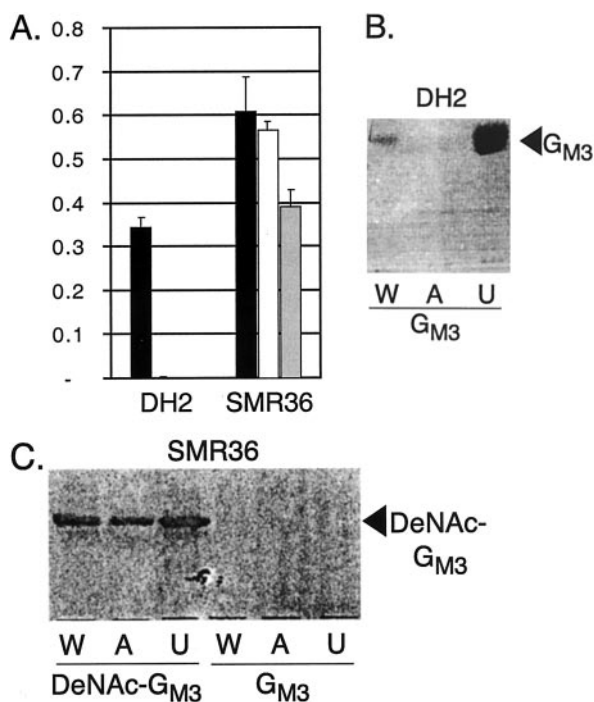


FIG. 2. Acid stability of neuraminic acid in de-*N*-acetyl- G_{M3} . Panel A, ELISA performed on a mixture of G_{M3} and de-*N*-acetyl- G_{M3} that was untreated (black bars) or incubated for 3 h at 80 °C in water (white bars) or for 3 h at 80 °C in 2 M acetic acid (gray bars) before application to ELISA plates. Monoclonal antibodies used are indicated. Panels B and C, HPTLC immuno-overlay of G_{M3} and de-*N*-acetyl- G_{M3} (DeNAc- G_{M3}) using monoclonal antibodies DH2 and SMR36, respectively. Gangliosides were incubated in water (W) or 2 M acetic acid (A) at 80 °C for 3 h or were untreated (U) before HPTLC plate application.

tibody R24 against G_{D3} did not bind to any of the CHO cell proteins, SGR37 reacted with two major bands (Fig. 1B, lane 3). Reactivity was not lost even after harsh periodate treatment (Fig. 1B, lane 5), indicating that the cross-reactive epitope is not even carbohydrate-dependent and likely represents polypeptide mimicry of the antibody epitope. The human melanoma cell line Melur, which has shown variable reactivity to SGR37, was subjected to the identical Western blotting procedure but did not show this cross-reactivity of SGR37 to proteins (data not shown). These observations of SGR37 cross-reactivity with a CHO cell polypeptide further emphasizes the need for definitive chemical evidence to prove that de-*N*-acetylgangliosides do exist in nature.

Neuraminic Acid in De-*N*-acetylated G_{M3} Is Resistant to Mild Acid Hydrolysis—In the course of studying a novel acid-resistant carboxylated structure of *N*-linked glycans of bovine lung (24, 25), we explored the possibility that it might be Neu. Although this hypothesis turned out to be incorrect, our studies led to novel findings regarding the acid stability of the Neu glycosidic linkage. Here, we use the mAbs SMR36 (19, 20) and DH2 (28), which recognize de-*N*-acetyl- G_{M3} and G_{M3} , respectively, to explore this question (antibody specificity is not an issue when using purified standards). A mixture of the monosialoganglioside G_{M3} (with a terminal Neu5Ac residue) and chemically de-*N*-acetylated G_{M3} (with a terminal Neu residue) was incubated at 80 °C for 3 h in either water or 2 M acetic acid (“mild acid”-treated). After evaporation of the solvent and re-suspension of the residue in methanol, aliquots of the solutions were spotted onto a 96-well plate in triplicate, and an ELISA assay was performed with the aforementioned monoclonal antibodies, as described under “Experimental Procedures.” As expected, the mild acid treatment, which is known to be sufficient to completely hydrolyze the acid-sensitive glycosidic link-

