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SERUM GANGLIOSIDES: ANALYTICAL METHODS AND ANALYSIS OF SERA FROM BREAST CANCER PATIENTS

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SERUM GANGLIOSIDES: ANALYTICAL METHODS AND ANALYSIS OF SERA FROM BREAST CANCER PATIENTS

By

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ABSTRACT

SERUM GANGLIOSIDES: ANALYTICAL METHODS AND ANALYSIS OF SERA FROM BREAST CANCER PATIENTS

By

Douglas Andrew Wiesner

Gangliosides are sialic acid-containing glycosphingolipids that are ubiquitous to the mammalian plasma membrane. Alterations in gangliosides in the tissue, and in some cases, the sera, due to aberrant glycosylation, have been associated with oncogenesis. These changes in the ganglioside profiles may be useful in the clinical diagnosis, prognosis and monitoring of the disease. In this study, we examined the profiles of sera from breast cancer patients and an appropriate control group of female to determine the nature of these profiles.

In order to adequately analyze the ganglioside profiles, new protocols for the isolation and detection of gangliosides needed to be developed. I found that modification of a three phase partition system was useful the isolation of gangliosides from as little as 1 ml of sera. For the analysis of the ganglioside mixtures, a system of 2-dimensional high-performance thin-layer chromatography and densitometry with an image analyzer was well suited for our analytical needs. A derivitization procedure for the analysis of components of gangliosides by gas chromatography was developed for component analysis of the ganglioside mixtures.

The gangliosides of sera of 19 women with breast cancer and 16 women of an appropriate control group was analyzed by two-dimensional high-performance thin-layer chromatography gas chromatography. We found a significant increase ($P < .05$)

in the level of the total circulating gangliosides of the cancer sera over that of control. In addition, we noted an increase in the level of complex (highly glycosylated) gangliosides as well as the ratio of b-series gangliosides over the a-series gangliosides in the sera of breast cancer patients. These results suggest a change in the biosynthetic pathways of gangliosides associated with cancer. There was no alteration in a specific ganglioside that could be classified as a tumor-associated antigen. Free sialic acid and the activity of sialidase determined in the sera or plasma of both groups to determine any possible contribution either might have in breast cancer. The free sialic acid levels were found to be unchanged when examined with a modified thiobarbituric acid assay. No sialidase activity was detected in either group when assayed with a coupled enzyme assay.

To my parents, who taught me to work hard to achieve my goals,

yet also to enjoy life.

To my fiancée, Alexandra, for reminding me everyday.

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LIST OF ABBREVIATIONS

Gangliosides abreviations, nomenclature, and structure - See Table 1, Chapter 1

Da - Daltons

Globo - Galα1-4Galβ1-4Glcβ1-1Cer, Ganglio - GalNAcβ1-4Galβ1-4Glcβ1-1Cer, Lacto - Galα1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer, Neolacto - Galα1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

DEAE - Diethylaminoethyl, HPLC - High-Performance Liquid Chromatography, HPAE - High pH Anion-Exchange, PAD - Pulsed Amperometric Detection, HPTLC - High-Performance Thin-Layer Chromatography, GC - Gas Chromatography, MS - Mass Spectometry, FAB - Fast Atom Bombardment, CID - Collision-Induced Dissociation, EIMS - Electron-Impact Mass Spectrometry, NMR - Nuclear Magnetic Resonance

TMS - Trimethylsilyl, DMSO - Dimethyl sulfoxide

NGF - Nerve Growth Factor, PDGF - Platelet-Derived Growth Factor, EGF - Epidermal Growth Factor

CM - Chloroform-Methanol, DIPE/ButOH - Diisopropyl ether/n-butanol

1- , 2-D - One-, Two-Dimensional, aq. - Aqueous

GSL - Glycosphingolipid, FAME - Fatty Acid Methyl Ester, HDA - Methyl Heptadecanoate, Glc - Glucose, Gal - Galactose, GalNAc - N-Acetylgalactosamin, GlcNAc - N-Acetylglucosamin, Fuc - Fucose, LCB - Long-Chain Base, NeuAc - N-Acetylneuraminic Acid and Sialic Acid, Cer - Ceramide

LBSA - Lipid-Bound Sialic Acid

ABBREVIATIONS (Cont'd)

BSA - Bovine Serum Albumen , PBS - Phosphate Buffer Solution, HRP-B - Horseradish Peroxidase conjugated to Cholera Toxin B-Subunit, 4-MU-NANA - 4-Methylumbelliferyl-α-D-*N*-Acetylneuraminate

EIA - Enzyme-linked Imunoadsorbance

TBA - Thiobarbituric Acid1

$A_{280,490}$ - Absorbance at 280 and 490 nanometers, respectively

CHAPTER 1

Literature Review

Introduction

Breast cancer is by far the leading cause of cancer among women and the second most prevalent among all cancers after lung cancer. Women have a one-in-eight chance of developing breast cancer over their lifetime. Although the mortality rate of breast cancer has remained stable over many decades at 27 deaths per 100,000 women, the incidence rate has shown an annual 1% increase over the last century (1). Survival after five years is close to 90% for those women with cancers caught at an early stage when the cancer remains localized in the breast tissue (1). It becomes obvious that early diagnosis and accurate staging of the tumor is key to a good prognosis. Much research is devoted to finding new and better ways to characterize the disease such that earlier diagnosis is possible and better prognosis follows. Moreover, many breast cancers elude monitoring strategies, making their prognosis difficult. New methods of monitoring these elusive types of cancers as well as better means of monitoring the more detectable cancers is an ongoing struggle in the battle against breast cancer.

A recent trend in the fight against all kinds of cancers is the analysis of glycosphingolipids associated with the cancers. Oncogenic transformation has long been associated with an alteration in the composition of glycosphingolipids, both of

the neutral type and gangliosides, in the plasma membranes of cancer cells (2). Numerous studies have indicated both direct and indirect roles of gangliosides in tumorigenesis (reviewed in 3, 4). Furthermore, gangliosides have been shown to be shed from these cancer cells into the local environment and eventually into the blood stream (5-7). Some researchers have tried to take advantage of this phenomenon by identifying the presence of unusual or increased levels of gangliosides in the plasma of cancer patients as a circulating tumor marker (6, 8). This type of circulating tumor marker can be extremely useful because it can be an indicator of the progress and severity of the disease. It can be particularly advantageous because it only would require a small aliquot of blood.

Ganglioside Structure and Metabolism

Over 300 different glycosphingolipids have been characterized with 79 different gangliosides being among them (9). Gangliosides are, by definition, sialic acid-containing glycosphingolipids with molecular weights from 1000 - 5000 Da. Gangliosides are typically amphipathic molecules, consisting of a hydrophobic tail and a polar head group. The polar head group is an oligosaccharide that is glycosidically linked to the hydrophobic tail. The oligosaccharide backbone found in mammals may consist of varying proportions of glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, or fucose. Sulfate can also be found as an O-linked ester. One or more sialic acid residues are bound to this carbohydrate backbone, giving it a net negative charge. It is believed that this net negative charge may confer gangliosides with much of their bioreactivity (10). The hydrophobic tail, called ceramide, consists

of a long-chain sphingoid base with a fatty acid of varying length (14-26 carbons) bound in an amide linkage. These biomolecules are ubiquitous components of the extracellular aspect of the plasma bi-layer membrane in mammalian cells; the highest concentrations are found in the brain. The structural similarity of ceramide to diacylglycerol allows it to orient itself in the membrane with the carbohydrate portion facing out into the extracellular matrix, often perpendicular to the orientation of the hydrophobic tail (11). The composition of both the ceramide and the oligosaccharide have influence on the activity of the molecule. Figure 1 shows the structure of GM_2 ganglioside.

The synthesis of gangliosides (nomenclature and structures are summarized in Table 1) occurs largely in the Golgi apparatus by the step-wise transfer of sugars from sugar nucleotides to the non-reducing end of the glycan portion of a glycosphingolipid (or ceramide) by specific glycosyltransferases. Most mammalian glycosphingolipids are found with four main core structures: globo (Galα1-4Galβ1-4Glcβ1-1Cer), ganglio (GalNAcβ1-4Galβ1-4Glcβ1-1Cer), lacto (Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer), and neolacto (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer). Most gangliosides have the ganglio core but are not restricted to this type of structure. The biosynthetic pathways of glycolipid core structures are shown in Fig 2 and the biosynthetic pathways of most gangliosides are shown in Fig. 3. Specific glycosidases are responsible for the step-wise degradation of glycosphingolipids in a reverse manner in which they were synthesized and occurs in the lysosomes. Deficiencies of any of the glycosidases can block the catabolism and cause an accumulation of lipid. Disorders such as Tay-Sach's disease (12) and Gaucher's

Table 1.
Summary of Ganglioside Nomenclature

Ganglioside	Structure
GM_4	NeuAcα2-3Galβ1-1Cer
precursor	
LacCer	Galβ1-4Glcβ1-1Cer
a-series	
GM_3	NeuAcα2-3Galβ1-4GlcCer
GM_2	GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
GM_1	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
GD_{1a}	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
GT_{1a}	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
b-series	
GD_3	(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GD_2	GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GD_{1b}	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GT_{1b}	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GQ_{1b}	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
c-series	
GT_3	NeuAcα2-8NeuAcα2-8NeuAcα2-3Galβ1-4GlcCer
GT_2	GlcNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GT_{1c}	Galβ1-3GlcNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GQ_{1c}	NeuAcα2-3Galβ1-3GlcNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
lacto series	
SPG	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4GlcCer
2-6SPG	NeuAcα2-6Galβ1-3GlcNAcβ1-3Galβ1-4GlcCer
SLe^a	NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4GlcCer
SLe^x	NeuAcα2-3Galβ1-3(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcCer
neolacto series	
SNHC	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcCer
Sialyl I	(NeuAcα2-3Galβ1-4GlcNAcβ1-3,6)G lβ1-4GlcNAcβ1-3Galβ1-4GlcCer
6C	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcCer
SdiY-2	NeuAcα2-3(Galβ1-4(Fucα1-3)GlcNAcβ1-3)$_2$Galβ1-4GlcCer

Figure 1. GM_2 Ganglioside. GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer.

Figure 1.

GM2 Ganglioside

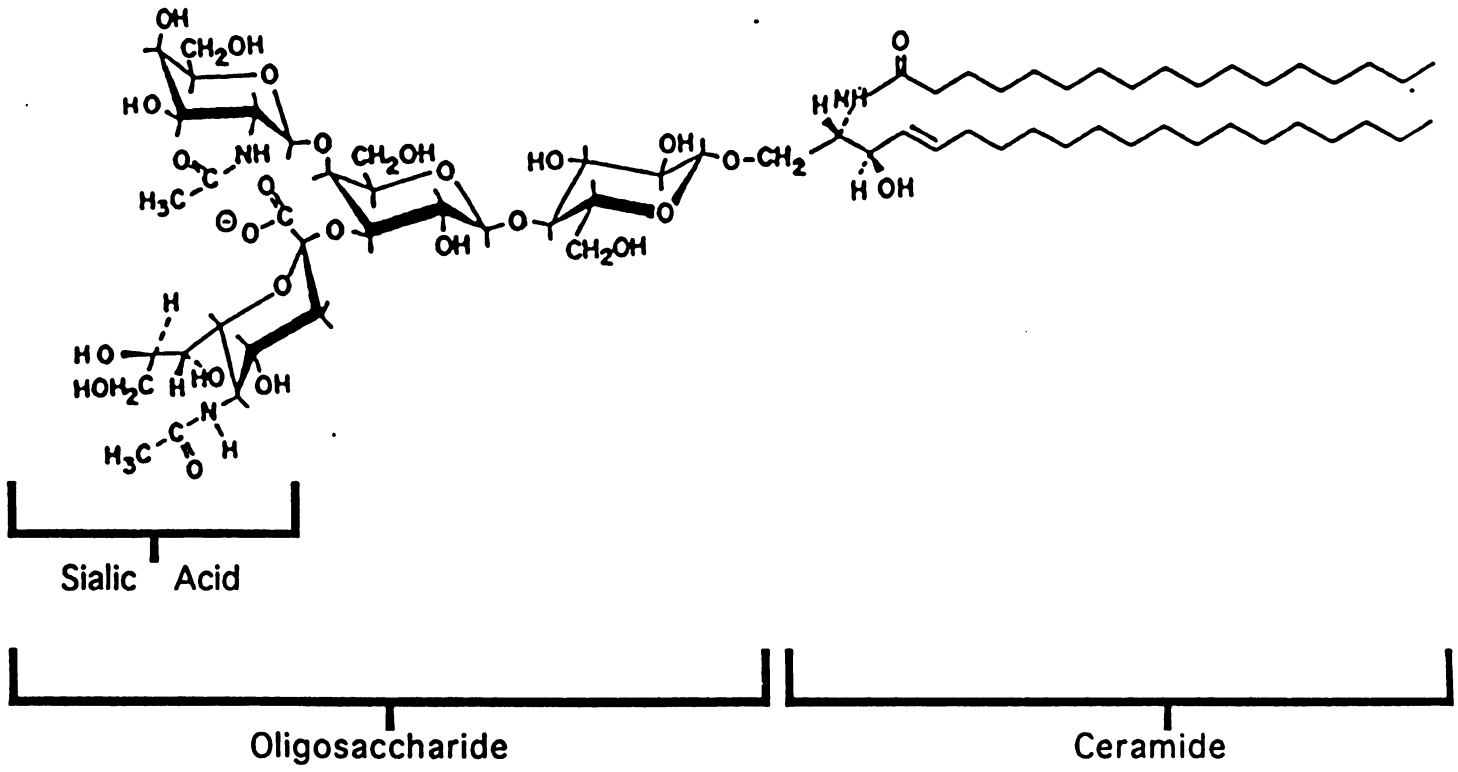


Figure 2. Biosynthesis of core glycolipid structures.

Figure 2 **BIOSYNTHESIS OF GLYCOLIPID CARBOHYDRATE CORE STRUCTUS**

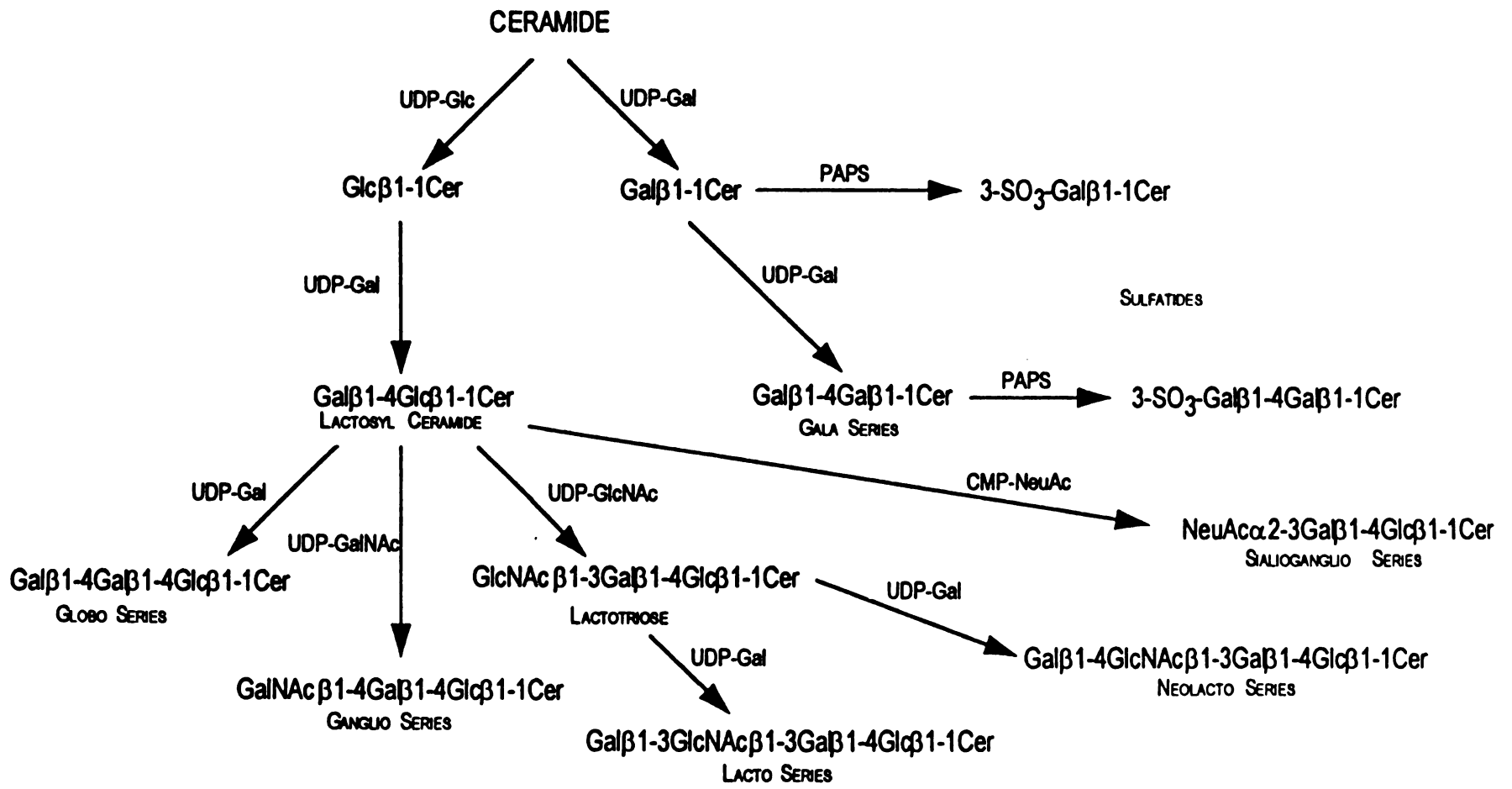
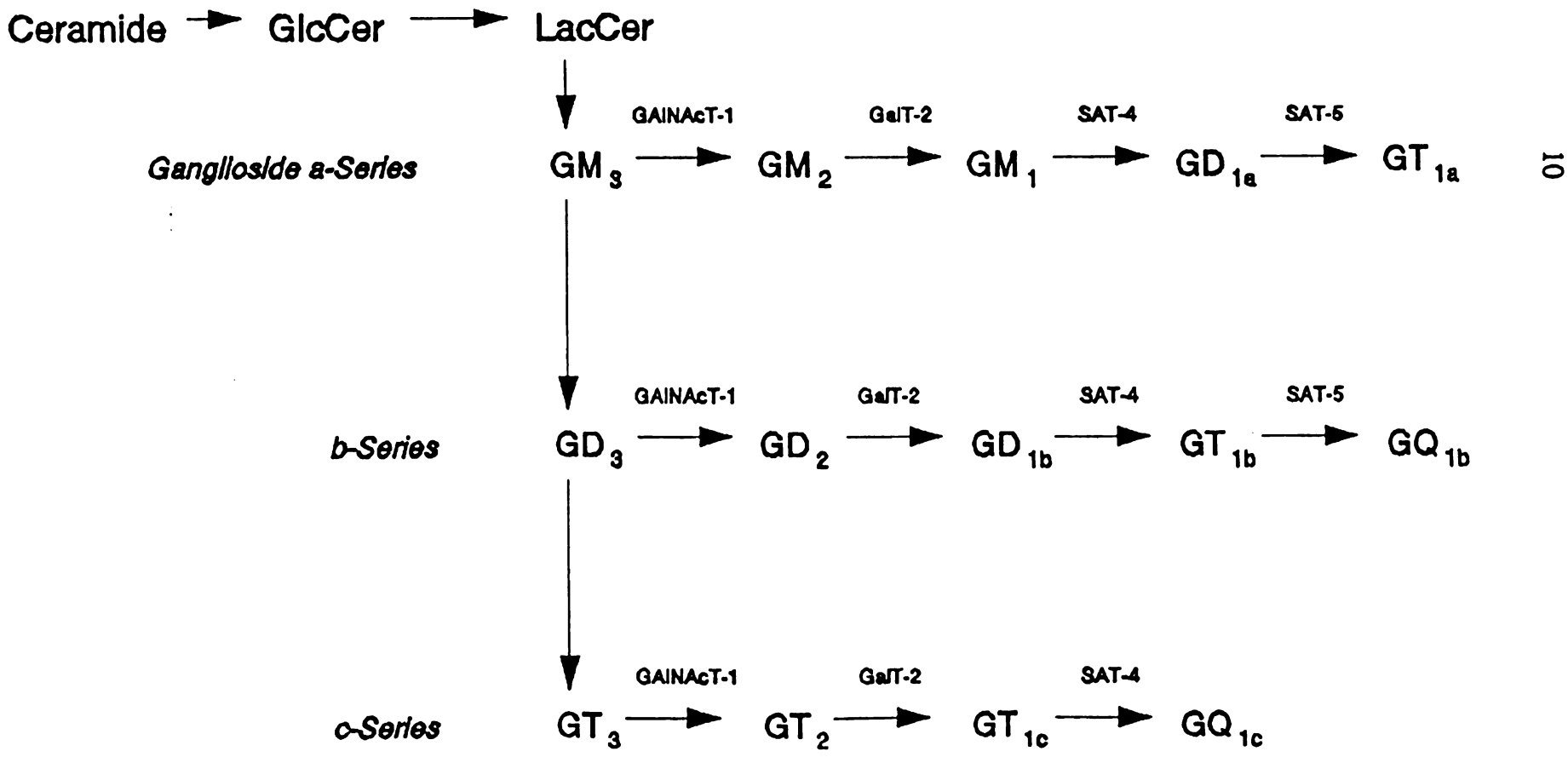


