Characterization of the Acid Stability of Glycosidically Linked Neuraminic Acid

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

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-acetylated 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 α2-3-linked Neu on chemically de-N-acetylated GM3 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 Neu2Me is hydrolyzed after 3 h in 10 mM HCl at 80 °C, whereas Neu5Ac2Me is 95% hydrolyzed after 20 min under the same conditions. Notably, Neuß2Me is hydrolyzed even slower than Neu2Me, 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) structure and are N-acetylated 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-GM3 specifically enhanced epidermal growth factor receptor signaling when added to cells in culture, whereas the conventional N-acetylated GM3 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

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1 The abbreviations used are: Neu, neuraminic acid; Neu5Ac, N-acetyleneuraminic acid; Neu5Ac2Me, Neu5Ac α-methyl glycoside; Neu5Gc, N-glycolyleneuraminic acid; Neu5Pr, N-propionylneuraminic acid; Neu2Me, neuraminic acid α-methyl glycoside; Neu2Me2, 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). GM1, glycosylphosphatidylinositol glycolipid 1; G2a, Neu5AcO2,3Galβ1,4Glcβ1,1-ceramide; G0, Neu5AcO2,8Neu5AcO2,3Galβ1,4Glcβ1,1-ceramide; Galβ1,3GalNAcβ1,4Neu5AcO2,3Galβ1,4Glcβ1,1-ceramide.
Acid Stability of Neu

17503

de-N-acetyl-G_{C37}, 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 \(^1\)H NMR data defining traces of de-N-acetyl-G_{C37} 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 SR37 or SM36 were prepared in collaboration with Dr. Tadashi Tai as previously described (19). Expired clinical grade mAb R24 (Celltech Ltd.) (27) was obtained 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-minimun 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. Neu5Ac2enMe2 was purchased from Sigma, and Neu82Me2 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_{C22} or G_{C32} (Matreya) was dissolved in 14.3 ml of 1 x 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 x acetic acid and dried on a rotary evaporator. Small volumes of water were added throughout to ensure complete evaporation of butanol. The de-N-acetylated 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 70–80% confluence. The media was removed, and cells were washed once in PBS and fixed in 2% parafomaldehyde 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 μg/ml final) and SR37 (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-aminio-9-ethylcarbazole) substrate system (Vector Labs). The substrate solution was removed, slides were briefly rinsed with PBS, and hematoxylin was added. Images were captured using the 400× objective on a Zeiss Axioskop microscope fitted with a Sony DYC-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 μl 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 × g. 10 μl 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 to fit the wells. Strips of polyvinylidene difluoride were rocked gently in either 2 × 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 μg/ml) and SR37 (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 included DH2 (hybridoma supernatant) and SM36 (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 SM36) (Bio-Rad). Wells were washed again 3 times for 5 min each and developed with citrate-phosphate buffer, pH 5.0, containing 400 μg/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 N 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-μl Hamilton syringe (4 μg of ganglioside/lane; phospholipid extract originating from 2 × 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 phosphomolybdic acid spray reagent (Sigma) according to the manufacturer’s directions. Individual gangliosides were visualized using the appropriate monoclonal antibody in an immuno-overlay assay.

Immunooverlay—This procedure was performed as described elsewhere (19, 31). Briefly, HPTLC plates were allowed to air dry after development and then rehydrated by immersing the plate for 1 min in hexane, 2% polysorbate methacylate in chloroform (84:16). The plate was then covered 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 μg/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 (100 × 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).

Analysis of Free Sialic Acids by 1,2-Diamino-4,5-methylene Dioxybenzene (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 × 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 Spectroflow 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 Neo2Me—De-N-acetylation of Neu5Ac2Me was accomplished using hydrazine. Two micromoles of lyophilized Neu5Ac2Me were dissolved in 0.2–0.3 ml anhydrous acetic acid, 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 tolune 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 Neo2Me was separated from surviving...
Acid Stability of Neu

Acid Stability of neuraminic acid in de-N-acetyl-GM₃
Panel A, ELISA performed on a mixture of G₃₄₃ and de-N-acetyl-GM₃ 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 G₃₄₃ and de-N-acetyl-GM₃ (DeNAc-GM₃) 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.

Acid Stability of Neuraminic Acid

Neuraminic Acid in de-N-acetylated G₃₄₃ Is Resistant to Mild Acid Hydrolysis—In the course of studying a novel acid-resistant glycoproteins with the help of monoclonal antibodies, we have discovered that de-N-acetylated G₃₄₃ is resistant to mild acid hydrolysis. This was confirmed by the lack of reactivity of the de-N-acetylated G₃₄₃ with a monoclonal antibody against Neu5Ac (SMR36) after incubation in 2 M acetic acid at 80 °C for 3 h. The absence of reactivity was confirmed by HPTLC immuno-overlay analysis.

Acid Stability under Acidic Conditions

Acid Stability of Neu5Ac and Neu5AcO in de-N-acetylated G₃₄₃
Upon hydrolysis of Neu5Ac in de-N-acetylated G₃₄₃ (DeNAc-GM₃) with a terminal Neu5Ac residue in 2 M acetic acid, the acid-sensitive glycosidic linkage of Neu5Ac was hydrolyzed, leading to the formation of Neu5AcO and Neu5Ac(OH)₂. The intensity ratio of Neu5AcO to Neu5Ac was measured as a function of time. The results showed that Neu5AcO was formed at a faster rate than Neu5Ac, indicating that Neu5AcO is more resistant to acid hydrolysis than Neu5Ac.