age of Neu5Ac (34), resulted in complete loss of DH2 reactivity (Fig. 2A). The lability of the glycosidic linkage of Neu5Ac is emphasized by the loss of DH2 reactivity even after heating in water; cleavage of the Neu5Ac α 2,3Gal linkage under these conditions is attributed to “auto-hydrolysis,” catalyzed by H⁺ ions from the Neu5Ac carboxyl group. In contrast, the SMR36 reactivity decreased only slightly upon mild acid treatment, indicating that the Neu α 2,3Gal glycosidic linkage was relatively resistant to acid hydrolysis under these conditions (Fig. 2A). This result is representative of several similar experiments in which the difference in SMR36 reactivity between control and acid-treated samples varied from no change to a 30% loss in signal. The discrepancy is likely due to variable ganglioside recovery after the mild acid treatment. A better quantitation of the relative acid stability of the glycosidic linkages of Neu5Ac and Neu is shown below using NMR on model compounds.

In a parallel experiment, gangliosides were spotted on a glass-backed silica gel HPTLC plate after the 3-h 80 °C incubation in acid or water. The plates were developed, and compounds were visualized by immuno-overlay. Again, de-*N*-acetyl- G_{M3} maintained SMR36 reactivity after mild acid treatment (Fig. 2C), whereas the mild acid-treated G_{M3} was not recognized by DH2 (Fig. 2B). As observed in the ELISA assay, even the incubation of G_{M3} at 80 °C in water resulted in significant Neu5Ac hydrolysis and reduction of DH2 reactivity. As an important control, we also noted that mild acid treatment of G_{M3} did not result in de-*N*-acetylation of the Neu5Ac, which would have been detected by the antibody SMR36 (Fig. 2C).

Neuraminic Acid α -Methyl Glycoside Shows Similar Glycosidic Linkage Stability under Acidic Conditions—Studying the acid hydrolysis of gangliosides is complicated by the fact that they form micelles in aqueous solution. To obtain more direct proof of the relative stability of the glycosidic linkage of neuraminic acid, we used ¹H NMR spectroscopy to analyze the acid hydrolysis of the α -methyl glycoside of Neu (Neu α 2Me, an analog of the sialic acid in de-*N*-acetyl- G_{M3}) at 80 °C as a function of time. One-dimensional ¹H NMR allowed us to monitor the disappearance of the 2-*O*-methyl group and the concomitant appearance of methanol, which would be produced upon hydrolysis of the glycosidic linkage (see Fig. 3A). Because of the interference that 2 M acetic acid produces in NMR, we initially used hydrochloric acid at 10 mM, which had a pH of 2.04 (2 M acetic acid, pH 2.08). The ¹H NMR spectrum of Neu α 2Me in 10 mM HCl in D₂O (Fig. 3A, bottom trace) showed the H-3ax and H-3eq signals known to be characteristic for sialic acids (35); their chemical shifts (see Table I) are in agreement with involvement in α -glycosidic linkage. The triplet observed at δ 3.265 was assigned to Neu H-5 (by two-dimensional ¹H NMR experiments; results not shown); the emergence of the H-5 signal from the envelope of other proton signals ($3.6 < \delta < 4.2$) is due to the presence of the free amino group at C-5. Finally, the signal for the 2Me group was observed at δ 3.376. Upon hydrolysis of Neu α 2Me, we observed a decrease in intensity of the 2Me signal, with a concomitant increase of a singlet at δ 3.340. The latter signal arises from methanol (CH₃OD), one of the products of Neu α 2Me hydrolysis. The intensity ratio of the 2Me and methanol signals in the ¹H spectra at various times of exposure to 80 °C was taken as a measure for extent of hydrolysis of the glycosidic linkage; the fraction of intact glycoside is plotted as a function of heating time in Fig. 4. During the course of hydrolysis, the other signals of Neu α 2Me (including the H-3 and H-5 signals) decreased in concert with the 2Me signal, whereas product signals appeared (including those at $4.2 < \delta < 4.5$, marked in Fig. 3A). In this regard classic studies reported that free Neu is unstable, undergoing a series of