Figure 3. Biosynthesis of Gangliosides.

GANGLIOSIDE BIOSYNTHETIC PATHWAY



disease (13) have been attributed to the accumulation of GM_2 ganglioside and glucosylceramide due to defects in β-N-acetylhexosaminidase and glucocerebrosidase, respectively.

Isolation of Gangliosides

Gangliosides can be isolated by first extracting all of the lipophilic material from the biological tissue or fluid. Partitioning or column chromatography is employed to isolate the ganglioside mixtures from the undesired lipid material, i.e. phospholipids, neutral glycosphingolipids, neutral lipids, etc. The removal of salts and small molecular weight contaminants is required at least once, and often twice, based on the particular method chosen for the above steps. Total lipid extracts of biological materials are typically obtained by dispersing the material in 10 to 20 volumes of varying ratios of chloroform and methanol, typically 2:1 or 1:1. It may be necessary to add a small bit of water if polysialylgangliosides are sought. After adequate mixing or homogenization, the mixture is centrifuged or filtered and the pellet extracted a second time. The supernatant fractions are combined and dried, leaving the total lipid extract. Another method reported to extract gangliosides quantitatively employs 9 vol of tetrahydrofuran:0.01 M phosphate buffer pH 6.8 (14).

To remove unwanted lipid material, one of two strategies is employed, that of partitioning or column chromatography. Most early purifications involving partition used a mixture of chloroform, methanol, and saline (15, 16) that created a biphasic solution in which the gangliosides were recovered in the aqueous phase and neutral lipids partitioned into the organic phase. While this type of preparation was good for crude separations, substantial losses of less polar gangliosides, such as GM_3, were seen. Furthermore, the

more polar neutral glycosphingolipids would partition with the gangliosides in the aqueous phase, thus requiring further purification. These problems made it unusable for small samples. Another method based on partitioning, developed by Ladisch *et al.* (17), employed diisopropyl ether:n-butanol:water (4/2/3). This method was more suitable for smaller samples with almost quantitative removal of phospholipids in two extractions with minimal loss of gangliosides. Removal of salts and low molecular weight contaminants was then achieved either by dialysis, size exclusion chromatography, or reverse phase-chromatography (18). Partitioning of these types leaves a semipure ganglioside mixture that is suitable for many applications.

When a much purer preparation of gangliosides is desired, column chromatography is employed. There are many ion-exchange methods based on diethylaminoethyl functional groups conjugated to a solid support (19-21). Gangliosides bind to the column while neutral and positively charged lipids are washed off. The gangliosides are eluted by adding salt to the eluant. This step is typically followed by mild alkaline methanolysis to destroy any lipids with O-linked fatty acids. After desalting by the methods described above, a column of silica gel is used to remove sulfatides (21). The result is a fairly pure mixture of gangliosides.

Isolation of individual ganglioside species can be achieved by additional chromatography on DEAE-Sephadex and silica gel (19-21), high-performance liquid chromatography (22, 23), or preparative high-performance thin-layer chromatography. Ganglioside mixtures purified by the above methods often are homogeneous with respect to the oligosaccharide moiety but heterogenous with respect to the ceramide portion. Reverse-phase chromatography can be used to separate different species of a particular ganglioside based on the hydrophobic tail heterogeneity.

Characterization of Gangliosides

Characterization of gangliosides depends on the nature of the gangliosides and the intent of the study. Mixtures of gangliosides are best analyzed by high-performance thin-layer chromatography (HPTLC) employing numerous sensitive methods of visualization. When a more homogeneous preparation is at hand, several different techniques are currently available including different types of mass spectrometry (MS), high-performance liquid chromatography (HPLC), gas chromatography (GC), proton and ^{13}C-nuclear magnetic resonance spectroscopy (NMR), as well as immunological techniques. These latter methods are capable of providing much information about the nature of the ganglioside composition, carbohydrate sequence, and linkage and anomerity of carbohydrates.

High-Performance Thin-Layer Chromatography

Chromatography on high-performance thin-layer chromatography (HPTLC) plates is widely used to separate mixtures of gangliosides and other glycolipids. HPTLC plates are glass plates precoated with a 0.25 mm layer of uniform superfine silica gel, which has a mean particle size of 5 μm and a mean pore diameter of 80 nm. The test sample is applied near the bottom of the plate and the plate placed in a preequilibrated chamber with an appropriate solvent to resolve the gangliosides of interest. The most common solvents consist of a mixture of chloroform, methanol and water, containing either ammonia, potassium or calcium chloride, in ratios varying from 60/40/9 to 50/40/10, respectively. The solvent is allowed to run the length of the plate, the plate dried, and the gangliosides visualized by a colormetric spray or, more recently, by an immunological technique.

Resorcinol-HCl (24) and orcinol-H_2SO_4 (25) are common colormetric sprays for the detection of sialic acid residues of gangliosides and glucose residues of glycosphingolipids, respectively. After heating, they react with the specific sugar to form a bluish purple spot. For preliminary identification of gangliosides, the mobility and reactivity can be compared with those of known reference compounds. A difficulty with this method is that more than one ganglioside may have the same number of carbohydrates and such mixtures may be difficult to separate. In addition, gangliosides with identical oligosaccharide structures may produce two or more bands due to differences in the ceramide moiety. These problems can be overcome by altering the solvent system such that the bands of interest are better resolved. Other techniques involve HPTLC of the acetylated derivative or use of alternate types of plates, such as borate-impregnated silica gel. Alternatively, newer methods allow for overlay techniques using antibodies developed for specific gangliosides or epitopes found on gangliosides (26-28) or using lectins of the same nature (29). An aluminum-backed HPTLC plate for this type of analysis is fixed with polyisobutylmethacrylate, immersed in a primary antibody solution or specific lectin solution, then soaked in the second radiolabeled antibody solution (27, 28) followed by autoradiography, or an avidin-biotin enzyme system (30).

When glycosphingolipids on HPTLC plates have been visualized by conventional methods using colormetric sprays, densitometric scanning methods have been developed to quantify the spots (31-33). The resorcinol-positive plate is scanned with a scanning densitometer in the transmittance mode with incident light at 580 nm. This gave linear response in the range of 0.5-10 nmol with as little as 100 pmol capable of routinely being detected (32). One study reported as little as 10 pmol of ganglioside being reproducibly

detectable. A more recent technique of quantification of spots on chromatograms is that of image densitometry (34). Chromatograms are scanned into a computer as a digital image of 1024 pixels by 1024 pixels. Specific software then computes the area of the spot and its optical density, and integrates this information. This method of quantification is applicable directly to resorcinol-positive thin-layer plates or to autoradiograms, which previously were difficult to quantify. The method is linear down to a sample size of 10 pmol. Quantification of individual gangliosides can be done by the method of Suzuki (35) in which gangliosides are visualized with iodine, scraped from the plate, and quantified by a modified resorcinol assay (24). This is also applicable for radiolabeled gangliosides by scrapping and counting with a scintillation counter.

Two-dimensional HPTLC is often used when added resolution is necessary. A ganglioside mixture is spotted in one corner of the plate, 1 cm from each edge, run in the first dimension in a suitable solvent, then allowed to dry. The plate is then turned 90° and run in the second dimension with a different solvent. This method takes advantage of the differential resolving properties of two different solvent systems. Many qualitative and semi-quantitative studies have been conducted to analyze interspecies differences in various tissues (36-38). One reason for the not-so-wide-spread use of 2-D HPTLC, despite its resolving power, is the lack of a suitable method of detection and quantification of the spots. One study (36) used sequential scanning densitometry across the entire plate, summing the areas of peaks corresponding to the same spot. This requires a special sequential scanning densitometer. Linearity and sensitivity has been reported to be from 0.1 to 6 nmol. Image densitometry, as described above, makes the quantification of spots run in two-dimensions as simple as in one-dimension. Furthermore, it is applicable to direct analysis of resorcinol-

positive plates as well as autoradiography from radiolabeled lipids or by the many overlay methods as described above (34).

High-performance Liquid Chromatography

Most methods involving the use of HPLC in the analysis of the oligosaccharide structure of gangliosides require the release of the intact oligosaccharide from the ceramide portion. Classically, this has been done by oxidation of the olefinic double bound by ozonolysis (39) or osmium tetroxide (40) followed by alkaline-induced degradation releasing the intact oligosaccharide. Another chemical method involves the use of trifluoroacetolysis (41). Recent characterizations of endoglycoceramidase (42), or similarly ceramide-glycanase (43, 44) have made enzymatic release of the oligosaccharide possible. Once the oligosaccharide has been freed, many different protocols involving HPLC are available, depending on the nature of the oligosaccharide. These are reviewed in 45. A difficulty in the analysis of carbohydrates, whether they be mono- or oligosaccharides, is the ability to detect them in a simple manner with some degree of sensitivity. They have no natural absorption in the ultraviolet or visible regions, nor can they be detected by fluorescence. While other physical types of detection are possible, they are neither specific nor sensitive. To overcome this, reacting the reducing end with a fluorophore such as pyridylamino derivatives (46, 47) or ultraviolet-absorbing 1-phenyl-3-methyl-5-pyrazolone derivatives are often employed. Another, more current method for the separation and detection of underivatized mono- and oligosaccharides involves the separation on high-pH anion exchange chromatography with electrochemical detection with a pulsed amperometric detector (23, 48-52).

Gas Chromatography and Mass Spectrometry

Gas chromatography (GC) is a useful tool for the estimation of components in glycolipids. GC can give information on the oligosaccharide composition and structure as well as sphingoid base and fatty acid moieties. Most methods require some type of derivatization in order to make the compounds volatile enough for analysis with gas chromatography. Small oligosaccharide molecules (2-3 sugars) released from glycolipids can be analyzed by GC as permethylated alditols (53). Larger oligosaccharides are best resolved by HPLC as described above. GC is most useful for the estimation of the components of glycolipids. Monosaccharides are released from the oligosaccharide either by acid-catalyzed hydrolysis or methanolysis. Hydrolysis is achieved using sulfuric acid (54), hydrochloric acid (55), or acetic acid with sulfuric acid (56), followed by conversion to alditol acetates (57, 58) and analysis by GC. This method is useful in the analysis of mixtures of free sugars because single peaks are observed for each saccharide. Problems with this type of determination include the need for different conditions of hydrolysis for different carbohydrates and, often, the complete destruction of sialic acid. Furthermore, hydrolysis often requires complex, time-consuming work-up procedures not suitable for small samples.

Methanolysis in dry methanolic hydrochloric acid (HCl), followed by trimethylsilylation and analysis by GC (59-66), is often used instead of hydrolysis. Typically, a sample of glycolipid is methanolyzed with 0.75 N methanolic HCL for 16-24 h at 80 °C followed by neutralization. Fatty acids can be removed by partitioning with hexane and can be run separately if desired. Trimethylsilylation of the methyl glycoside with a mixture of hexylmethyldisilazane, trichlorosilane and pyridine, now conveniently found in commercial preparations, produces a derivative suitable for GC. The derivatized

carbohydrates are run on a non-polar gas chromatography column, producing multiple peaks due to different anomers of the methyl glycoside. Quantification is based on the area of all or one of the resultant peaks, relative to an internal standard, often mannitol. Identification is based on comparison of retention times of known standards and retention indices. A recent microscale method has reduced the sample volume and methanolysis time to 1.5 h (67). This method also makes use of peracetylation to produce a stable derivative with useful chromatographic properties.

Many of the above methods can and have been used in conjunction with mass spectrometry. Pure monosaccharides as well as mixtures can be analyzed with gas chromatography and electron impact mass spectrometry (GC-EIMS) as TMS (60, 68-72), permethylated (73-77) and peracetylated derivatives (57, 63, 73). Mass spectral data give more substantial evidence of the presence of a particular saccharide over basic comparisons of retention times with known standards, which is the case with gas chromatography alone. Intact oligosaccharides, released from the ceramide, can also be analyzed by GC-EIMS but this approach often requires previous derivatization such as permethylation (78). Structural information is possible with the use of methylation analysis followed by GC-EIMS. Oligosaccharides are released from the ceramide portion by a method stated above and subjected to methylation with methyl iodide and methylsulfinylmethanide in dimethyl sulfoxide (DMSO) (79). This procedure methylates all free hydroxyl groups, acetamide residues, and carboxyl groups. This permethylated product is hydrolyzed to form partially methylated monosaccharides. This mixture is reduced with sodium borohydride, producing partially methylated alditols, which are acetylated with acetic anhydride. The products contain O-acetyl groups at positions of substitution in the oligosaccharide and on carbons

attached to ring oxygens. These products are readily analyzed by comparison of their retention times and fragmentation patterns obtained with GC-EIMS. Information on positions of glycosidic linkages and ring size of sugar substituents is possible. Methylation analysis is widely used in the analysis of all types of glycoconjugates (60, 76, 80). GC-EIMS of intact oligosaccharides following permethylation is also possible (78) and yields information on the sequence as well as the linkages.

Both Sweeley and Dawson (81) and Samuelsson and Samuelsson (82) used GC-EIMS to analyze TMS derivatives of intact glycolipids. Information was obtained on the sequence of sugar and ceramide, yet no molecular ion was seen. The analysis of glycolipids as large as tetrahexosylceramide (83) was possible. Permethylation of glycolipids analyzed by GC-EIMS (77, 84) provided better information on the exact ratios of sugars and sequence up to eight carbohydrates. Urdal and Hakomori (85) used direct probe EIMS instead of GC-EIMS of the permethylated derivative of a tumor associated ganglioside to determine differences in the fatty acid moiety. The development of soft ionization techniques such as fast-atom bombardment mass spectrometry (FAB-MS) (86) with collision-induced dissociation (22, 87, 88) permitted analysis of underivatized gangliosides. Costello (88) has developed a systematic nomenclature for the identification of fragment ions observed with CID-FAB-MS.

Gangliosides in Cellular Interaction, Signal Transduction, and Oncogenesis

Cellular Interaction

Gangliosides and other glycosphingolipids found on the surface of the plasma membrane have been associated with several cell binding events. Several studies have found cell-surface gangliosides to be integral components in cell-cell adhesion. The compaction of

morula, one of the first cell-binding events in ontogenesis, is modulated by Le^x glycolipid (89). The Le^x glycolipid has been shown to strongly bind other Le^x glycolipids and glycoconjugates containing the Le^x determinant. It weakly binds the Le^y and nLc_4 determinants. The ganglioside GM_3 can bind asialo-GM_3 (lactosyl ceramide) (90) as well as Gg_3Cer with strong affinity (91). While these interactions are not as strong as some protein-protein bonds, the observed clustering of gangliosides on the cell surface, instead of being homogeneously distributed on the surface, suggests that there is strength in numbers (90). Hakomori has postulated that the interaction of these glycolipids is not the main type of adhesion of cells, but precedes a stronger binding event. The patches of gangliosides or other glycolipids on the surfaces allow cells to come in contact and remain oriented long enough for the more stable binding takes place (90).