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 (Neu2Me), an analog of the sialic acid in de-N-acetylated G₃₄₃ 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 Neu2Me 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 1) 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 < δ < 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 3.4. After incubation in 2 M acetic acid at 80 °C for 3 h, the signal for the 2Me group decreased in intensity, indicating that Neu2Me is resistant to mild acid hydrolysis.

Acid Stability of Neu5Ac and Neu5AcO in de-N-acetylated G₃₄₃
Upon hydrolysis of Neu5Ac in de-N-acetylated G₃₄₃, the acid-sensitive glycosidic linkage of Neu5Ac was hydrolyzed, leading to the formation of Neu5AcO and Neu5Ac(OH)₂. The intensity ratio of Neu5AcO to Neu5Ac was measured as a function of time. The results showed that Neu5AcO was formed at a faster rate than Neu5Ac, indicating that Neu5AcO is more resistant to acid hydrolysis than Neu5Ac. 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-GM₃ maintained SMR36 reactivity after mild acid treatment (Fig. 2C), whereas the mild acid-treated G₃₄₃ was not recognized by DH2 (Fig. 2B). As observed in the ELISA assay, even the incubation of G₃₄₃ 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 G₃₄₃ did not result in de-N-acetylation of the Neu5Ac, which would have been detected by the antibody SMR36 (Fig. 2C).
Acid Stability of Neu

We are currently pursuing the structural characterization of intra-molecular degradation reactions in acidic conditions (17). 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 1. We found that the 2-O-methyl signal decreased by 47% in the Neu2Me 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 Neu5Ac2Me 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) and O-linked Neu. In 0.1 M HCl, Neuβ2Me was 50% hydrolyzed after ~16 h at 80 °C (Fig. 4B), whereas Neuα2Me 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-Acetylation—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-acetylated neuraminic acid (and other acid-sensitive lipid species) in the biological lipid extracts, while leaving the de-N-acetylated-gangliosides relatively intact (and at the same time not artifactually generating them). The surviving de-N-acetylated-gangliosides could then be chemically N-acetylated, rendering them sensitive to mild acid.

Amino sugars can be chemically N-acetylated under the appropriate conditions with several acyl-anhydrides (19, 38, 39). To confirm that N-acetylation of de-N-acetyl-gangliosides restores an acid labile sialic acid, we subjected mild acid pretreated de-N-acetyl-G_{M_3} to acylation with acetic anhydride or propionic anhydride using acetic acid or propionic acid as controls. Subsequently, the samples were re-submitted to mild acid hydrolysis, and the released sialic acids were derivatized with DMB. The fluorescent peaks corresponding to the DMB derivatives of the N-acetylated sialic acids (sialic acid quinoxalinones, SiaQ) are clearly resolved using reverse-phase HPLC for separation (Fig. 5, note that the starting de-N-acetyl-G_{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

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**Table 1.**

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<tr>
<th>Condition</th>
<th>Neu2Me</th>
<th>Neu5Ac2Me</th>
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<tr>
<td>10 mM HCl, 3 h</td>
<td>95%</td>
<td>47%</td>
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<tr>
<td>10 mM HCl, 20 min</td>
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Fig. 2. NMR spectroscopic analysis of the stability of the glycosidic linkage of sialic acid α-methyl glycosides under acidic conditions. 

**A**

![1H NMR spectra of Neu2Me (panel A) and Neu5Ac2Me (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 Neu2Me sample. The horizontal brace (panel A) indicates new signals seen only after exposure to acid and heat.](image)

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**B**

![1H NMR spectra of Neu5Ac2Me at 80 °C, 10 mM HCl.](image)
Acid Stability of Neu

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$^1$H chemical shifts of atoms used to monitor the hydrolysis of sialic acid methyl glycosides

Chemical shifts are reported at 27 °C relative to TSP. At the pH values of the D$_2$O 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.

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<th>Compound</th>
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<td>1.0</td>
<td>1.839</td>
<td>2.436</td>
<td>3.351</td>
<td>3.310</td>
<td></td>
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![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 Neu5AcO2Me (circles) and Neu2Me (squares) in 10 mM HCl at 80 °C. Panel B, time course of glycosidic linkage hydrolysis of Neu2Me 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).](image)

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-acetylated 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. 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).

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 PA).

To further examine the validity of this approach, an aliquot of synthetic de-N-acetyl-G$_{33}$ 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-acetylated 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 Neu5Q were resolved by C18 HPLC analysis with fluorescence detection 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.

![Fig. 5. HPLC analysis of sialic acids derived from N-acylation of de-N-acetyl-G$_{33}$. De-N-acetyl-G$_{33}$ (80 ng) was treated with acetic acid (HOAc), acetic anhydride (Ac$_2$O), propionic acid (HOPr), or propionic anhydride (Pr$_2$O) 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.](image)
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-GM3) (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. 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% 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
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 Neu in the melanoma with mild acid treatment to eliminate contaminating lipid. We have also used phospholipase C in combination with the ion trap mass spectrometer under the original mobile phase conditions (8% acetonitrile, 7% methanol). A molecule of mass of 440 Da was detected (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 Neu in the melanoma with mild acid treatment to eliminate contaminating lipid.

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 derivative of such a fraction would be extremely difficult. Our approach for 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. Therefore, if Neu5Pr in the melanoma arose from some endogenous biosynthetic route, 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-acetylation of Neu, efficiency of DMB derivatization, and recovery on HPLC. Efficiency of N-acetylation 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-methylene 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 glycosphosphatidylinositol-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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REFERENCES
Acid Stability of Neu