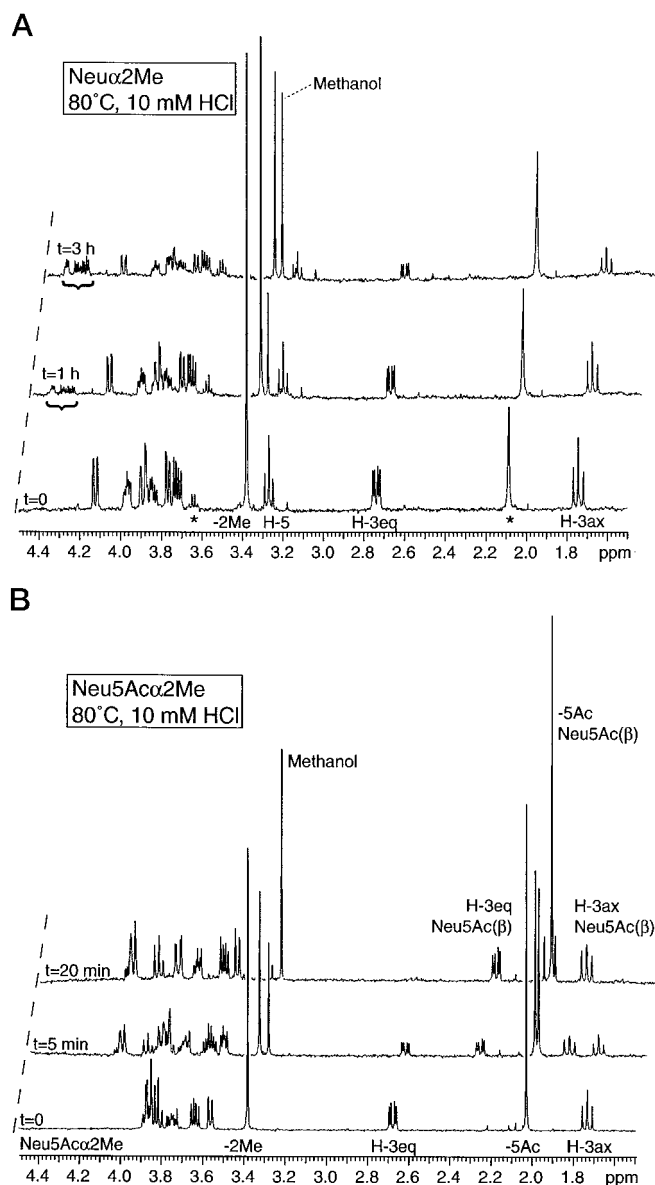


FIG. 3. NMR spectroscopic analysis of the stability of the glycosidic linkage of sialic acid α -methyl glycosides under acidic conditions. ^1H NMR spectra of Neu α 2Me (panel A) and Neu5Ac α 2Me (panel B) in 10 mM HCl were obtained before ($t = 0$), and after incubation at 80 °C for the time periods indicated. The Sia reporter groups and the signal for free methanol (produced by hydrolysis) are marked. Signals labeled by asterisks (panel A) are attributed to impurities in the Neu α 2Me sample. The horizontal brace (panel A) indicates new signals seen only after exposure to acid and heat.

intra-molecular degradation reactions in acidic conditions (17). We are currently pursuing the structural characterization of the product(s) by NMR spectroscopy and other methods.

For comparison, we monitored the hydrolysis of Neu5Ac α 2Me in 10 mM HCl at 80 °C (Fig. 3B). The progress of the conversion of the glycoside into methanol and free Neu5Ac was traced by the decrease in intensity of the substrate 2Me, H-3eq, 5Ac, and H-3ax signals and the concomitant appearance and increase in intensity of methanol and of the H-3ax, H-3eq, and 5Ac signals at δ 1.880, 2.312, and 2.046, characteristic for free Neu5Ac in the β -configuration (35). Actually, Neu5Ac free in aqueous solution occurs as a mixture of its β and α anomers in ratio 92:8; the minor intensity H-3 and 5Ac signals of the α anomer are indeed visible in the spectrum taken after 20 min (Fig. 3B, top trace); their chemical shifts have been included in

Table I. We found that the 2-*O*-methyl signal decreased by 47% in the Neu α 2Me sample that had been treated in 10 mM HCl for 3 h at 80 °C (Fig. 3A). In contrast to this relative resistance to mild acid hydrolysis, the *N*-acetylated analog Neu5Ac α 2Me was 95% hydrolyzed in 10 mM HCl at 80 °C after just 20 min (Fig. 3B). These data provide more solid evidence that the conversion of the acetamido group at C-5 of sialic acid to a free amino group confers stability to the glycosidic linkage under acidic conditions.