Interaction of GM_3 with lactosyl ceramide is stronger in the presence of integrins (91), suggesting that gangliosides and other glycolipids have some interaction with various extracellular proteins. Evidence of a role for gangliosides in cell adhesion to substrata was found when the antibodies against GD_2 and GD_3 were able to inhibit the attachment and spreading of human melanoma cells to a number of extracellular proteins including fibronectin, vitronectin, collagen, laminin and Arg-Gly-Asp-containing synthetic peptides (92). Gangliosides, GD_{1a} and GT_{1b}, were able to inhibit fibronectin-mediated adhesion to fibronectin-collagen complexes (93). Spiegal *et al.* (93) reported that complex gangliosides (the more highly glycosylated or sialylated) have an important role in fibronectin organization. Furthermore, GD_{1a} has been shown to bind laminin, suggesting a role for gangliosides in neural cell adhesion (95).

In addition to the cell-cell and cell-strata interactions, gangliosides, as well as other

glycolipids, can act as receptors for various hormones and bacterial and viral toxins (90, 96, 97). Gangliosides have been associated with the binding of the hormones such as thyroid-stimulating hormone (98), chorionic gonadotropin (99), as well as serotonin (100). GM_1 has been well characterized as the receptor for the B-subunit of Cholera toxin (12).

Signal Transduction and Cell Growth

Despite the topological arrangement of gangliosides on the outer leaflet of cells, there is a growing body of evidence suggesting that gangliosides act in transmembrane signal transduction. Gangliosides appear to influence cell transmembrane signaling in a variety of different mechanisms including 1) their interaction with plasma membrane ion channels, 2) direct interaction with plasma membrane growth factor receptor-associated kinases, and 3) through degradative products that inhibit protein kinase C (101). It is through these mechanisms that gangliosides are postulated to be involved in cell-cycle control.

The addition of exogenous gangliosides has been shown to stimulate Na^+-K^+-ATPase activity in rat brain mitochondrial membranes (102) and the binding of GM_1 to the B subunit of cholera toxin causes proliferation in lymphocytes and fibroblasts by inducing a rapid and sustained increase in intracellular calcium (103). Moreover, changes in ganglioside composition and metabolism have been associated with differentiation (104, 105). Several lines of evidence suggest that these compositional changes are more than just a phenotypic trait and that there is a specific role for gangliosides in differentiation. Exogenously added gangliosides can either stimulate or inhibit cell growth depending on the cell type and ganglioside added. Specifically, the addition of GM_1 or GT_{1b} enhances the effect of nerve growth factor (NGF) on PC-12 pheochromocytoma cells (106) and GQ_{1b} appears to have

NGF-like activities by itself (58). Cells may exhibit a glycolipid contact response that is closely related to contact inhibition of cell growth, suggesting that gangliosides may be involved in the regulation of cell growth. Synthesis of specific gangliosides can often accompany density-dependent growth inhibition. This has been seen with GM_3 in NIL cells (107), GD_{1a} in 3T3 cells (108), and GM_3 and GM_1 in human fibroblasts (109).

Gangliosides may modulate cell growth through various growth factor receptors. Studies have found that exogenously added GM_3 and GM_1 to 3T3 fibroblasts inhibited their growth (110). The addition of these gangliosides had little or no effect on the growth factors binding to their respective receptors. It was later demonstrated that GM_3 interacts directly with the EGF-r and GM_1 or GM_3 with PDGF-r and inhibits the autophosphorylation of the receptor (111, 112). Furthermore, various metabolites of GM_3 have different modulating effects on the receptor. De-*N*-acetyl-GM_3 activates the tyrosine kinase of EGF-r and is a strong promoter of cell growth (111, 112). On the other hand, lyso-GM_3 (de-*N*-acyl-GM_3), is a potent inhibitor of EGF-r autophosphorylation and cell growth (113). Parallels have been drawn between the growth modulating effects of GM_3 metabolites and that of the phosphatidylinositol cascade (90).

Oncogenic Transformation

Oncogenic transformation has been characterized by aberrant synthesis and organization of gangliosides (3). Alterations in the compositions of gangliosides are a result of incomplete synthesis due to down regulation or blockage of certain biosynthetic pathways. Precursor or less complex gangliosides can accumulate as a direct result. Also, neo-synthesis, the synthesis of novel gangliosides with a different core structure, and retrogenic

synthesis, the reappearance of fetal lipids through activation of quiescent fetal glycosyltransferases (3, 75), of gangliosides is possible. A possible result is an increase in the less complex gangliosides with the disappearance of the more complex. Similarly, the emergence of novel gangliosides in the form of different core structures or biosynthetic pathways may accompany oncogenic transformation. Table 2 is a list of gangliosides that have been well characterized in particular cancers. It is not yet clear as to what the role of aberrant ganglioside expression is in the biology of cancer, however, it is easy to see the potential functions gangliosides may have when one considers their suspected and understood functions in normal cells as stated above.

The aberrant expression of gangliosides on the surface of cancer cells has also been suggested to have an immunosuppressive function, possibly allowing gangliosides to escape immune surveillance Gangliosides of cancer tissues have been demonstrated to inhibit natural killer cell activity (114), T-cell response (140) and block the effect of IL-2 (141). The various postulated effects of gangliosides on the immune system have been reviewed in 142. Of particular interest when considering the immunosuppressive aspects of gangliosides is the phenomenon of their shedding from the surfaces of tumor cells into the local environment and eventually the blood stream. Neuroblastoma and melanoma have both been shown to shed gangliosides from their surface at a high rate (5, 7, 129). In particular, neuroblastoma has elevated levels of GD_2 in the tumor tissue as well as elevated level in circulation (6, 8). The levels of GD_2 in the plasma or serum of neuroblastoma patients was closely correlated with the progression of the disease and has been suggested as a circulating tumor marker (6, 8, 130). Furthermore, shed gangliosides of neuroblastoma were

Table 2

TUMOR-ASSOCIATED GANGLIOSIDE ANTIGENS

Chemical Structure	IUPAC-IUB symbols	Svennerholm abbreviation	Tumors	References
NeuAcα2-3Galβ1-4Glcβ1-Cer	II^3NeuAc-LacCer	GM_3	various	7, 115-119
NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ-1Cer	$II^3(NeuAc)_2$-LacCer	GD_3	melanoma	74,92,115,115,120, 121
			colorectal and pancreatic lymphoma	122 123, 124
			neuroblastoma	7, 125
			brain tumors	126,132
NeuAc-9-0-Acα2-8NeuAcα2-3Galβ1-4Glcβ-1Cer	II^3NeuAc-9-0Ac-NeuAc-LacCer	9-0Ac-GD_3	Melanoma	116
GalNAcβ1-4Galβ1-4Glcβ1-Cer ↑3 2αNeuAc	II^3NeuAc-Gg_3Cer	GM_2	Lung Cancer Melanoma Glioma Nueroblastoma	126 127 128 22
GalNAcβ1-4Galβ1-4Glcβ1-Cer ↑3 2αNeuAcα2-8αNeuAc	II^3NeuAc-$_2Gg_3$Cer	GD_2	Neuroblastoma Other Brain tumors Glioma Melanoma	5,22,129-131 79, 123, 126, 132 128 116, 120, 133, 134

Table 2. (Cont'd)

Chemical Structure	IUPAC-IUB symbols	Svennerholm	Tumors	References
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer ↑6 2αNeuAc	IV^6NeuAc-nLc_4-Cer	6′-isoLM1	Colonic adenoarcinoma Pancreatic adenocarcinoma	135 136
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-Cer ↑3 2αNeuAc	IV^3NeuAc-Lc_4-Cer	3′-isoLM1	Lung Carcinoma Glioma	137 138
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-Cer ↑3 2αNeuAcα2-8NeuAc	IV^3Sia-III^6Sia-Lc_4-Cer	3′8′-IsoLD1	Glioma	139
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer ↑3 ↑4 2αNeuAc 1αFuc	IV^6NeuAcIII^4Fuc-nLc_4Cer	SLe^a	Colorectal Adenocarcanoma Pancreatic Cancer	117 117
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer ↑3 ↑3 2αNeuAc 1αFuc	IV^6NeuAcIII^4Fuc-nLc_4Cer	SLe^x	Colorectal Adenocarcanoma Pancreatic Cancer	117 117

demonstrated to be immunosuppressive in a lymphoproliferative assay *in vitro* (131).

Sialic Acid and Sialidase

Sialic acids are acetylated derivatives of neuraminic acid (2-keto-5-amino-3,5-dideoxy-D-nonulosonic acid) and are widely found in nature as mucins, glycoproteins, gangliosides, and milk oligosaccharides (10, 143-146). Gangliosides are defined as sialic acid-containing glycosphingolipids and the N-acetylneuraminic acid is thought to be the only homolog found in humans (147). The presence of sialic acid on glycoconjugates has been demonstrated to have a wide range of biological functions. These include structural conformation (10, 146, 148, 149), recognition and masking of binding sites (10, 146), and to confer mitogenic properties as well as the binding of various substrates (119, 148). Concentrations of sialic acid, defined as total serum sialic acid (all sialic acid containing glycoconjugates and free sialic acid) or lipid-associated sialic acid (gangliosides), have been suggested to be useful in the diagnosis, prognosis, and the monitoring of various disease states including several cancer types (150-163).

Sialidases (N-acetylneuraminosyl glycohydrolases (EC 3.2.1.18)) are the enzymes that cleave sialic acid residues from their oligosaccharide bases. Sialidases have been characterized in humans from the plasma membrane, cytosol, lysosome, as well as in the medium of cultured human fibroblasts (164, 165). There have been reports of trace sialidase activity in plasma (166), yets its presence and activity are inconclusive in either healthy individuals or cancer patients. Activity of sialidase towards gangliosides was found in transformed hamster cells that was not found in the

normal cells (167). It was postulated that this sialidase activity directed towards extracellular gangliosides may be involved in disrupting cell-cell binding events necessary for transformation.

Breast Cancer and Gangliosides

Breast cancer is one of the most common and most dreaded cancers in women. It has an unpredictable course and the risk of metastases continues for 20 years or more. It accounts for 18% of cancer deaths in females. It is typically detected by self examination or mammography and staging of the cancer is based on tumor size, presence in lymph nodes and the degree of metastasis. The earlier the tumor can be discovered and determining the degree to which it has spread is important in the prognosis (168). The need for better methods of detection as well as basic understanding of the disease continues to be an ongoing struggle.

Several studies have examined the utility of sialic acid in the circulation for the diagnosis and staging of breast cancer (152, 154-156, 169-171). The results of these studies typically indicate that sialic acid can be a useful marker for breast cancer yet there are problems associated with these studies and therefore the approach is not yet fully accepted (158, 172). The little work done that examines the ganglioside content in the breast tumor tissue shows abberrant glycosylation and an alteration in the ganglioside profile (173). There is evidence for a correlation between sialic acid concentration and progression of disease; however, a causal relationship has not been established. If these alterations in the ganglioside profiles can possibly be understood and made useful to the physcian, it may be a great boon to the many women fated to

suffer the disease.

Statement of the Problem

Breast cancer is a tragic, and often times fatal disease. There can never be too much information available to the physician when attempting to diagnose and treat patients with this disease. Circulating gangliosides have been demonstrated to be useful in the diagnosis and prognosis of other cancers (130, 174). This study seeks to determine the utility of circulating gangliosides as tumor markers for the diagnosis and monitoring of breast cancer. Additionally, by defining the circulating ganglioside profile associated with breast cancer, some insight may be gained in the workings of the disease. In order to thoroughly assess this potential, the following goals must be met:

1. Identify or develop protocols for the isolation and purification of gangliosides from small amounts of serum.
2. Identify or develop protocols for the quantification of gangliosides at the picomole level.
3. Identify or develop protocols for the separation of complex ganglioside mixtures using one- or two-dimensional high-performance thin-layer chromatography.
4. Create a map of relevant gangliosides by one- or two-dimensional high-performance thin-layer chromatography to aid in identification of unknown gangliosides.
5. Analyze a suitable amount of samples of plasma or serum from breast cancer patients and appropriate control group women.
6. Analyze the free sialic acids levels and activity of sialidase in the blood of breast

cancer patients and appropriate control group women.

7. Compare the two test groups' ganglioside profiles as well as the free sialic acid concentration and sialidase activiy and discuss any anomalies.

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CHAPTER 2

Isolation of Gangliosides from Serum or Plasma

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Abstract

In order to determine the most appropriate method for the isolation of gangliosides from small samples of plasma or sera (1 ml), several different techniques were considered. Solvent partitioning, column chromatography, and solid-phase extraction were carefully examined and each step evaluated for its potential usefulness in an overall procedure. We determined that a modified partitioning method using diisopropyl ether, butanol and water was the most useful when combined with gel filtration. Gangliosides could be isolated from small volumes of plasma or sera in good yield and were suitable for analysis by high-performance thin-layer chromatography (HPTLC). It was, however, necessary to further purify gangliosides mixtures from plasma or sera with a DEAE-Sephadex column and gel filtration when analysis by gas chromatography was desired.

Introduction

To determine the usefulness of serum gangliosides as relevant circulating tumors markers, a suitable method for their isolation was required. As a primary goal in this study, the method had to be suitable for the isolation of gangliosides from relatively small amounts of blood serum. Currently available methods are useful for many types of applications, yet fall short of the requirements outlined by this study. Most methods yield a total-lipid extract from the biological tissue or fluid by thorough mixing and/or homogenization in a mixture of chloroform and methanol. Lipids are solubilized and precipitated proteins removed by centrifugation. The classic Folch (1) partition and its many modifications partition the total-lipid extract with mixtures of chloroform, methanol, and saline, with the gangliosides partitioning into the aqueous phase and phospholipids and other undesired components partitioning into the organic phase. After lyophilization, salts and other low molecular weight contaminants are removed by dialysis or size-exclusion chromatography. This method provides a crude mixture of gangliosides suitable for many uses or ready for further clean up with additional column chromatographic procedures. Problems with this method are the need for large sample sizes and losses of less polar gangliosides (GM_3) through partitioning into the organic phase. Other methods for the isolation of a ganglioside fraction from the total lipid extract involve various types of weak-anion exchange, affinity, and size exclusion column chromatography. The most popular method involves the use of a DEAE-Sephadex A-25 to remove neutral and positively charged contaminants from the ganglioside fraction. Mild alkaline methanolysis is then used

to destroy any remaining phospholipids, with dialysis, size-exclusion or reverse-phase chromatography used to remove salts and low molecular weight contaminants. Chromatography on silicic acid removes residual negatively charged contaminants to produce a relatively pure mixture of gangliosides. While this method of isolation produces mixtures of gangliosides pure enough for most applications, scaling down of the method to accommodate the small samples required by this study would be required. Ladisch and Gilliard (2, 3) found that gangliosides could be isolated from the total lipid extract with good recovery by partitioning between diisopropyl ether, n-butanol and distilled water (6:4:5). The gangliosides were recovered in the aqueous phase and phospholipids in the organic phase. After desalting on a small column of Sephadex G-50, the sample was ready for analysis. This method is simple and suitable for smaller samples; however, losses of the less polar ganglioside into the organic phase become critical as the sample size decreases. Furthermore, while the degree of purity was acceptable, often contaminants would make one-dimensional thin-layer chromatography (1-D HPTLC) and quantitation by densitometry difficult if not impossible. All but the Folch method were examined thoroughly for use in this study. The reason the Folch method was not considered was the admitted loss of gangliosides which would most likely be exacerbated when scaled down. Criteria for choosing a particular method were simplicity, ability to handle small (i.e. 1 ml serum) and numerous samples, and potential for clinical usefulness. It was determined that none of the current methodologies, as is, would be suitable without some degree of modification. The following summaries are of studies conducted to evaluate various methods and modifications in an attempt to optimize each potentially useful step in an

overall procedure.

Material and Methods

Materials

Fresh outdated plasma was obtained from the American Red Cross (Lansing, MI) and was stored at -70 °C until used. All solvents were of analytical grade or better. DEAE-Sephadex A-25 and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden) and SepPak C_{18} cartridges were obtained from Waters (Milford, MA). The Bond Elut aminopropyl and silica gel cartridges were from Analytichem (Harbor City, CA). High-performance thin-layer chromatography plates (HPTLC) were obtained from Merck (Darmstedt, Germany). Reference gangliosides were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Isolation of Total-Lipid Extract

The isolation of gangliosides along with other lipid material from sera or other biological fluid was easily achieved by extraction with 10 vol of chloroform:methanol in varying proportions. We found that two extractions with chloroform:methanol (C:M, 1:1), first for 4 hours and then for 4 to 20 hours, gave quantitative recovery of gangliosides in the total-lipid mix (4). Proteins and other insoluble materials were removed by centrifugation after extraction. The additional removal of partially soluble proteins and salts was achieved by reducing the volume to one-quarter and the temperature to -70 °C followed by centrifugation. Modifications included lyophilization of serum prior to total-lipid extraction with the intent to speed up future

drying steps. Also, dialyzing the plasma against water was examined to remove small molecular weight contaminants at an early stage. Lastly, rotary evaporation was examined versus evaporation under a stream of nitrogen with heating for the initial removal of solvent.

Isolation of Gangliosides by Column Chromatography.