It has been well established that glycosidic linkages in the equatorial position of pyranoses are less stable than axial glycosidic linkages (36, 37). We decided to test if this result held true for neuraminic acid by comparing the α - and β -methyl glycosides (glycosidically bound sialic acid in aqueous solution adopts the $^2\text{C}_5$ conformation, where the α -glycosidic linkage is equatorial, and the β is axial). Subjecting both methyl glycosides to acid and heat revealed that the β -linked Neu was indeed hydrolyzed at a significantly slower rate than the α -linked Neu. In 0.1 M HCl, Neu β 2Me was 50% hydrolyzed after \sim 16 h at 80 °C (Fig. 4B), whereas Neu α 2Me was close to 50% hydrolyzed after 3 h at 80 °C in 10 mM HCl (Fig. 4A). Further studies are needed to identify the mechanism(s) responsible for this observed difference. Regardless, these experiments demonstrate that the α -glycosidic linkage of Neu is much more acid stable than that of α -glycosidically linked Neu5Ac.

Glycosidically Linked Neuraminic Acid Can Be Hydrolyzed and Detected after N-Acylation—To date, there has been no chemical proof of the presence of Neu in a ganglioside fraction that had not been previously subjected to alkaline hydrolysis (which can artifactually generate Neu from Neu5Ac). The primary reason for this lack of proof is that naturally occurring de-*N*-acetylated gangliosides are very minor components of complex mixtures of lipids. We therefore applied our new knowledge of their relative acid stability to develop a modified purification scheme. We reasoned that mild acid treatment would degrade gangliosides containing *N*-acylated neuraminic acid (and other acid-sensitive lipid species) in the biological lipid extracts, while leaving the de-*N*-acetyl-gangliosides relatively intact (and at the same time not artifactually generating them). The surviving de-*N*-acetyl-gangliosides could then be chemically *N*-acylated, rendering them sensitive to mild acid.

Amino sugars can be chemically *N*-acylated under the appropriate conditions with several acyl-anhydrides (19, 38, 39). To confirm that *N*-acylation of de-*N*-acetyl-gangliosides restores an acid labile sialic acid, we subjected mild acid pretreated de-*N*-acetyl- $\text{G}_{\text{M}3}$ to acylation with acetic anhydride or propionic anhydride using acetic acid or propionic acid as controls. Subsequently, the samples were re-subjected to mild acid hydrolysis, and the released sialic acids were derivatized with DMB. The fluorescent peaks corresponding to the DMB derivatives of the *N*-acylated sialic acids (sialic acid quinoxalinones, SiaQ) are clearly resolved using reverse-phase HPLC for separation (Fig. 5, note that the starting de-*N*-acetyl- $\text{G}_{\text{M}3}$ preparation contains a small amount of Neu5Ac that had survived the original de-*N*-acetylation reaction). The area under the peak corresponding to Neu5PrQ is within 7% of the area of the Neu5AcQ peak. This demonstrates that glycosidically bound Neu5Ac and Neu5Pr are very similar in their sensitivity to hydrolysis under mild acid conditions.

The molecular identity of the fluorescent adducts was confirmed by mass spectrometry (as described under "Experimental Procedures"; data not shown). Because Neu5Pr is not a naturally occurring form of sialic acid, we reasoned that *N*-propionylation could be used for tagging the free amino group of neuraminic acid on de-*N*-acetyl-gangliosides. The addition of

TABLE I
¹H chemical shifts of atoms used to monitor the hydrolysis of sialic acid methyl glycosides

Chemical shifts are reported at 27 °C relative to γTSP. At the pH values of the D₂O solutions used in this study (pH < 4), the methanol signal was found at δ 3.340. The methyl signal of acetic acid (*i.e.* free acetate in acidic solution) was found at δ 2.081, whereas the chemical shift of acetone was 2.217. ND, not determined.

Compound	pH	Chemical shift of				
		H-3ax	H-3eq	H-5	2Me	5Ac
Neu5Acα2Me	2.0	1.732	2.678	ND	3.382	2.030
Neu5Ac (β)	2.0	1.880	2.312	ND		2.046
Neu5Ac (α)	2.0	1.705	2.702	ND		2.029
Neuα2Me	2.0	1.738	2.734	3.265	3.376	
Neuβ2Me	1.0	1.839	2.436	3.351	3.310	

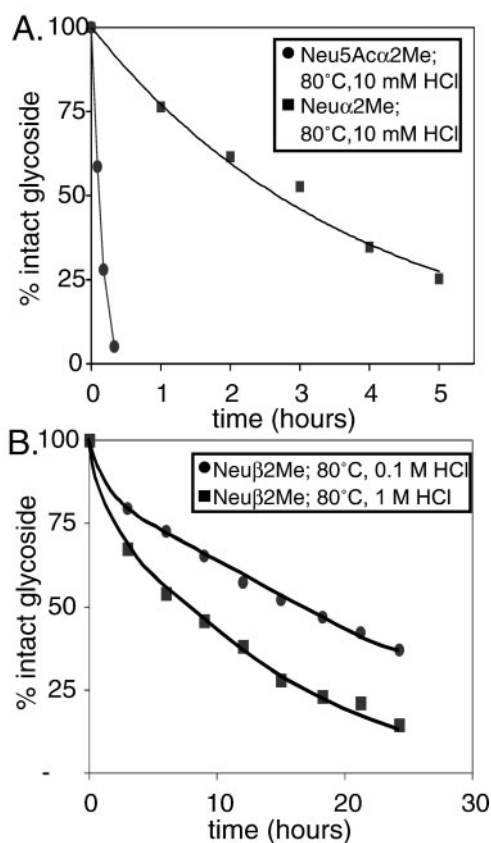


FIG. 4. Kinetics of the acid hydrolysis of the glycosidic linkage of sialic acid methyl glycosides. Panel A, time course of cleavage of the glycosidic linkage of Neu5Acα2Me (circles) and Neuα2Me (squares) in 10 mM HCl at 80 °C. Panel B, time course of glycosidic linkage hydrolysis of Neuβ2Me in 0.1 M HCl (circles) and in 1 M HCl (squares). The degree of intactness of the methyl glycoside at a given time was derived from the intensity ratio of the 2-*O*-methyl and methanol signals in the corresponding ¹H NMR spectra (*cf.* Fig. 2).

the *N*-propionyl group not only allows subsequent mild acid hydrolysis of the sialic acids but also gives a product of unique molecular weight and HPLC retention time that can be easily differentiated from any endogenous *N*-acylated sialic acids in biological samples that survive the initial hydrolysis.

Phospholipase C Can Be Used to Eliminate Phospholipids from Total Lipid Extracts of Biological Origin—Another formidable challenge in the identification of de-*N*-acetyl-gangliosides has been the physical dominance of contaminating phospholipids in total lipid extracts. We have previously found that phospholipids interfere with de-*N*-acetyl-ganglioside migration on HPTLC plates, with their elution from DEAE-HPLC columns, and can block their recognition by monoclonal antibodies.³ On the other hand, we found that the typical phospholipid saponification procedures used in ganglioside purification protocols will cause some chemical de-*N*-acetylation (data not shown).

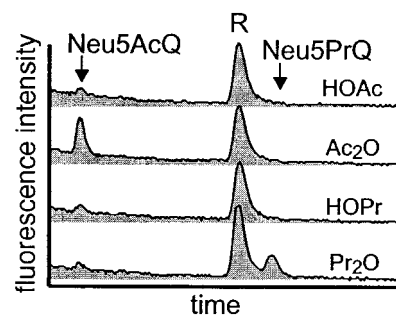


FIG. 5. HPLC analysis of sialic acids derived from *N*-acylation of de-*N*-acetyl-G_{M3}. De-*N*-acetyl-G_{M3} (80 ng) was treated with acetic acid (HOAc), acetic anhydride (Ac₂O), propionic acid (HOPr), or propionic anhydride (Pr₂O) before mild acid hydrolysis, DMB derivatization of released sialic acids, and C18 HPLC analysis with detection of the fluorescent adducts. Peaks corresponding to DMB-derivatized Neu5Ac (Neu5AcQ) and Neu5Pr (Neu5PrQ) are indicated with arrows. R (reagent) indicates a fluorescent peak that forms independently of sialic acids.

Alternative methods utilizing two-phase solvent partitioning also pose a problem because of unpredictable behavior of de-*N*-acetyl-gangliosides due to the interactions with phospholipids.

To bypass these problems, we have developed an alternative approach to degrade phospholipids enzymatically using a broad-spectrum PLC, which also has activity against sphingomyelin (40). An example of the degradation is shown in Fig. 6. Total lipid extracts from B16 mouse melanoma tumors were treated with PLC from *Bacillus cereus*, which results in significant but not total degradation of phospholipids (Fig. 6, lane P). Phospholipid degradation was enhanced by the addition of mild acid treatment (Fig. 6, lane P/A).