The use of DEAE-Sephadex chromatography for the purification of gangliosides was originally designed for 20 ml of plasma (5). A subsequent report suggested modifications on how to scale down by a factor of three (4). We extended this scaling down to accommodate 1 ml of serum with the use of pasteur pipets or champagne columns (60 mm X 5 mm I.D. columns with 10 ml reservoir). The DEAE-Sephadex resin was washed 3 times with chloroform:methanol:0.8 M sodium acetate (aq.), 30:60:8 (Solvent A), then allowed to soak over night in the same solvent. The resin was then washed 3 times with chloroform:methanol:water, 30:60:8 (Solvent B). Columns of approximately 0.3 and 1 ml of resin were poured into pasteur pipets and 2 ml of resin into a champagne column. These columns were fitted with glass wool plugs forced in rather tightly in order to restrict the flow to a fairly slow flow rate. The resin was washed with 15 ml more of solvent A. Samples of the total lipid extract were applied in very small volumes, i.e. < 1 ml, and were reapplied twice (the application volume is collected and reapplied to the top of the column) to ensure total adsorption of the gangliosides to the column. The columns were washed with 5 to 15 ml more of solvent A to remove neutral and positively charged materials, then the ganglioside fractions were eluted with 5 to 15 ml of

solvent B. After reducing the eluates to dryness under a stream of nitrogen, the samples were subjected to mild-alkaline methanolysis, by taking up in small volumes of 0.1 N sodium hydroxide in methanol and incubated at 37 °C for 2-3 hours. This was to destroy any acidic phospholipids still present. The reaction mixtures were neutralized with acetic acid and extracted twice with equal volumes of hexane to remove any residual neutral lipids and fatty acid methyl esters (FAME). The solvent was again removed with a stream of nitrogen and oil-pump vacuum to ensure removal of all traces of solvent.

Several techniques were employed to remove salts and low molecular weight contaminants, including dialysis, size-exclusion chromatography on small Sephadex G-50 columns. and solid-phase extraction cartridges. Dialysis was done with prewashed dialysis tubing (0.32 ml/cm, 12-14, kDa cutoff) cut to 2-3 ml length and was carried out for 2 days against water with numerous water changes. The dialysate was then lyophilized and the residue taken up in a small volume of chloroform: methanol (1:1) and further clarified by centrifugation if necessary. Alternatively, salts could be removed by size exclusion chromatography with a small 10 ml column of Sephadex G-50. The samples of ganglioside (< 30 nmol) were applied to the top of the column in a small volume (0.3 ml), allowed to flow at a slow rate (<0.5 ml/min) with the gangliosides collected in the void volume. The void volume was determined by blue dextran and the total volume by sodium chloride detected with silver nitrate. The ganglioside fraction was lyophilized and further clarified if necessary. The use of SepPak C_{18} cartridges to remove salts was also considered. The SepPaks were fitted with either glass or plastic 10 ml syringes and washed with

10 ml water, methanol:water, methanol, chloroform:methanol, methanol, methanol:water and 0.1 M sodium chloride each. Samples were applied to the cartridge in a small volume of 0.1 M sodium chloride (< 1 ml) and reapplied twice to ensure complete adsorption to the cartridge. The cartridge was then washed with 2 ml of 0.1 M sodium chloride then 40 ml of distilled deionized water. The gangliosides were then eluted with 2 ml of methanol and 10 ml of chloroform:methanol (1:1), dried under a stream of nitrogen, and clarified as above if necessary.

Two methods were evaluated for the purification of serum gangliosides, based on solid-phase extraction techniques on commercially available cartridges. The first method utilized a weak anion exchanger, Bond Elut aminopropyl cartridge. The cartridge was washed twice with 10 ml of methanol and 10 ml of chloroform. Sample was applied in 2 ml of chloroform:methanol (85:15) and reapplied twice to ensure adsorption. The flow was gravity fed. The cartridge was then washed with 10 ml of chloroform, dichloromethylene:methanol (8:2) and methanol. Gangliosides were eluted with 0.5 N ammonium bicarbonate in methanol. The sample was dried and desalted on a SepPak C_{18} cartridge, as outlined above. Mild alkaline methanolysis was carried out as before and the sample was again desalted on a SepPak C_{18} cartridge.

Another solid-phase extraction method attempted was based on silicic acid chromatography. A Bond Elut silica gel cartridge was washed twice with chloroform and methanol and the sample applied to the cartridge in 1 ml of chloroform and reapplied twice. The cartridge was washed with 10 ml of chloroform,

acetone:methanol (9:1), methanol, with the gangliosides being eluted with methanol:water (9:1). The eluant was dried and subjected to mild alkaline methanolysis with subsequent desalting on a SepPak C_{18} cartridge.

Isolation of Gangliosides by Diisopropyl ether:n-Butanol:Water Partition.

A method of isolating gangliosides from small samples from biological tissues and fluids, described by Ladisch *et al.* (2), used a three-phase partition mixture of diisopropyl ether, n-butanol, and water. The dried lipid extract was dispersed in 2 vol of diisopropyl ether:n-butanol (6:4) with sonication and vortexing. Water (1 vol) or 0.1 M sodium chloride(aq.) was added and the mixture was sonicated and vortexed for 2 min. The phases were separated with centrifugation at 1000 x g for 10 min. The upper, phospholipid-containing organic phase was carefully removed with a 9 in. pasteur pipet. To quantitatively remove phospholipids from the sample, the lower ganglioside-containing aqueous phase was reextracted with fresh organic solvent. The mixture was sonicated and vortexed a second time for two minutes and centrifuged to separate the phases. After the upper organic layer was removed, the lower aqueous phase was lyophilized to concentrate and remove all traces of solvent. The purified gangliosides were desalted on a 10 ml Sephadex G-50 column, as stated above, with the gangliosides collected in the void volume. The ganglioside fraction was lyophilized and taken up in a small volume of chloroform:methanol (1:1) with clarification by centrifugation if necessary. The authors stated that the gangliosides were now pure enough for analysis by HPTLC but we found frequent interfering impurities and low yields when applied to samples of 1 and 2 ml of serum or plasma.

Several modifications were attempted in order to increase the purity and the yield. Modifications of the partitioning step itself consisted of repeating the partition step a third time and altering the ratio of diisopropyl ether:n-butanol to 7:3. A third partition was used to remove any potential phospholipids not removed in the first two steps. The increase of diisopropyl ether in the organic mixture was derived from the authors' original report that the ratio of 6:4 was an optimization of both recovery of gangliosides and the extraction of phospholipids. The ratio of 7:3 reported a slightly higher recovery of gangliosides with a decrease in the removal of phospholipids. The ratio of 7:3 was used with two partitioning steps and mild alkaline methanolysis, as described above, was used to destroy any phospholipids that still remained after the partitioning. The use of both a 10 ml column of Sephadex G-50 and SepPak C_{18} cartridges, both as described above, were compared for their ability to remove salts. Furthermore, a second Sephadex G-50 chromatography step was examined for its ability to increase the purity of the mixture. Lastly, a small column of DEAE-Sephadex A-25 (0.3 ml), utilized as stated above, was employed to remove residual neutral glycolipids, with a subsequent desalting by an above method.

Results and Discussion

The individual isolation procedures were analyzed and compared by one-dimensional high-performance thin-layer chromatography (1-D HPTLC). The purified gangliosides from serum were quantitatively applied to a HPTLC plate 1 cm from the edge and run in pre-equilibrated chambers until the solvent just reached the top. The plates were visualized with resorcinol spray and compared. Table 1 lists

the many different isolation procedures attempted. These include the main ganglioside purification step as well as the desalting method. Any additional steps have also been listed. They have been rated according to the yield and degree of purity of the procedure.

Modification of Total-Lipid Extraction

The initial step in the purification of gangliosides from serum typically involves extraction of all lipid material with chloroform:methanol in various ratios. We found that the use of 10 vol of chloroform:methanol per ml of serum was adequate for quantitative extraction of gangliosides from the serum. The use of lyophilization prior to the extraction was attempted so as to make subsequent drying steps more rapid. Dialysis of the serum and lyophilization was also examined as an additional purification step. We found that the recovery of gangliosides was considerably reduced when lyophilization, with or without prior dialysis, was used in conjunction with a particular isolation procedure. Based on this, the basic isolation of total-lipid material by chloroform:methanol 1:1 was employed with no modifications.

Chromatographic Isolation of Gangliosides

Many researchers have successfully obtained substantially pure preparations of gangliosides using procedures based on DEAE-Sephadex A-25 ion-exchange. DEAE-Sephadex removes the neutral and positively charged species and alkaline methanolysis is then used to destroy residual negatively charged phospholipids. Iatrobeads (silicic acid) chromatography is often used to remove other negatively

Table 1. Isolation of Circulating Gangliosides. All isolation based on 1 ml of plasma. Abbreviations: C:M -chloroform:methanol; Seph.- Sephadex; MAM - mild-alkaline methanolysis; DIPE/ButOH - diisopropyl ether/n-butanol; DEAE Seph - diethylaminoethyl Sephadex; (++++) - Excellent and (---) Very Poor.

Table 1.

Total-lipid extraction	Isolation of Gangliosides	Desalting	Other	Yield	Purity
2x 10 vol C:M 1:1	DEAE-Seph + MAM	Seph G-50		+	+ +
		SepPak C_{18}		+	+ +
		Dialysis		+	+ +
	Bond Elut NH_2	SepPak C_{18}	MAM + SepPak C_{18}	- - -	- - -
	Bond Elut Silica Gel + MAM	SepPak C_{18}		- - -	- - -
	2 x DIPE/ButOH 6:4	Seph G-50		+ + +	+ + +
		SepPak C_{18}		+ +	+ + +
	3 x DIPE/ButOH 6:4	Seph G-50		+ + +	+ + +
	2 x DIPE/ButOH 6:4	2 x Seph G-50		+ +	+ + +
		Seph G-50	DEAE Seph + G-50	+ +	+ + + +
	2 x DIPE/ButOH 6:4 + MAM	Seph G-50		+ + +	+ + +
	2 x DIPE/ButOH 7:3 + MAM	Seph G-50		+ + + +	+ + +
	2 x DIPE/ButOH 7:3 + MAM	Seph G-50	DEAE Seph + G-50	+ + +	+ + + +
Lyophilization	2 x DIPE/ButOH 6:4	Seph G-50		-	
Dialysis				-	
Rotary Evap.				+	

charged lipids such as sulfatides and free fatty acids. This procedure was based on larger samples of plasma (10-50 ml); however, the authors state that it can successfully be scaled down to one-third the size (4). We attempted to repeat this result using 1 ml of plasma and column sizes of approximately one-fifth (2 ml), one-tenth (1 ml), and one-thirtieth (0.3 ml) the original size. Furthermore, the last Iatrobead chromatography was not used because the sample would be pure enough for HPTLC with only the previous steps. The potential contaminants of free fatty acids and sulfatides do not appear purple with resorcinol spray and can be removed from the area of interest by 2-D HPTLC (See below). The results following these modifications were not encouraging. We found that while the purity was acceptable, the recoveries were less than what was necessary for our studies. The nature of the losses was unknown. The first wash of the DEAE-Sephadex did not appear to have significant amounts of gangliosides as determined by HPTLC, yet it was difficult to be certain about this point, due to the amount of neutral and positively charged lipids. The addition of higher salt-containing solvents did not increase the yield. While this method had potential usefulness for the isolation of small concentrations of gangliosides, it was abandoned for the sake of time and simplicity.

The use of solid-phase extraction for the isolation of biological materials is an emerging science that has found many uses due to its intrinsic simplicity and facility. We attempted to use this technology to isolate gangliosides from serum. The procedure is based on the method of ion-exchange chromatography, as is the procedure based on DEAE-Sephadex. The Bond Elut cartridges are made of aminopropyl functional groups on a solid support of silica gel as the weak anion-

exchanger. The negatively charged gangliosides are bound to the solid-phase while contaminants are eluted with a variety of solvents of increasing polarity and salt concentration. We found the protocol to be very poor both in terms of recovery and purity. Resorcinol-positive purple bands corresponding to gangliosides were barely visible amidst all the non-resorcinol positive spots. It was determined that small homemade columns of DEAE-Sephadex, albeit not perfect, were far superior in terms of both purity and yield, than commercially available solid-phase extraction cartridges.

Isolation of Gangliosides by Diisopropyl Ether:n-Butanol Partition.

In 1983, Wong and Ladisch (6) found that a technique to delipidate serum by a partitioning between diisopropyl ether, butanol and water (7) was not useful for the removal of gangliosides with the other lipid material. In fact, they found that gangliosides partitioned almost quantitatively into the aqueous phase while phospholipids and less polar neutral lipids partitioned into the organic phase. Taking advantage of this observation, they produced a protocol for the isolation of gangliosides from plasma or serum (2, 3). The authors stated that this method was suitable for qualitative work when used on 1 ml samples and qualitative and quantitative if 2 ml of plasma or serum were used. Furthermore, the authors admitted that there was approximately a 14% loss of GM_3, the least polar of plasma gangliosides, into the organic phase. With GM_3 being the most abundant ganglioside in plasma, 50-70% of the total, this becomes important. Senn *et al.* (8) studied this method extensively and found that the loss of GM_3 into the organic phase increased

with decreasing sample size. They found that with samples of plasma as large as 5 ml, up to 24% of the GM_3 was preferentially partitioned into the organic phase. This suggests that samples of plasma or serum that were smaller than 5 ml would suffer substantial losses. In addition, they found the need for an additional step of DEAE-Sephadex chromatography to remove residual neutral glycosphingolipids in order to obtain HPTLC chromatograms clean enough for densitometry. Our findings were similar to those of Senn *et al.* (8). We experienced similar problems with purity and recovery of small samples. Several attempts were made at modifying the Ladisch procedure to increase recoveries from smaller amounts of serum because of the many desirable characteristics of this method. These approaches have been summarized in Table 1.

Initial modifications, additional partitions and/or additional chromatographic steps on Sephadex G-50, were attempted to increase the degree of purity but met with no success. Passing the semipure mixture obtained from the partition over a small DEAE-Sephadex column and subsequent desalting on Sephadex G-50 was very useful in removing many contaminants; however, they led to a slight decrease in the recovery. Ladisch and Gilliard (3) observed that altering the ratio of DIPE and n-ButOH to 7:3 from 6:4, respectively, increased slightly the partition of gangliosides into the aqueous phase. This came at the cost of increased phospholipids partitioning into the aqueous phase. We utilized this information and did the partition with DIPE:n-ButOH ratio of 7:3, compensating for the increased phospholipid contamination by utilizing mild-alkaline methanolysis to destroy the residual phospholipids. The resulting FAMEs were removed by extraction with hexane. We

found that after desalting on a Sephadex G-50 column, our recoveries were much greater and had higher proportions of GM_3. The apparent decrease in the polarity of the organic phase resulted in a more quantitative partition of the less polar gangliosides, such as GM_3, into the aqueous phase. Furthermore, the troublesome interfacial fluff that makes quantitative removal of the organic phase difficult was rarely encountered, aiding in the complete removal of the organic phase.

Conclusion

The goal for this particular part of this study was to identify or develop method(s) for the isolation of gangliosides from serum. It was to be simple, quantitative, and able to handle large numbers of small amounts in a reasonable amount of time. After examining several different methods and their potential modifications, it was determined that a modified method of Ladisch *et al.* (3) best fulfilled the requirements. Briefly, 1 ml aliquots of plasma or serum were placed in 15 ml screw-capped test tubes. Methanol (5 ml) was added with vortexing and sonication to produce a fine suspension. Chloroform (5 ml) was added with vortexing and sonication, then the mixture was placed on a bed rocker in a cold room for at least 6 h. The samples were centrifuged at 2600 rpm for 20 min to pellet the insoluble material. The supernatants were removed to 100 ml round bottom flasks and rotary evaporated untill only a small volume was left. These were then transferred to 15 ml test tubes with small amount of chloroform:methanol (1:1). The pellets were reextracted with 10 ml of chloroform:methanol for an additional 12 h. Again, the supernatant was removed and rotary evaporated to a small volume, and

combined with the previous supernatants. The volumes were then adjusted to approximately 5 ml and the tubes placed in a -70 °C freezer for at least 4 hours to precipitate any slightly soluble proteins or peptides. After centrifugation, the supernatants were collected and dried under a stream of nitrogen and oil-pump vacuum. The residues were taken up in 2 ml diisopropyl ether:n-butanol (7:3) with vortexing and sonication untill a fine suspension was achieved. Distilled, deionized water (1 ml) was added and the mixtures vortexed and sonicated for at least 2 min. The mixtures were centrifuged for 10 min to separate the phases. A 9 in pasteur pipet was used to remove the upper organic phase. The lower aqueous phase was reextracted with 2 ml of fresh organic solvent, vortexed and sonicated, centrifuged, and the organic phase removed. The lower, ganglioside-containing, aqueous phases were lyophilized to concentrate and remove any traces of organic solvent. The semipure mixtures of gangliosides were then subjected to mild-alkaline methanolysis in 0.1 N sodium hydroxide in methanol for 2 h at 37 °C. After neutralizing with 1 N acetic acid, the samples were extracted twice with hexane to remove any fatty acid and other neutral contaminants. The samples were quickly dried under a stream of nitrogen, taken up in 0.3 ml water, and applied to 10 ml Sephadex G-50 columns. The columns were run at a flow rate of <0.5 ml/min and the gangliosides were collected in the void volume, as determined by blue dextran. After lyophilization, clarification and concentration, the samples were ready for analysis by HPTLC. However, for analysis by gas chromatography, an additional step using DEAE-Sephadex was necessary to remove the residual neutral glycolipids and other contaminants. This step also required desalting on a 10 ml Sephadex G-50 column.

We found these methods to be quite suitable for the needs of this study.

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CHAPTER 3

Characterization of Gangliosides by Two-Dimensioal High-Performance Thin-Layer Chromatography (HPTLC)

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Introduction

Thin-layer chromatography has become a universal tool for the separation and identification of gangliosides. Technological advances in the sorbents and solvent systems, as well as the means of detection and quantitation, have had a tremendous role in the development of glycolipid science. Commercial high-performance thin-layer plates allow for analysis of much smaller amounts of lipid with much better resolution than earlier home-made thin-layer plates. Scanning densitometry has simplified the quantitation of minute quantities of gangliosides that have been resolved by one-dimensional high-performance thin-layer chromatography (1-D HPTLC) with reproducible results. More recently, image densitometry has simplified HPTLC even more. Gangliosides analyzed on 1-D HPTLC and visualized by a resorcinol-HCl spray can easily be resolved and quantitated with image densitometry. The HPTLC plate is scanned by a camera scanner and digitized into a computer image. Then, specialized software computes the size and optical density of the bands and integrates them to produce an integrated optical density. Image densitometry also makes the analysis of two-dimensional high-performance thin-layer chromatography (2-D HPTLC) much easier and useful. Resorcinol-positive spots can be detected and quantitated in the same manner as 1-D HPTLC. This becomes important when one realizes the qualitative differential resolving power of 2-D HPTLC without a suitable method for quantitative analysis.

In this portion of the study, both 1-D and 2-D HPTLC are examined for their usefulness in analyzing gangliosides isolated from plasma. Various solvents and

running conditions are explored for use with both 1- and 2-D HPTLC. An image densitometer, or image analyzer, is described and its usefulness explained. In addition, a map of standard gangliosides as well as some rare gangliosides found in serum and tumor tissue is created using 2-D HPTLC. This map will be useful in the identification of gangliosides in complex mixtures such as those found in human serum.

Materials and Methods

All gangliosides were obtained from Sigma (St Louis, MO) except as follows. Ganglioside GM_4 was isolated from chicken liver according to the method of Shiraishi and Uda (1). Gangliosides GD_3, GM_{1b}, GT_{1a}, and GT_2 were a gift from Dr. R.K. Yu of the Medical College of Virginia, Virginia Commonwealth University. Gangliosides 6C, sialyldi-Y-2, sialyl Le^x, sialyl Le^a, 2,6-sialylparagloboside, sialylnorhexaosylceramide, and sialyl I were a gift from Dr. E. Nudelman of the Biomembrane Institute. Ganglioside N-glycoyl-GD_3 was a gift of Dr. S. Handa of the Tokyo Medical and Dental University.

All solvents were of analytical grade or better. High-performance thin-layer plates (Kiesel gel 60, 10 x 10 cm) were obtained from Merck (Darmstadt, Ger). The resorcinol spray was made according to Svennerholm *et al* (2, 3). Briefly, 5 ml of a stock solution of resorcinol (2 g of resorcinol recrystallized from benzene in 100 ml of water) is added to 40 ml of concentrated hydrochloric acid containing 0.125 ml of 0.1 M copper sulfate and the total volume brought to 50 ml with distilled, deionized water. This reagent is stable at 4 ^{o}C until it turns green. The stock solution is stable

for many months at 4 °C.

High-Performance Thin-Layer Chromatography

All HPTLC plates were prerun in methanol-diethyl ether (1:1) and preactivated by heating at 110 °C for 30 min prior to use. Plates were used immediately after preactivation.

For 1-D HPTLC, a solution of gangliosides was spotted in bands 5 mm wide, 1.5 cm from the bottom edge of the plate in microdroplets with a 10 μl Hamilton syringe. By placing the bands 2 mm apart, up to 13 different samples could be analyzed on the same plate. In many cases, 1500 pmol of GM_4 ganglioside is run with the test samples for quantitation purposes. Several different chromatography solvents were tested in order to determine the most suitable solvent system for our purposes, as follows: a) chloroform-methanol-0.2 % $CaCl_2 \cdot 2H_2O$ (aq) (60:40:9), b) chloroform-methanol-2.5 N NH_3(aq) (60:40:9), c) chloroform-methanol-0.1 M KCl (aq) (60:40:9) and d) chloroform-methanol-2.5 N NH_3 and 0.1 M KCl (60:40:9). In all cases, the solvent (35 ml) was placed in a wicked (Gel Blot Paper, Schleicher and Schuell, Keene, NH) metal chromatography chamber (Aldrich, Milwaukee, WI.) and allowed to equilibrate for at least 2 h. The plate was placed in the chamber and allowed to develop until the solvent front reached a line 1.5 cm from the top of the plate. The plate was removed and thoroughly dried in a 34 °C drying oven. After the plate was fully dried, it was ready for visualization and analysis.

In the case of 2-D HPTLC, the plates were prepared in a similar way as described above. Ganglioside mixtures were spotted in one corner, 1.5 cm from each

edge. The spotting was done conveniently with a 10 μl Hamilton syringe. Ganglioside GM_4 (1500 pmol) was applied directly on top of the spot containing the ganglioside mixture. Once again, several different solvent systems were considered to determine the one best-suited to the needs of this study. The different solvent systems considered were a) chloroform-methanol-0.2% $CaCl_2$(aq) (50:40:10) and chloroform-methanol-2.5 N NH_3 (aq) (50:40:10) (4), b) chloroform-methanol-0.25% KCl and chloroform-methanol-2.5 N NH_3 and 0.25% KCl(aq) (50:40:10) (4), *c)* *n*-propanol-0.1% $CaCl_2$(aq) (80:20) and *n*-butanol-pyridine-0.1%$Cacl_2$(aq) (60:45:15) (5), and d) chloroform-methanol-0.2% $CaCl_2$(aq) (50:40:10) and *n*-propanol-11.4 N NH_3(aq) (2:1) (6). The solvent for the first dimension is listed first and the second dimension listed second. In order to achieve the highest degree of reproducibility between plates in different runs and within the same run, the running conditions were made as similar as possible. Taking this idea further, up to 10 plates were run at one time. This was done in multiple 600 ml Pyrex beakers, wicked with Gel Blot paper, and containing exactly 35 ml of solvent each. Watch glasses (6 in. diam.) were conveniently used as lids and the spouts taped to insure a proper seal. The chambers were allowed to equilibrate for at least two hours prior to use. The plates were placed in the chambers and allowed to develop until the solvent front just reached a line etched in the surface, 1.5 cm from the edge of the plate. At that point, the plates were removed and allowed to dry thoroughly in a 34 °C drying oven. In the meantime, the pyrex beakers were emptied and the wicks removed. After the beakers had dried, new wicks were placed in the beakers along with 35 ml of the second dimension solvent. The chambers were again allowed to equilibrate for at least 2 hr

before use. The dried plates were then rotated 90° from the first direction and placed in the chambers and developed until the solvent front reached a second line etched 1.5 cm from the edge of the plate. The plates are removed and dried in the drying oven.

Visualization of Gangliosides

The visualization procedure for gangliosides on HPTLC plates was the same for both 1- or 2-D HPTLC. A non-destructive method involved placing the plate in an iodine chamber, which stains the gangliosides and other lipids yellow. After marking the presence of ganglioside, the iodine can be removed by allowing the plate to sit in the open air for the iodine to evaporate from the spots. Coomassie Brilliant Blue staining of the gangliosides is also possible (7). Plates are submersed in a 20% methanol solution containing 0.03% Coomassie Brilliant Blue R. Destaining is done in a 20% methanol solution. Resorcinol-HCl spray, a destructive method which is specific for sialic acid, is the most useful for the analysis of gangliosides. Plates are sprayed lightly with the resorcinol spray in an air-powered atomizer, covered with a clean glass plate, and placed in a 110 °C oven for 10 minutes. Gangliosides are visualized as deep bluish-purple bands or spots.

Two-Dimensional Mapping

A two-dimensional map of standard gangliosides was created with HPTLC for use in identifying gangliosides found in human serum. After a core framework was developed with a mixture of standard gangliosides, the more unusual gangliosides

were included and their migrations, relative to the framework, recorded. When necessary, the BioImage Visage 110 image analyzer was used to map and compare gangliosides on HPTLC plates using overlap and triangulation software.

Image Densitometry

Image densitometry was utilized to quantitate the resorcinol-positive spots from both 1- and 2-D HPTLC plates. The plates were scanned by a Visage 110 BioImage image analyzer (Millipore, MA). The resulting computer image was 1024 pixels by 1024 pixels, with 155 different color shades. An integrated optical density value was obtained by measuring the optical density and integrating it over the area of the spot. The linearity of the relationship between the image densitometric response and the ganglioside content was assessed using mixtures of gangliosides GM_3, GM_1, GD_3, and GD_{1a}. The densitometric response was relative to that of the internal standard GM_4.

Results and Discussion

Both one- and two-dimensional high-performance thin-layer chromatography were considered for the characterization of gangliosides isolated from human serum and plasma. One-dimensional HPTLC is inherently simpler and more able to handle multiple samples but this can be at the cost of resolving power. Up to 13 different samples can be assayed on a single plate in one dimension while 2-D HPTLC can be run on only one sample per plate. The value of 2-D HPTLC comes as much greater resolving power over 1-D HPTLC. Figure 1 shows the two-dimensional map created

for the identification of gangliosides isolated from serum, along with a photograph of an actual 2-D HPTLC plate in which gangliosides from 1 ml of serum have been run. Table 1 lists all the gangliosides, their source, and means of identification. By looking at the standard map, one can easily see the resolving power of two-dimensional chromatography. Very complicated mixtures of gangliosides can be resolved and identified using 2-D HPTLC. The solvent system of chloroform-methanol-0.2% $CaCl_2$(aq) (50:40:10) in the first dimension and n-propanol-11.3 N NH_3 (aq) (2:1) in the second could potentially separate 25 different species of gangliosides, as shown in Figure 1. This solvent system was chosen over the others mentioned in the materials section for its exceptional resolving power and its reproducibility. Furthermore, when using this solvent system, gangliosides that were identical with respect to the oligosaccharide portion of the molecule were observed as a single band. Many other solvents resolve gangliosides that are alike in the oligosaccharide into two or more bands based on their fatty acid chain length and presence or absence of hydroxyl groups. This can help simplify an otherwise complicated mixtures of gangliosides differing in their fatty acid composition. The 2-D pattern of spots was very consistent from run to run when certain precautions stated above were followed. Of particular importance was the removal of the plate as soon as the solvent front reached the mark. Allowing the solvent to "flow over the top of the plate" could cause distortions that were difficult to resolve. For the one-dimensional analysis, the solvent of chloroform-methanol-2.5 N NH_3 and 0.25% KCl (aq) (60:40:9) was found to be the most suitable, for reasons stated below, in terms of linearity and reproducibility.

Figure 1. Two-Dimensional Map of Gangliosides. Standard gangliosides were mapped out in two dimensions using chloroform-methanol-0.2% $CaCl_2$ (aq) (50:40:10) in the first dimension and n-propanol-11.3 N NH_3(aq) (2:1) in the second. Gangliosides found in serum or plasma, as shown in the photograph, have been shaded, while other gangliosides were left clear. Unidentified non-ganglioside material is represented as dark spots.

Figure 1a

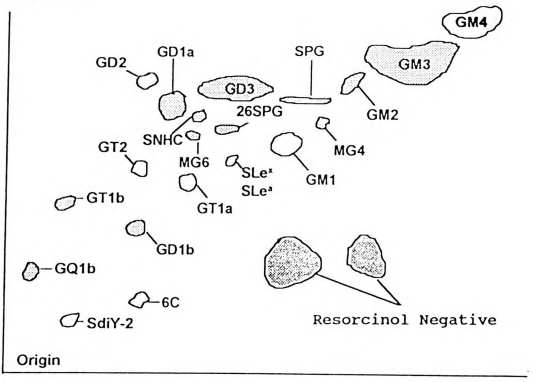


Figure 1b

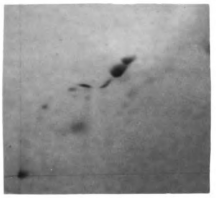
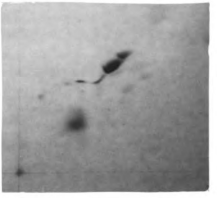


Figure 1c



mixture of gangliosides ranging from 10 pmol to 5 nmol per component The densitometric responses from the image analyzer were plotted and the results can be seen in Figures 2 and 3 for 1- and 2-HPTLC, respectively. The lines represent the results of a linear regression of the points corresponding to increased amounts of ganglioside based on lipid-bound sialic acid content. In all cases the values of R^2 (variance) were better than 0.99. However, when one compares Figure 2 with Figure 3, the superiority of 2-D HPTLC over 1-D can be seen in terms of molar densitometric response. The 2-D standard curve shows an equimolar response for equal moles of lipid-bound sialic acid. The 1-D curve shows a differential response for different gangliosides containing equal amounts of sialic acid. This could be a result of different band sizes or geometries following migrations. This conclusion was reported by Mullin *et al.* (8), who found that different solvents and different distances the solvent front travels can affect the densitometric response. They further reported that this was not due to a difference in extinction coefficients of the resorcinol reaction of sialic acids of different gangliosides on silica gel. Our results were similar to theirs. We also found that equimolar amounts of ganglioside that were spotted on an HPTLC plate in increasing size bands and not developed in a solvent had increasing densitometric response, when visualized with resorcinol (Figure 4). Furthermore, the solvent chloroform-methanol-0.2% $CaCl_2$ (aq) (60:40:9) produced curves like Figure 5 similar to the report of Mullin (8) while chloroform-methanol-2.5 N NH_3 and 0.25% KCl (aq) (60:40:9) produced much more linear plots (Figure 3.). These results suggest that solvent systems that differ in their salt content can have a considerable effect on the migration patterns and densitometric response.

Figure 2. Standard curve of gangliosides analyzed by 2-D HPTLC, based on lipid-bound sialic acid content. The solvent in the first dimension was chloroform-methanol-0.2% $CaCl_2$(aq) (50:40:10) and n-propanol-11.3 N NH_3(aq) (2:1) in the second. Gangliosides were visualized with resorcinol and quantitated by image densitometry based on 1.5 nmol GM_4 added as an internal standard. The inset box is an enlargement of the region of the plot from 0 to 1 nmol. Lines represent linear regressions of the points and are identified in the legend.

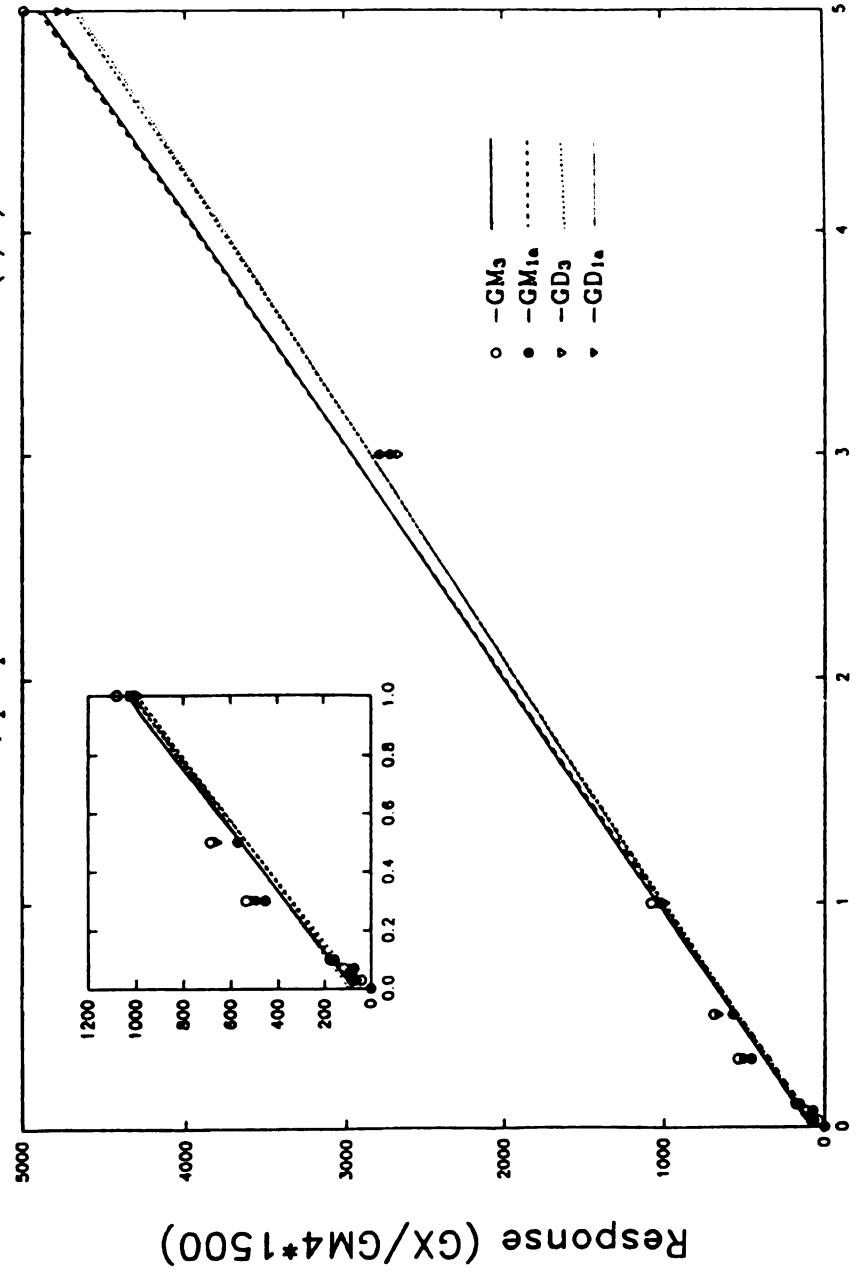

Standard Curve for 2-D HPTLC
Solvents: 1D; chloroform-methanol-0.2%CaCl2 (50/40/10)
2D; propanol-11.3 N ammonia (2/1)
Response (GX/GM4*1500)
Nanomoles of Lipid-Bound Sialic Acid
-GM3
-GM1a
-GD3
-GD1a

Figure 2.

Figure 3. Standard curve of gangliosides analyzed by 1-D HPTLC, based on lipid-bound sialic acid content. The plate was developed in chloroform-methanol-0.25% KCl and 2.5 N NH_3(aq) (50:40:10). Gangliosides were visualized with resorcinol and quantitated by image densitometry based on 1.5 nmol GM_4 added as an internal standard. The inset box is an enlargement of the region of the plot from 0 to 1 nmol. Lines represent linear regressions of the points and are identified in the legend.

Figure 3.