To further examine the validity of this approach, an aliquot of synthetic de-*N*-acetyl-G_{D3} was spiked into a total lipid extract from human kidney, and the extract was subjected to PLC treatment and mild acid hydrolysis. Phospholipid head groups and hydrolyzed *N*-acylated sialic acids were then eliminated using a Microcon spin filter (3000 Da molecular mass cut-off). The retentate was then recovered and treated with propionic anhydride to *N*-propionylate the surviving de-*N*-acetyl sialic acids. The newly *N*-propionylated sialic acids were now susceptible to mild acid cleavage and could be isolated in the flow through after spin filtration. After DMB derivatization, the SiaQ were resolved by C18-HPLC with fluorescence detection and the NeuPr adduct detected (data not shown). A human kidney extract that was not spiked with synthetic de-*N*-acetyl-G_{D3} was subjected to the same purification procedure in parallel with the spiked sample. The Neu5Pr adduct could not be detected in this negative control. This indicates that Neu was not artifactually generated during the course of the purification.

Naturally Occurring Neuraminic Acid Can Be Identified in a Mild Acid and Phospholipase C-treated Human Melanoma Lipid Extract—We next applied this new approach to search for

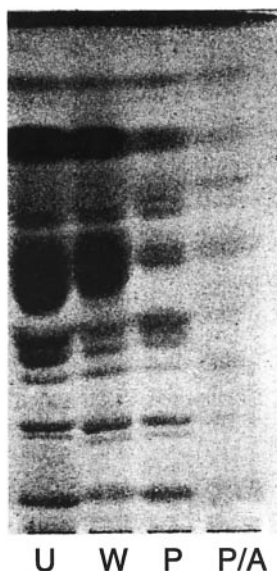


FIG. 6. **Phospholipase and acid degradation of lipids in biological extracts.** HPTLC of total lipid extracts from B16 mouse melanoma cells that were untreated (*U*), incubated for 3 h at 80 °C in water (*W*), treated with PLC (100 milliunits) for 3 h at 37 °C (*P*), or treated with PLC and then incubated for 3 h at 80 °C in 2 M acetic acid (*P/A*). Phospholipids were visualized using the phosphomolybdic acid spray reagent.

de-*N*-acetyl-gangliosides in several tissues and cell lines. These included 25 different normal mouse tissue samples, human lung (0.2 g) and kidney (0.5 g), a tumor of B16 mouse melanoma cells (0.5 g) grown in nude mice (B16 cells have been reported to contain de-*N*-acetyl- G_{M3}) (5), U937 cells (0.2 g) (reportedly containing cyclic sialic acid on glycoproteins, for which Neu is the proposed precursor) (22), and three primary human melanomas (0.2, 0.6, and 2.4 g) that were positive to varying degrees for SGR37 reactivity.⁴ In our hands, the three human tumors were the only samples for which we had observed positive staining using the anti-de-*N*-acetyl-ganglioside antibodies.

Of the 32 samples subjected to our purification scheme, one human melanoma sample gave a fluorescent HPLC peak that co-eluted with the DMB-derivatized Neu5Pr (Neu5PrQ) standard and was not present in the propionic acid-treated control sample (Fig. 7A). Notably, this tumor was the largest (2.4 g) and stained most intensely with SGR37 (data not shown), which suggested that it would be the richest source of de-*N*-acetyl-gangliosides. Fig. 7A shows two traces, each representing 5% of tumor lipid extract. A significant amount of Neu5Ac appeared to be retained in the sample despite the acid treatment and spin filtration. This may be because of the known difficulty of taking the acid hydrolysis reaction to completion in gangliosides (41). The fluorescent peak corresponding to Neu5PrQ from human melanoma TB365 was subsequently isolated by subjecting 85% of the extract to the purification procedure, with some minor changes to accommodate the increase in lipid mass. Spin filtration units had a tendency to clog easily, so dialysis was used to eliminate *N*-acylated Sias after the first acid hydrolysis. Additionally, the second spin filtration was replaced with anion and cation exchange as described under "Experimental Procedures" to specifically isolate negatively charged molecules that had been acid-released after propionic anhydride treatment.

HPLC conditions were also altered to achieve better separation of Neu5PrQ from other sialic acid adducts. Decreasing the acetonitrile concentration in the mobile phase from 8 to 7%

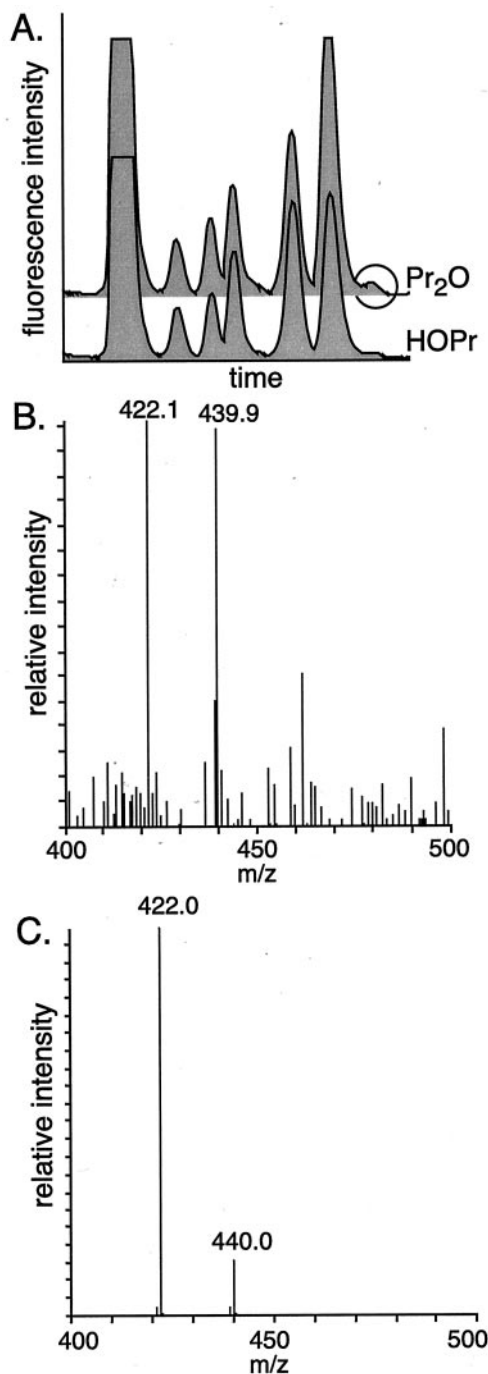


FIG. 7. **Detection of Neu5Pr in a propionic anhydride-treated lipid extract from a human melanoma tumor.** A total lipid extract from melanoma tumor TB365 was subjected to sequential PLC and mild acid treatment as described under "Experimental Procedures," dialyzed, and then subjected to *N*-propionylation and sialic acid analysis. *Panel A*, C18 HPLC elution profile of DMB adducts formed after treatment with propionic anhydride (Pr_2O) or propionic acid ($HOPr$) and mild acid release. The peak corresponding to Neu5PrQ is indicated with a circle. *Panel B*, ESI ion-trap mass spectrum of the 34–39 min fraction of the propionic anhydride treated TB365 sample. *Panel C*, mass spectrometry profile after secondary collision-induced dissociation of the m/z 440 ion in *panel B*.

resulted in increased retention time of Neu5PrQ (from ~22 to ~35 min) and improved resolution of individual fluorescent peaks (data not shown). The appropriate fraction (34–39 min) was collected as it eluted from C18-HPLC and re-run on the HPLC. This second run was necessary because the prevalence of other sialic acid adducts in the sample caused some HPLC

⁴ Nissi Varki, unpublished observations.

column overloading. A fluorescent peak corresponding to Neu5PrQ elution was clearly seen in the second C18-HPLC run of the TB365 sample (data not shown). This peak was collected and run a third time on a C18-HPLC column that feeds directly into the ion trap mass spectrometer under the original mobile phase conditions (8% acetonitrile, 7% methanol). A molecule co-eluting with the authentic Neu5Pr standard and having a mass of 440 Da was detected (Fig. 7B). The identity of the molecule was further confirmed by collision-induced dissociation, which resulted in conversion to 422 Da (Fig. 7C). The loss of 18 mass units is typical of dehydration seen in sialic acid quinoxalinones subjected to such secondary bombardment (42). This represents the first chemical proof for the presence of native Neu in a ganglioside fraction. Our inability to detect Neu5Pr in many other samples processed in parallel to the positive sample indicates that Neu is not being artifactually generated over the course of our purification and work-up procedures but, rather, occurs endogenously in detectable amounts only in this human melanoma. It could still be argued that artifactual chemical generation of Neu is only detectable in samples with the highest amount of sialic acids, such as the positive melanoma. However, the lack of detectable Neu in mouse brain serves as proof against this. Although the Neu5Pr-positive melanoma sample was somewhat larger in mass than the Neu5Pr negative mouse brain, the sialic acid content of the lipid fraction of these samples is similar (only a 1.5-fold difference). Therefore, if Neu5Pr in the melanoma arose from some unexpected artifact of chemical de-*N*-acetylation occurring during the purification of sialic acid rich samples, it would also have been detected in the mouse brain.