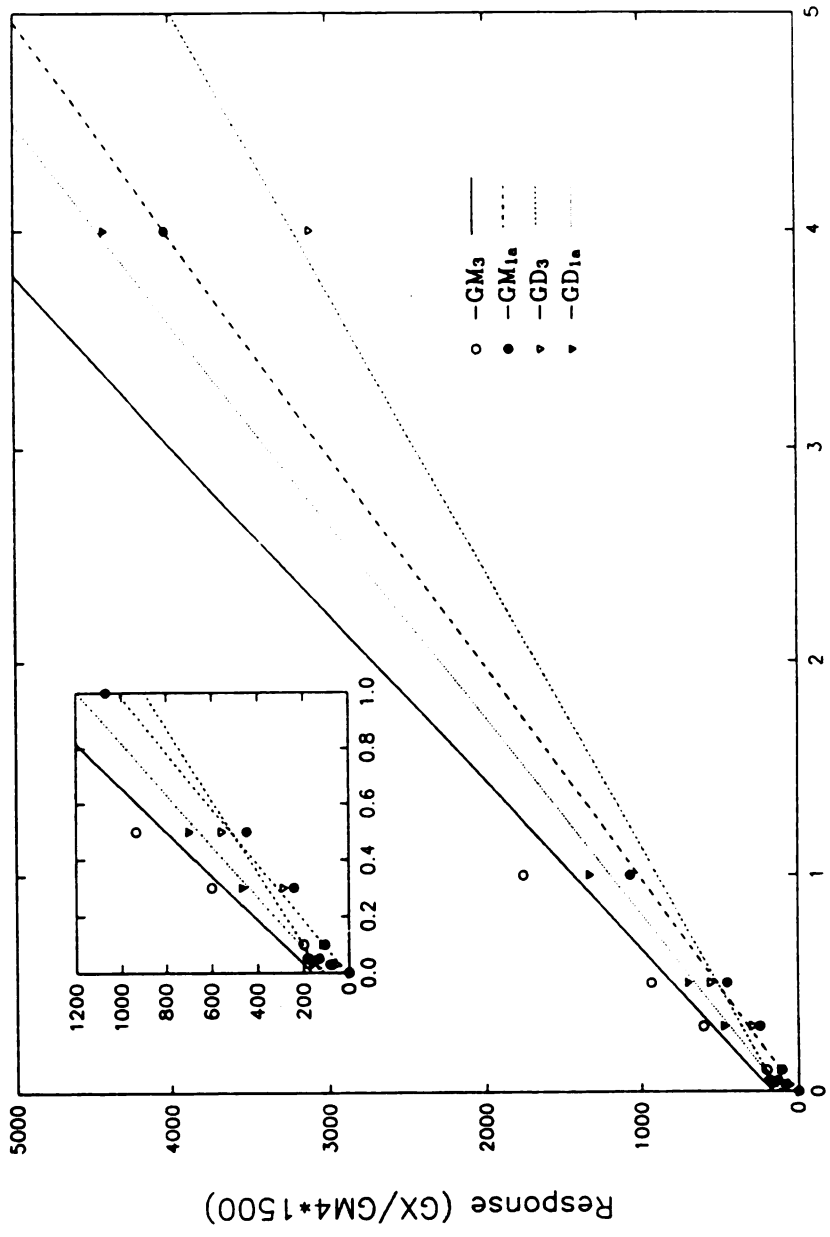
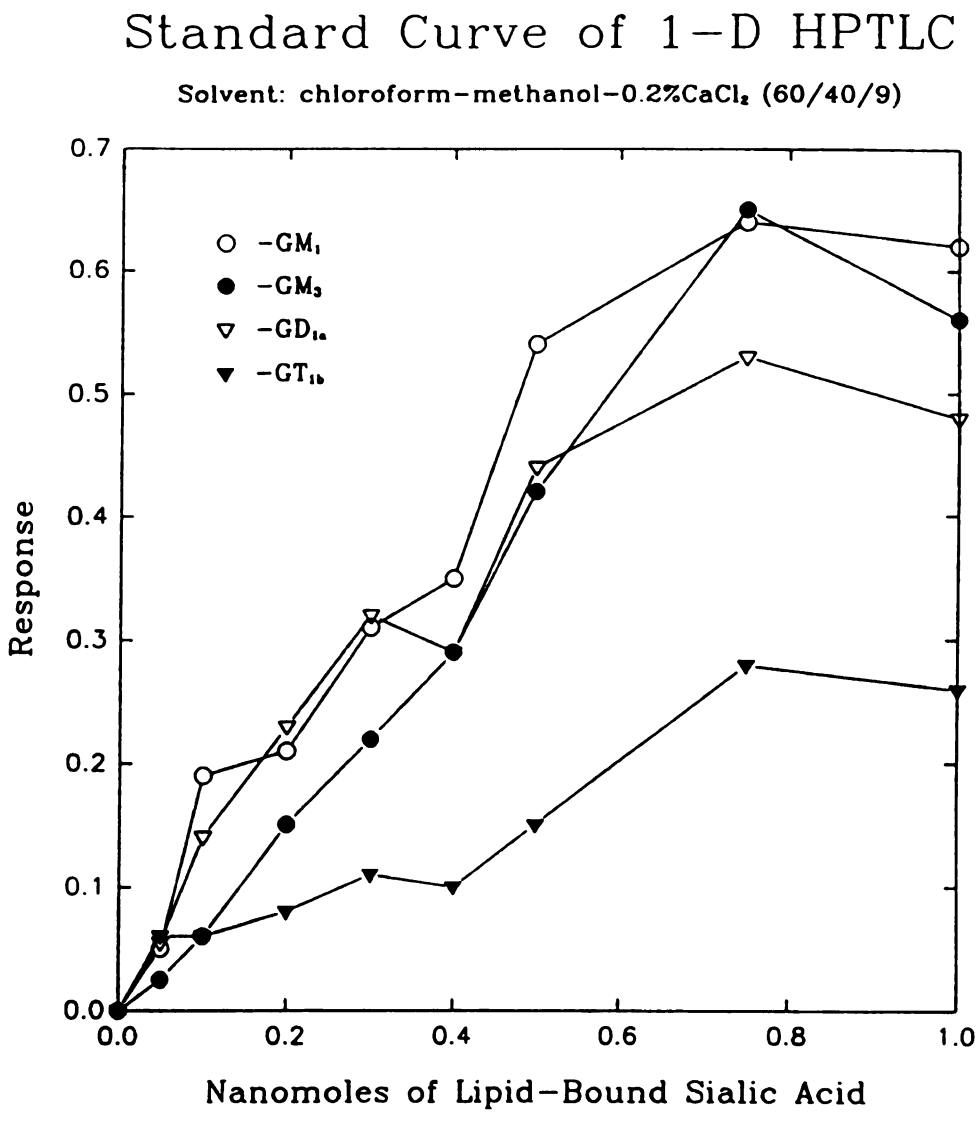

Standard Curve of 1-D HPTLC
Solvent: chloroform-methanol-0.25% KCl and 2.5N NH3 (50:40:10)
Response (GX/GM4*1500)
Nanomoles of Lipid-Bound Sialic Acid
-GM3
-GM1a
-GD3
-GD1a

Figure 4. Standard curve of gangliosides analyzed by 1-D HPTLC, based on lipid-bound sialic acid content. The plate was developed in chloroform-methanol-0.2% $CaCl_2$ (60:40:9). Gangliosides were visualized with resorcinol and quantitated by image densitometry.

Figure 4.


Standard Curve of 1-D HPTLC
Solvent: chloroform-methanol-0.2%CaCl₂ (60/40/9)
○ -GM₁
● -GM₃
▽ -GD₁ₐ
▼ -GT₁ᵦ
Response
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.0
0.2
0.4
0.6
0.8
1.0
Nanomoles of Lipid-Bound Sialic Acid

Alkaline solvent systems have been suggested to improve the resolution of HPTLC (4, 6). Our results show that the use of both potassium salt and ammonia in the solvent system can improve the linearity of linear response when quantitated by resorcinol and densitometry. Figure 2 shows that in the 2-D HPTLC with a solvent system that contains salt in the first dimension and is alkaline in the second, somehow the shape and geometry of the bands are controlled in a uniform way so as to give an equimolar response of lipid-bound sialic response for equal moles of lipid-bound sialic acid.

Identification and Quantitation of Gangliosides from Human Serum

Gangliosides isolated from 1 ml of serum were concentrated into the bottom of a 6 ml conical centrifuge tube. GM_4 (1500 pmol, 3 μl) was added and the entire sample was placed on the corner of the plate in a quantitative manner. The plate was run in two-dimensions as described above, stained with resorcinol and analyzed by image densitometry. Figure 1 shows a photograph of a typical 2-D HPTLC plate of gangliosides isolated from serum. Figure 1 also shows the map created in with 2-D HPTLC and the location and identification of gangliosides found in serum. Further information as to the quantitation of the gangliosides will discussed in detail in Chapter 6.

Conclusion

In this study we have presented a useful, reliable method for the thin-layer chromatographic analysis of gangliosides isolated from serum or plasma. We suggest

that the methods described here would also be useful for the analysis of other biological tissues or fluids. The solvent system of chloroform-methanol-0.2% $CaCl_2$ (aq) (50:40:10) in the first dimension and n-propanol-11.3 N NH_3 (aq) (2:1) adequately resolved the 13 or more different gangliosides found in serum. This, combined with the use of image densitometry, provided linear responses from 30 pmol to 5 nmol lipid-bound sialic acid. As little as 10 pmol was sometimes detectable but not reproducibly. Spots corresponding to gangliosides having lipid-bound sialic acid concentrations lower than 100 pmol should be viewed with caution due to high standard deviations. Image densitometry was also useful in developing the map from which the identifications of unknown gangliosides were made. The images obtained by scanning the 2-D HPTLC plates are easily saved indefinitely on magnetic tapes. Actual plates must be covered and frozen, yet still fade after time. These images can be compared to each other and even overlaid on the computer for help in locating the many spots found on the map. When necessary, the computer can compensate for minor distortions when two plates were being overlaid and compared. This method of analysis was useful in characterizing gangliosides isolated from the sera of breast cancer patients and an appropriate control group, as will be discussed in greater detail in Chapter 6.

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CHAPTER 4

Microscale Analysis of Glycosphingolipids by Methanolysis, Peracetylation and Gas Chromatography.[1]

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ABSTRACT

A method is described for the analysis of pure samples of individual glycosphingolipids by microscale methanolysis, peracetylation, and gas chromatography. Solvolysis of glycosphingolipids in dry methanolic HCl and peracetylation were conducted in a single 4.5 cm sealed capillary tube (2 mm i.d.), after which the products were directly injected into a gas chromatograph. Total-component analysis (i.e. analysis of the sugar, fatty acid, and sphingosine moieties) was possible after a 45 min chromatographic run. Time-course studies of the acid-catalyzed methanolysis of GM_1 ganglioside at 80, 110, and 150 °C showed that methanolysis was complete after 2 h at 110 °C. Rates of methanolysis of individual components were compared and the release of the fatty acid moiety from the long-chain base was shown to be the slowest reaction. The methanolysis of all glycosidic bonds was complete in 0.5 h. Peracetylated methanolysis products were very stable over time and provided for good gas chromatographic detection of subnanomolar amounts of hexose, hexosamine, fatty acid, sialic acid, and long-chain sphingoid base components. Recoveries of fucose and N-acetylglucosamine were determined with reference samples of fucosylα1-2lactose and lacto-N-fucosylpentaose II. Applications of the method are presented for the component analysis of a gift mixture of 6C ganglioside and sialyldi-Y-2 ganglioside and analysis of GM_3 isolated from human plasma.

Introduction

Glycosphingolipids (GSLs)[3] are of interest because of the several biological roles they have been proposed to have, such as in cellular interaction, differentiation, and oncogenic transformation (1). More than two hundred GSLs have been discovered and characterized (2). Analyses of their levels in physiological fluids and tissues are difficult, especially because of their presence as mixtures in very low amounts.

While it has been reported that the carbohydrate composition of glycosphingolipids cannot be determined by a single method (3), there are a number of different approaches for such analyses. Typical methods used for analysis of the carbohydrate moiety of glycolipids involve either acid-catalyzed hydrolysis or methanolysis. Hydrolysis is achieved using sulfuric acid (4), hydrochloric acid (5), trifluoroacetic acid (6), or acetic acid with sulfuric acid followed by conversion to alditol acetates and analysis by gas chromatography (GC) (7). Problems with this type of determination include the need for different conditions of hydrolysis for different carbohydrates and, often, the complete destruction of sialic acid. Furthermore, hydrolysis often requires complex, time-consuming work-up procedures.

Methanolysis in dry methanolic hydrochloric acid (HCl), followed by re-N-acetylation followed by trimethylsilylation and analysis by GC, is often used instead of hydrolysis and is the current method of choice in many laboratories. However, complete methanolysis may require 12-24 h, depending on the methanolysis conditions, and different strategies are often required for the complete recovery of

different components of both the carbohydrate and ceramide parts. To avoid losses of the methyl glycosides during concentration, neutralization of the methanolysis mixture is required. This involves either trituration with silver carbonate (8, 9) or passing over an ion-exchange column (10-12), both of which are cumbersome and unsuitable with small samples. The problem of neutralization has been addressed somewhat for glycoproteins in a report by Chaplin (13) but other difficulties with this procedure and its application to GSLs have not been adequately addressed.

In this paper, we describe a somewhat different approach for the methanolysis, peracetylation, and gas chromatography of glycolipids, designed especially for use with very small samples. The method requires only 0.5 h for complete release of all sugar components and 2 h for total methanolysis of the amide linkage of fatty acid to the sphingoid base, with no need for neutralization. The use of peracetylation instead of trimethylsilylation for derivitization prior to GC results in stable derivatives with useful chromatographic properties.

Materials and Methods

Materials

All gangliosides were obtained from Sigma (St. Louis, MO.) unless otherwise stated. The gangliosides 6C (NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-Cer) and Sialyldi-Y-2 (NeuAcα2-6Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-Cer) were a generous gift of Dr. E. Nudelman of the Biomembrane Institute, Seattle WA. 2'-Fucosyllactose (2'FL; Fucα1-2Galβ1-4Glc) and lacto-N-fucopentaose II (LNFP; Galβ1-3(Fucα1-

4)GlcNAcβ1-3Galβ1-4Glc) were obtained from Oxford Glycosystems (Rosedale, NY). N-acetylneuraminic acid (sialic acid, NeuAc), glucose, and heptadecanoic acid were also obtained from Sigma. All solvents were of reagent grade or better, while chloroform and methanol were HPLC grade. Pyridine was distilled before use.

Methanolic HCl, 0.75 N, was prepared by bubbling gaseous HCl through methanol, then titrated with base to obtain the normality. This concentrated stock solution was stored at -20 °C and diluted with methanol to 0.75 N as necessary. The stock solution was titrated periodically to determine any loss of HCl. The stock reagent was stable for up to one month.

Acid-Catalyzed Methanolysis and Peracetylation.

Samples (10 pmol to 10 nmol) of a neutral glycosphingolipid or ganglioside were dissolved in chloroform:methanol (1:1) and transferred into capillary tubes, 2.5 cm x 2 mm, with one end sealed. The solvent was quickly removed from the tubes by placing them in a high vacuum (i.e. oil pump). Dry 0.75 N methanolic HCl (25 μl) and methyl acetate (5 μl) were added and the tops of the tubes were sealed in a flame. The sealed tubes were placed in an oven at the stated temperature for an appropriate time. Following heating, the tubes were allowed to cool briefly, one end scored and carefully cracked open. The methanolic HCl was quickly removed with a high vacuum. Methyl heptadecanoate (HDA, derived by solvolysis of heptadecanoic acid with methanolic HCl), 2.1 nmol in 5 μl of methanol, was added as the internal standard. Again, the methanol was removed *in vacuo*. Pyridine:acetic anhydride (1:1, 5 μl), made fresh daily, was added, the tubes were resealed in a flame, and the

peracetylation was allowed to proceed for at least 1.5 h at room temperature.

Gas Chromatography

Tubes were scored at one end and cracked open just prior to injection of aliquots into the gas chromatograph. The sample, in pyridine:acetic anhydride, was injected (1 to 3 μl) into an Hewlett Packard (Avondale, PA) 5890 gas chromatograph fitted with a 60 m DB-1 column (J & W Scientific, Folsom, CA) and a flame ionization detector. After injection, the temperature was held at 150 °C for 15 min, then raised to 300 °C linearly at 4 °C per min. Hydrocarbons (12-20, 24-30, even) were coinjected for use in the calculation of retention indices. Peak areas were measured with a Hewlett Packard 3392A integrator.

Derivatization Time and Temperature Optimization

Methanolysis of GM_1 ganglioside (8 nmol) was carried out in duplicate at temperatures of 80, 110, and 150 °C. The reaction was stopped after periods of 0 to 24 h by removing the tubes from heat, brief cooling, opening, and solvent removal *in vacuo*. The samples were analyzed by gas chromatography following peracetylation for 2 h. Yields of glucose, galactose, N-acetylgalactosamine (GalNAc) , fatty acids, sialic acid, and long-chain sphingoid base (LCB) were relative to that of the internal standard. Absolute amounts of these products can be derived from the results, provided accurate molar responses of each relative to internal standard is known.

Stability of the peracetylated derivative was determined by conducting the methanolysis of GM_1 for 2 h, peracetylation, and injecting at increasing times

following derivitization.

Isolation of GM_3 from Plasma

Gangliosides were isolated from plasma using a modified method described by Ledeen and Yu (14). Heparinized plasma was extracted twice with 20 vol of chloroform:methanol (1:1), the supernatants were combined and solvent removed under a stream of nitrogen. The total lipid extract was fractionated on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) and the ganglioside fraction was subjected to mild-alkali-catalyzed methanolysis in methanolic 0.1 N NaOH to destroy glycerophospholipids. After dialysis, the semipure mixture was applied to an Iatrobeads column (15 ml) to remove acidic contaminants. The pure gangliosides were then applied to high-performance thin-layer chromatography (HPTLC) plates (E. Merck, Darmstedt, Germany) which were developed in chloroform:methanol:2.5 N NH_3 (50:40:10). Ganglioside bands were identified by placing the dry plate in a chamber containing iodine crystals until the bands became visible. After allowing the iodine to sublime off, the GM_3 band was then scrapped off and eluted from the gel with chloroform:methanol:water (5:5:1). Dissolved silica and other contaminants were then removed with a 10 cm Sephadex G-50 column, eluted with water.