Very recently, another group studying heptafluorobutyrate derivatives of acid-released sialic acids provided the first evidence of naturally occurring Neu on a glycoprotein, ovine submaxillary mucin (23). However, this study used traditional acid hydrolysis conditions for release and, thus, may have underestimated the amount of Neu present. In our current studies, we compared the overall amount of Neu5PrQ detected to the amount of Neu5AcQ by assuming that the two forms of sialic acid behave similarly with regard to efficiency of extraction, extent of acid hydrolysis after *N*-acylation of Neu, efficiency of DMB derivatization, and recovery on HPLC. Efficiency of *N*-acylation reactions of Neu were also assumed to be similar to those achieved for our standards (39%). Using these assumptions, we calculate that Neu is about 0.06% of the Neu5Ac content in the total lipid fraction of this human melanoma. Based on these calculations, we concluded that purification of the intact ganglioside from this mixture using current methodology would be extremely difficult. Our approach for purifying the soluble sugar has the advantage of selectively isolating small molecules that resist acid hydrolysis but can be released by mild acid after treatment with propionic anhydride. This specific set of criteria greatly minimizes other contaminants in the preparation. Isolating and characterizing an intact de-*N*-acetyl-ganglioside or its *N*-propionylated form from biological samples will require further technological innovations.

Conclusions and Perspectives—Here we have presented a cautionary note regarding nonspecific cross-reactivity of a monoclonal antibody thought to be specific for de-*N*-acetyl-gangliosides, a novel finding regarding the acid stability of the glycosidic linkage of neuraminic acid, and a further difference in this stability when comparing the α - and β -methyl glycosides of the sugar. We have also used phospholipase C in combination with mild acid treatment to eliminate contaminating lipid species and definitively prove the existence of de-*N*-acetyl-gangliosides. We obtained definitive evidence for the existence of Neu by the following criteria: (a) initial resistance to mild

acid; (b) mild acid release after *N*-propionylation; (c) DMB derivatization (specific for α -keto acids); (d) HPLC fractionation (with collection of fractions at the elution time specific for the Neu5Pr product); (e) mass spectrometric proof for the Neu5Pr product; (f) secondary mass spectrometric proof for the Neu5Pr product; and (g) the absence of Neu5Pr in the propionic acid treated control. The approaches presented in this paper should be useful in future studies that seek proof of naturally occurring neuraminic acid. Additionally, using PLC as a purification tool for PL degradation will aid in the study of alkali labile *O*-acetyl groups known to exist on some gangliosides in lipid extracts.

Despite these advances, the low level of expression of de-*N*-acetyl-gangliosides pose daunting technical problems in their purification and characterization. However, this low level does not argue against their biological importance. The *in vitro* effects of adding synthetic de-*N*-acetyl-gangliosides have been impressive and appear to be specific (5–7). There is also accumulating evidence for the transient organization of gangliosides into “glycosphingolipid-enriched microdomains” (GEMs) (43–45) or into glycosylphosphatidylinositol-enriched “rafts” (46, 47) within the plasma membrane. A recent report suggests that such microdomain formation can correspond to a decreased threshold for signaling in T-cells (48). Such a system would allow potentially potent activators of signaling to exist in the membrane in an inactive state of disorganization. However, upon raft assembly, local concentrations of trace molecules like de-*N*-acetyl-gangliosides could be elevated to effectual levels.

Exploration of such possibilities would be greatly aided by the identification and cloning of the putative enzyme(s) responsible for the de-*N*-acetylation. In preliminary studies, we have been unable to detect such an activity in extracts from tissues and cells using a synthetic ganglioside analog. It is possible that the specific substrate we are using is not recognized by the enzyme or that other co-factors are needed. An alternative approach for isolating the de-*N*-acetylase would be expression cloning. De-*N*-acetyl-ganglioside-specific monoclonal antibodies are available, and most cells express the precursor *N*-acetylated gangliosides abundantly. Overall, de-*N*-acetyl-gangliosides remain a challenging but potentially important class of molecules for further study.

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