Results and Discussion

Gas Chromatography of Peracetylated Methanolysis Products of GM_1

Figure 1 shows a typical chromatogram of the peracetylated methanolysis products of GM_1 ganglioside (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer). GM_1 was chosen because it contains most of the components (except fucose and N-acetylglucosamine which have unique peaks) that one would likely find when studying a mixture of gangliosides. The separation and response of the different peaks is apparent. Table 1 shows the peaks and retention indices corresponding to the O-acetylated methanolysis products of galactose, glucose, N-acetylgalactosamine, sialic acid (N-acetylneuraminic acid), d18:1 and d20:1 sphingosines, and several fatty acids. For comparison, Table 1 also reports the retention indices from peaks corresponding to O-acetylated methyl fucoside, N-acetylglucosaminide, O,O,N-triacetyl dihydrosphingosine, and 1,3,4-tri-O-acetyl-N-acetyl phytosphingosine although they do not appear in this sample of GM_1. All peaks are easily detectable and quantitatable. A mix of hydrocarbons, C14 to C30, except for C20, was coinjected for use in the calculation of retention indices. None of the hydrocarbon peaks interfered with any of the peaks of interest except for overlap of dihydrosphingosine and C28 hydrocarbon. This can be overcome by the removal of C28 from the hydrocarbon mix if dihydrosphingosine is suspected. The use of methyl heptadecanoate as the internal standard was chosen to avoid interference with any of the glycolipid products. This internal standard had one centrally located peak in the chromatogram and was stable under the running conditions. Samples ranging from 10 pmol to 10 nmol were found to be reproducible and the peaks were linear in

Figure 1. Chromatogram of the peracetylated methanolysis products from GM_1 ganglioside. See Table 1 for peak identification and text for derivatization and chromatography conditions.

Figure 1.

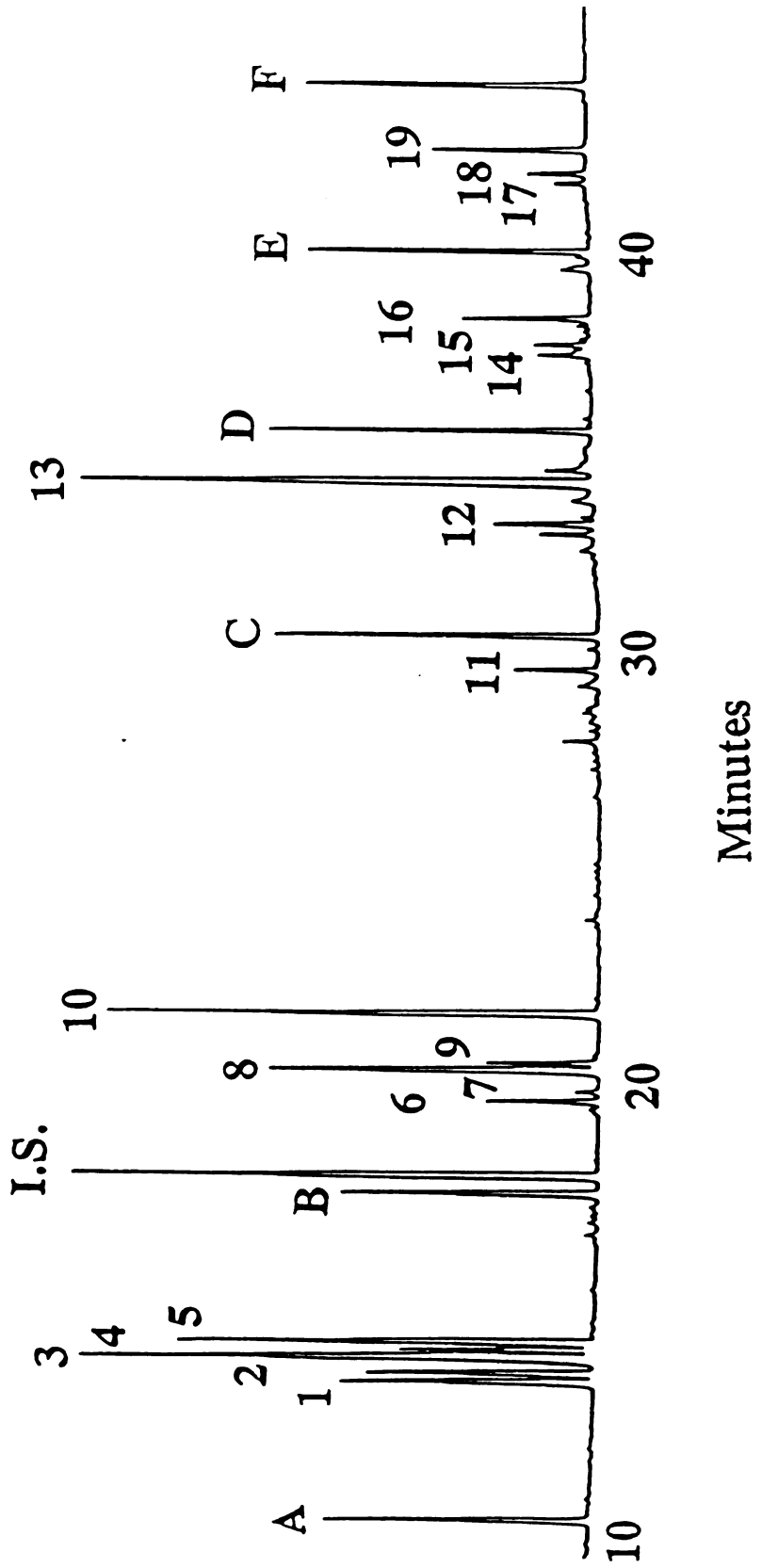

A
1
2
3
4
5
B
I.S.
6
7
8
9
10
C
11
12
13
D
14
15
16
E
17
18
19
F
10
20
30
40
Minutes

TABLE 1.

Gas Chromatography of Peracetylated Methanolysis Reference Compounds and Products from GM_1 Ganglioside.

Parent Structure	Derivative Structure	Peak[a]	Retention Index[b]
Fucose	Methyl α-L-fucopyranoside[c]		1613
	Methyl β-L-fucopyranoside[c]		1614
Galactose	Methyl n?-D-galactofuranoside	1	1886
	Methyl α-D-galactopyranoside	2	1891
	Methyl β-D-galactopyranoside	3	1999
Glucose	Methyl α-D-glucopyranoside	4	1904
	Methyl β-D-glucopyranoside	5	1910
N-Acetylgalactosamine	Methyl 2-acetamido-2-deoxy-n?-D-galactofuranoside(?)	6	2067
	Methyl 2-acetamido-2-deoxy-n?-D-galactofuranoside(?)	7	2073
	Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	8	2088
	Methyl 2-acetamido-2-deoxy-β-D-galactopyranoside	9	2094
N-Acetylglucosamine	Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside[c]		2101
Palmitic Acid	Methyl Palmitate (C18:0)	10	2127
	Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside[c]		2138
Arachidic Acid	Methyl Eicosanate (C20:0)	11	2331
Behenic Acid	Methyl Docosanate (C22:0)	12	2520
Sialic Acid	Methyl (methyl 5-acetamido-3,5-dideoxy-D-glycero-D-galacto-α-nonulo-pyranosid) onate	13	2565
Sphingosine	O,O,N-triacetylsphing-4-enine (d18:1)	14	2689
	3-O-methyl-O,N-diacetylsphing-4-enine[d]	15,16	2702,2731
	5-O-methyl-O,N-diacetylsphing-3-enine[d]		
Sphinganine	Dihydrosphingosine (d18:0)[c]		2800
4-Hydroxysphinganine	Phytosphingosine (t18:0)[c]		2879
C_{20}-Sphingosine	O,O,N-triacetyleicosasphing-4-enine (d20:1)	17	2891
	O-methyl-O,N-diacetyleicosasphing-4-enine[d]	18	2902
	O-methyl-O,N-diacetyleicosasphing-3-enine[d]	19	2931
	Hydrocarbons C18-20	A,B	1800,2000
	Hydrocarbons C24-30	C,D, E,F	2400,2600, 2800,3000

[a]Refers to Fig. 1
[b]Based on co-injected hydrocarbons;
[c]Not found in GM_1, but provided for comparison;
[d]The two O-methyl sphingosines (C18) are 3-O-methyl-Δ^4-sphingosine and 5-O-methyl-Δ^3-sphingosine; their order of elution from the GC column is unknown. The same two peaks were observed for eicosasphingosine (d20:1)

response. Actual recovery of pure glycosphingolipid when compared to recoveries of authentic methyl glycosides was found to be better than 95%.

The multiple peak profiles observed in Figure 1 are a result of the methanolysis reaction of carbohydrates which produces anomeric mixtures of methyl α- and β-glycopyranosides along with some glycofuranosides, as in the case of galactose. Fucose and N-acetylglucosamine also have multiple peak profiles representing different anomeric and ring forms resulting from methanolysis. With this method, fucose had only two major peaks as opposed to other reports (13,15). These peaks have all been identified in Table 1. Sphingosine (trans-Δ^4-sphingenine, d18:1) has three peaks resulting from methanolysis. The first two (see Table 1) are a result of O-methylation of the hydroxyl at the C3 carbon of the sphingosine molecule. The third is the peracetylated derivative of he sphingoid base. This was also seen with the 20 carbon homolog (4-eicosphingenine, d20:1). Both phytosphingosine and dihydrosphingosine had only one peak, which gives evidence of the need for the double bond adjacent to the hydroxy group for O-methylation. Other possible peaks in all cases were minor and not considered.

The ratio of the combined areas of the three galactose peaks to the combined areas of the two glucose peaks was 1.9. In the same way, the sialic acid peak and the sum of the four GalNAc peaks relative to the glucose peaks were 1.0 and 0.8, respectively. The total area of the fatty acid peaks was 1.36 relative to glucose and the total of the sphingoid base peaks was 0.31 relative to glucose. Thus, there was good agreement in the molar ratios of galactose, sialic acid, and glucose. Low galactose/glucose ratios, described previously (9, 14), were believed to have been due

to side reactions that result when complex methods are used for HCl removal. The use of peracetylation instead of trimethylsilylation allows galactose and N-acetylgalactosamine to be run together without overlap (free amine groups are, by nature of the reaction condition, converted to N-acetyl derivatives). The ratio of GalNAc peaks to glucose was somewhat low (0.81) but still in the stoichiometric range with glucose. The slightly low ratio could be a result of the multiplicity of peaks. There was no evidence for partially methanolyzed disaccharides containing N-acetylgalactosamine. Quantititation could be based on the sum of the peaks or a ratio with just the largest GalNAc peak being used for calculation. The four-peak profile has been shown to be useful in confirming the presence of GalNAc over other aminosugars such as N-acetylglucosamine, which has only two major peaks..

The fatty acid ratio was slightly high, which we attribute at least partially to the fact that the fatty acids are more reduced than sugars and consequently have a higher molar response in the flame ionization detector. The sphingoid bases had the lowest response of all, but the peaks were useful for qualitative and quantitative purposes. We assume the low response is due to side reactions (16) and decomposition.

Certain fatty acids interfere with certain sugars. That is, palmitate and galactose, unsaturated C18 fatty acids and N-acetylgalactosamine, and C24 fatty acids and the sphingoid bases overlap. This problem can be easily avoided by removal of the fatty acid methyl esters with hexane after methanolysis and prior to the addition of the internal standard. This modification has no effect on the recovery of the sugar moieties. The fatty acid fraction can be analyzed separately, if necessary.

Time and Temperature Optimization of Methanolysis Conditions

Aliquots of GM_1 were placed in the reaction tube with the methanolic HCl and methyl acetate, sealed, and subjected to temperatures of 80, 110, and 150 °C for times ranging from 0 to 24 h. Figure 2 shows the time course for methanolysis at 110 °C. It is apparent that the release of all sugars is complete after only 0.5 h while methanolysis of the ceramide is complete after about 2 h. At 80 °C, total release was achieved after 5 h; at 150 °C, release of the sugars was quite rapid but so was degradation of the peracetylated methyl glycosides (data not shown).

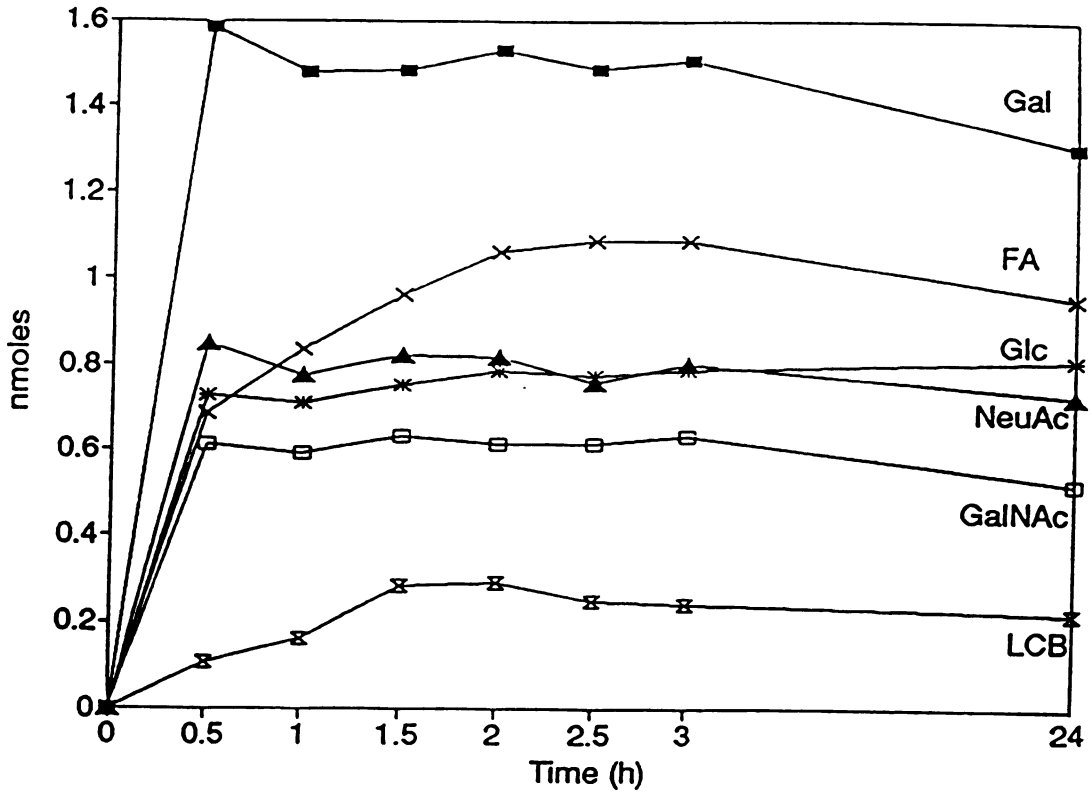
Standard conditions in the literature for methanolysis of glycosphingolipids has generally been 18-24 h at 80 °C. One report (17) described that the need for such long methanolysis times was due to the difficulty in breaking the glucose-ceramide bond. Our data show that in the sealed capillary tubes methanolysis of the glucose-to-ceramide bond was complete after only 0.5 h and that the limiting reaction was the release of the fatty acid from the sphingoid base. A possible explanation of the decreased time may be that in the sealed tube, the relatively small volume of the tube and head space after sealing maintains the HCl concentration in the methanol during the reaction.

Stability of Peracetylated Methanolysis Products.

To assess the stability of the peracetylated methanolysis products, samples of GM_1 were derivatized and run at increasing times following peracetylation. We found that the GC areas of the representative peaks were consistent over a period of one week. It was also determined that peracetylation was complete after only 2 h (data

Figure 2. Methanolysis time course of GM_1. Gal: galactose; Glc: glucose; FA: fatty acid; NeuAc: sialic acid; GalNAc: N-acetylgalactosamine; LCB: sphingoid base.

Figure 2.


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
nmoles
Gal
FA
Glc
NeuAc
GalNAc
LCB
0
0.5
1
1.5
2
2.5
3
24
Time (h)

not shown)

Glycoconjugates Containing Fucose and N-Acetylglucosamine

The method was evaluated for use with compounds containing fucose and/or N-acetylglucosamine. Because pure samples of appropriate glycosphingolipids were not available to us, commercial samples of fucosylα1-2lactose (2'-FL) and lacto-*N*-fucopentaose II (LNFP) were used for this study. Stock solutions of 2'-FL (2 mg/ml) and LNFP (1 mg/ml) were prepared in water. Aliquots of 2'FL (2.5 μl; 10.3 nmol) and LNFP (10μl; 11.7 nmol) were analyzed in triplicate after lyophilization in the standard methanolysis tubes. Methanolysis was at 110°C for 2 h.

Although pure gangliosides were not available to us, a very small amount of an equimolar mixture of ganglioside 6C (NeuAcα2-6Galβ1-4GlcNAcβ1-4Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcCer) and Sialyldi-Y-2 (NeuAcα2-6Galβ1-4(Fucα1-3)GlcNAcβ1-4Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcCer) was subjected to the standard procedure.

Table 2 shows the results of the carbohydrate analysis of 2' FL and LNFP as well as authentic GM_1 Ganglioside. Table 3 shows the carbohydrate analysis of the sample containing equimolar amounts of 6C and sialyldi-Y-2 gangliosides. The recovery (75-80%) of the fucose moiety was always below that expected. This may be attributed in part to the increased volatility of fucose (one less OH group than glucose) as well as, perhaps, to some degree of degradation. There recoveries and ratios of glucose and galactose were very close to expected except for cases in which an acetylated aminosugar was linked to a galactose residue. The de-N-acetylation of

TABLE 2.

Carbohydrate Analysis of Reference Oligosaccharides and GM_1

Compound	Component	Actual (nmol)	Recovery (nmol)	% Yield	Ratio to Glucose
2'FL	Fuc	10.3	8.3	81	0.82
	Gal	10.3	10.2	99	1.0
	Glc	10.3	10.1	98	--
LNFP	Fuc	11.7	8.8	77	0.77
	Gal	23.4	21.2	91	1.81
	Glc	11.7	11.7	100	--
	GlcNac	11.7	9.5	81	0.81
GM_1	Gal	1.62	1.50	93	1.90
	Glc	0.81	0.79	98	--
	GalNAc	0.81	0.62	75	78
	NeuAc	0.81	0.79	98	1.00

TABLE 3.

Carbohydrate Analysis of an Equimolar Mixture of 6C and Sialyldi Y-2 Gangliosides Plasma GM_3 Ganglioside

Ganglioside	Component	Ratio to Glucose	Theoretical Ratio to Glucose	Relative Yield
6C and Sialyldi-Y-2	Fuc	1.13	1.5	0.75
	Gal	3.13	3	1.0
	Glc	1	--	--
	GlcNAc	2.06	2	1.0
	NeuAc	1.0	1	1.0
GM_3	Gal	1.04	1	1.0
	Glc	1.0	--	--
	NeuAc	1.0	1	1.0
	Fatty Acid	1.75	1	1.75
	Sphingoid Base	0.43	1	0.43

the aminosugar during methanolysis makes its glycosidic bond more resistant to cleavage by methanolysis, hence a decrease in both the acetylated aminosugar and adjoining galactose. Moreover, it appears form our limited data that a de-N-acetylated galactosamine linkage may be more resistant to cleavage than a de-N-acetylated glucosamine linkage. The detection of disaccharides containing hexosamine and galactose were not found; however, such compounds may have retention times that were greater than the time span of our GC run. This problem was not seen in the analysis of the mixture of 6C and sialyldi-Y-2 gangliosides, which gave stoichiometric ratios (Table 3). The problem of varying recoveries can be addressed by the co-running of appropriate standards, using the same experimental conditions, and determining a conversion factor for each component, as should be done in any case. With these considerations, we believe that a wide variety of glycolipids and other glycoconjugate can be analyzed by this procedure.

Component Analysis of GM_3 Ganglioside isolated from human plasma.

GM_3 ganglioside was isolated from human plasma as described. Following methanolysis, the fatty acid methyl esters were removed by partitioning twice with hexane in the reaction tube. This was achieved with the use of a 10 μl Hamilton syringe. The upper phase was pulled into the barrel and expelled into the lower phase. After brief centrifugation to separate the layers, the upper hexane phase was removed. Methyl heptadecanoate (2.1 nmol in 5 μl) was added to the remaining lower phase as the internal standard, followed by peracetylation (5 μl pyridine:acetic anhydride, 1:1) for 2 h.

Gas chromatography and subsequent analysis of the peak areas from the plasma GM_3 gave the component ratios shown in Table 3. The observed ratios of the sugars were in good agreement with the composition of GM_3. The fatty acid ratio to glucose was higher than expected, beyond that explained by a higher detector response. This was perhaps due to slight phospholipid contamination. The types and relative abundance of fatty acids found in GM_3 in our sample agreed with reported data (18, 19), with palmitate and stearate (C16:0 and C18:0) being the most abundant and tetracosanoate and tetracosenoate (C24:0 and C24:1) being the next most abundant components.

Conclusion

Gas chromatography of neutral glycosphingolipids and gangliosides is often the first step in understanding the structures of these complex glycolipids. The oligosaccharide portion of the molecule has always drawn the most attention. Alterations in the structures of the oligosaccharide moieties with oncogenic transformation has been known for some time, as well as the antigenic specificity of this part of the molecule (1, 20). More recent studies have focused on the fatty acid and sphingoid base moieties and their importance in transmembrane signaling events. Spatial locations dictated by the ceramide moiety may determine how the course of enzymatic glycosylations (21). This is suggested by the fact that certain oligosaccharides tend to be associated with specific fatty acid mixtures. Furthermore, the presence of α-hydroxy fatty acids may also have an effect on the glycosylation pathway (20) and thus define certain facets of the neoplastic state. The need for a

simple, rapid, and comprehensive method for the analysis of the many different components of glycolipids is evident. We believe the method proposed herein will greatly facilitate the analysis of these interesting biomolecules.

ACKNOWLEDGEMENTS

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FOOTNOTES

[1] This work supported by NIH Grant #DK12434.

[2] To whom correspondence should be sent.

[3] Abbreviations used: GSL, glycosphingolipid; GC, gas chromatography; GM_3, NeuAcα2-3Galβ1-4Glcβ1-1Cer; HDA, heptadecanoic acid; GM_1, Galβ1-4GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer; 6C, NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-Cer; Sialyldi Y-2, NeuAcα2-6Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-Cer; 2'-Fucosyllactose; (2'FL) Fucα1-2Galβ1-4Glc, lacto-N-fucopentaose II, (LNFP) Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc, Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Cer, ceramide; LCB, long-chain base; HPTLC, high-performance thin-layer chromatography.

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CHAPTER 5

Potential Methods for Characterization of Gangliosides

The use of high-performance thin-layer chromatography and gas chromatography are well suited for the analysis of gangliosides isolated from biological tissues and fluids and they have been very useful in our studies of gangliosides from sera. However, these methods were not the only methods considered or tried. Other methods attempted were oligosaccharide analysis using high-pH anion-exchange high-performance liquid chromatography with pulsed amperometric detection (HPAE HPLC-PAD) and radioisotope labeling and autoradiography. In this chapter, these methods will be discussed briefly along with the work done and the observations made.

Oligosaccharide Analysis with HPAE HPLC-PAD

High pH anion-exchange high-performance liquid chromatography (HPAE HPLC) was developed for the analysis of mono- and oligosaccharides (1-6). It is an aqueous based HPLC system run in buffers of very high pH. Carbohydrates form oxyanion species at a pH of about 13, and are therefore separatable by anion-exchange chromatography used in this system. Pulsed amperometric detection (PAD) can successfully detect carbohydrates in the picomolar region. The alcohols of carbohydrates are easily detected by the electrochemical oxidation, with free radical intermediates adsorbing to the anode surface, reducing electrode current. Deposits

are removed by oxidative desorption with a pulse of high positive potential. Mono- and oligosaccharide analysis could be completed in under 2 h with identification done by the comparison of retention behavior with that of known standards. Ganglioside standards as well as complex mixtures of gangliosides could be analyzed in this way following the release of the oligosaccharides from their ceramide tails either chemically or enzymatically. The oligosaccharide mixtures could then be separated by HPAE-HPLC, identified and quantitated with the PAD, giving information on the oligosaccharides of the ganglioside mixture.

Experimental

Oligosaccharides from authentic gangliosides or mixtures of gangliosides isolated from plasma or serum were cleaved from their ceramide tails in one of several ways. One method explored was an enzymatic procedure that used endoglycoceramidase from *Rhodococcus* sp. (7, 8) or ceramide glycanase from earthworm (9) or leech (10) to cleave the intact oligosaccharide moiety from ceramide. Another method made use of ozonolysis of the olefinic double bond of sphingosine and alkali-induced degradation of the resulting aldehyde. If monosaccharides were desired, hydrolysis in trifluoroacetic acid was performed. After a brief clean-up procedure, the sample was injected into a Dionex Bio-LC fitted with a CarboPac PA-1 column and a Model PAD 2 detector. Samples were eluted in a buffer of 0.1 N sodium hydroxide and a gradient of 0 to 150 mmol of sodium acetate. Peak identifications were made by comparison with the retention times of authentic standards as well as reported values. Quantitation was based on the internal

standard, mannose.

Discussion

High pH anion exchange high-performance liquid chromatography coupled with pulsed amperometric detection has successfully been used to analyze mono- and oligosaccharides derived from glycoproteins (1-4, 6). The ability to resolve isomers differing only in anomeric configuration and/or linkages is useful in the structural analysis of many diverse oligosaccharides. However, there were some difficulties when this method was applied to the quantitative and qualitative analysis of gangliosides mixtures isolated from plasma.

A necessary first step for analysis of gangliosides is the release of the oligosaccharide moiety from the ceramide tail. Aside from the expected problems associated with clean-up, the enzymatic procedures suffered from a lack of specificity. It has been shown that the specificity of these enzymes can vary towards different ganglioside oligosaccharide structures (7) . Gangliosides with larger oligosaccharide moieties were not as rapidly hydrolyzed as their less complex counterparts. Therefore, it would be necessary to determine how the enzyme would behave towards a diverse mixture of gangliosides such as found in plasma or serum. Chemical release of the carbohydrate head group using ozonolysis would only be useful for species containing sphingosine in their ceramide moiety as the double bond at carbon 4 is necessary for the reaction to proceed. The yield of the ozonolysis reaction would have to be determined and considered.

The use of the HPAE HPLC-PAD system has great potential for structural

analysis of oligosaccharides. However, the day to day fluctuations in the performance of the instrument made quantitative and qualitative analysis difficult. Even with extreme care, retention times would vary unpredictably from run to run and more so day to day. Identification was based on retention indices, requiring daily running of retention standards to determine the current retention behavior and detector response. Quantitation on this system would be difficult due to each oligosaccharide having its own response factor, requiring several standard curves for estimations of oligosaccharide concentrations in a mixture. Analysis of the ceramide moieties would either have to be omitted or determined by another means. This method is clearly useful for semiqualitative and structural analysis of pure oligosaccharides and glycoconjugates, however, it becomes complicated when applied to the qualitative and quantitative analysis of complex mixtures of gangliosides.

Radioisotope Labeling, HPTLC, and Autoradiography

Costello *et al.* (11, 12) found that the use of microscale reactions carried out in the vapor phase for the analysis of glycosphingolipids increased the sensitivity when analyzed with fast atom bombardment mass spectrometry. Specifically, the reduction of amides to amines in the sphingosine moiety gave a much higher molecular ion signal. The first step in the reduction was the glycosphingolipid being reduced with borane vapor (BH_3). Next, solvolysis of the borane complexes was done in methanolic HCl vapor. Finally, oxidation of the organoborane was achieved with ammonia/hydrogen peroxide vapor. These reactions reduce amides to amines as well as reducing any olefinic bonds. It was observed that this reaction could be used

to label gangliosides with tritium by the reduction of amides, as well as any olefinic bonds, with tritiated borane. The labeled gangliosides could then be analyzed with HPTLC and autoradiography. This method had the potential of being extremely sensitive and able to detect minute alterations in ganglioside profiles.

Experimental

Gangliosides disolved in chloroform-methanol (1:1) were coated onto the inside of a capillary tube and dried in a vacuum. The ganglioside-containing capillary tube was placed in a special apparatus designed for vapor phase reactions (13). The apparatus was evacuated and the sample was reduced with the vapor of 1N borane (BH_3, BD_3, or BT_3) in tetrahydrofuran under reduced pressure for 2 h at 45 ^{0}C. The resulting borane complexes were solvolyzed in the apparatus in the same manner as above in methanolic HCl (0.5 M) replacing the borane/tetrahydrofuran for 3 h at room temperature. The methanolic HCl was evaporated under a vacuum and replaced with of 30 % hydrogen peroxide and 3 N NH_3 to oxidize the samples for 1 hour at 45 ^{o}C. These reactions were all conducted in the same apparatus with the excess reagents removed by evacuation before the next one is added.

Discussion

This method was under consideration for its ability to label minute mixtures of gangliosides without dramatic alteration of the structure. This would allow for a increase in the level of sensitivity of gangliosides detectable by conventional means far below the 50 pmol level. This is desirable in order to detect very minor

gangliosides that might be of interest in biological tissues and fluids. While this method could be potentially very useful in these studies, there were some important issues to be addressed.

The first and the most serious problem was in the protocol. In order for the reaction to proceed with a suitable yield, extremely high specific activities of tritiated borane gas would have to be used. In order to safely use a highly radioactive gas, already potentially harmful and reactive, would require an elaborate gas-train assembly to contain the reaction and the reagents. Several different designs were considered but none was found that satisfactorily dealt with the problem without complicating it.

As was stated, hydroboration and oxidation will reduce amides to amine and double bonds to single bonds. The original procedure was designed to be applied to very simple, pure glycosphingolipids, such as galactosylceramide, which may have one amine and possibly two double bonds, to be analyzed by mass spectrometry. The degree of unsaturation was not a concern. Mixtures of gangliosides isolated from biological sources most likely will have much more complex gangliosides containing one or more N-acetylated hexosamines. Furthermore, there is the possibility of heterogeneity in the ceramide moiety in both the sphingoid base and fatty acid, which may have some degree of unsaturatation. Since any amide bond or olefinic bond is capable of being reduced and therefore labeled, it is possible that GT_{1b} could have anywhere form 6 to 11 different sites for the reduction reaction while GM_3 might have only 2. Each tritium atom contributes to the detection signal and this could make efforts at quantitation difficult. Moreover, each little change in the structure,

albeit small, will give the ganglioside different migration properties on thin-layer. In essence, it would make an already highly complex mixture of gangliosides even more complicated. It was for these reasons that this method was not pursued.

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CHAPTER 6

Analysis of Circulating Gangliosides of Breast Cancer Patients and an Appropriate Control Group.

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Abstract

Gangliosides were isolated from the sera of recently diagnosed breast cancer patients and from individuals of an appropriate control group of women. Quantitative and qualitative analyses were by two-dimensional high-performance thin-layer chromatography (2-D HPTLC) and gas chromatography (GC). The profiles of the isolated gangliosides were determined by 2-D HPTLC, visualized with resorcinol, and quantified by digital image densitometry. The profile of the cancer patients was compared to that of the control, revealing an overall increase in total lipid-bound sialic acid and a specific increase in polysialogangliosides. Furthermore, an increase was noted in the b-series biosynthetic pathway over the a-series in the cancer sera, as compared to the controls. Gas chromatographic analysis of the peracetylated methanolysis mixtures derived from the total ganglioside fraction of cancer patients supported the HPTLC data, with an increase in the total sialic acid and galactose residues. No unusual gangliosides were found in either the normal or breast cancer gangliosides profiles. The observed increase in the polysialogangliosides, specifically the b-series gangliosides, is consistent with the idea that they may have a role in the metastasis of breast cancer.

Introduction

A recent trend in the research of many cancer types is the analysis of glycosphingolipids associated with the cancers. Oncogenic transformation has long been associated with an alteration in the composition of glycosphingolipids, both of the neutral type and gangliosides, in the plasma membranes of cancer cells (1-3). Numerous studies have indicated both direct and indirect roles of gangliosides in tumorigenesis (4-7). With the development of monoclonal antibodies against tumor-associated antigens, the presence of complex, more highly glycosylated or sialylated ganglioside tumor markers became more apparent. Magnani *et al.* (8) identified the first monoclonal antibody-defined ganglioside of colon cancer and a large number of antibodies to tumor-associated ganglioside antigens have subsequently been identified (Reviewed in 9, 10). Furthermore, gangliosides have been shown to be shed from these cancer cells into the local environment and eventually into the blood stream (11-14). Some researchers have tried to take advantage of this phenomenon by identifying the presence of unusual or increased levels of gangliosides in the plasma of cancer patients as a circulating tumor marker (12, 15, 16). Circulating tumor-associated antigens as well as tumor-associated tissue gangliosides have been examined for their use in antibody therapy (17, 18). A circulating tumor marker of this type may also be useful in the diagnosis and monitoring of breast cancer as well as potentially in the treatment of cancer. In this study, we examined the ganglioside content of several plasma and serum samples obtained from women suffering from ductal cell carcinoma of the breast and an appropriate control group to ascertain

whether such a circulating tumor marker may be present as well as to describe the nature of the ganglioside profile from serum samples of breast cancer patients.

Materials and Methods

Materials

All solvents were of reagent grade or better, while chloroform and methanol were HPLC grade and pyridine was distilled from a reagent stock before use. N-acetylneuraminic acid (sialic acid, NeuAc), glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and heptadecanoic acid were obtained from Sigma. DEAE-Sephadex A-25 and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). High-performance thin-layer chromatography plates (HPTLC) were obtained from Merck (Darmstedt, Germany). Reference gangliosides were obtained from Sigma (St. Louis, MO) unless otherwise noted. Ganglioside GM_4 was isolated from chicken liver according to the method of Shiraishi and Uda (5). Gangliosides GD_3, GM_{1b}, GT_{1a}, and GT_2 were a gift from Dr. R.K. Yu of the Medical College of Virginia, Virginia Commonwealth University. Gangliosides 6C, sialyldi-Y-2, sialyl-Le^x (SLe^x), sialyl-Le^a (SLe^a), 2,6-sialylparagloboside (SPG), sialylnorhexaosylceramide, and sialyl I were a gift from Dr. E. Nudelman of the Biomembrane Institute.

Resorcinol spray was made according to Svennerholm *et al.* (19). Briefly, 5 ml of a stock solution of resorcinol (2 g of resorcinol recrystallized from benzene in 100 ml of water) is added to 40 ml of concentrated hydrochloric acid containing 0.125 ml of 0.1 M copper sulfate and the total volume brought to 50 ml with distilled,

deionized water. This reagent was stable at 4 °C until it turns green. The stock solution was stable for many months at 4 °C.

Methanolic HCl, was prepared by bubbling gaseous HCl through methanol, then titrating the acidic methanol with base to obtain the normality. This concentrated stock solution was stored at -20 °C and diluted with methanol to 0.75 N as necessary. The stock solution was titrated periodically to determine any loss of HCl. The stock reagent was stable for up to one month.

Serum samples were obtained from women recently diagnosed with ductal cell carcinoma of the breast. All patients were in the early stages and had not received any treatment yet. Serum was obtained by allowing the patient blood to clot and immediately separating the serum from the red cells by centrifugation . The serum was stored at -70 °C prior to use. Plasma samples for the control group were obtained from women 22 to 63 years of age who were not currently menstruating. The blood was drawn after an overnight fast and treated with citrate to avoid clotting. The samples were also immediately separated and the plasma stored at - 70 °C prior to use.

Isolation of Gangliosides from Serum and Plasma.

A modified method of Ladisch *et al.* (20) was used for the extraction of gangliosides from serum and plasma. Briefly, 1 ml of serum or plasma was extracted twice with 10 ml of chloroform:methanol (C:M, 1:1), with the gangliosides collected in the supernatant and thoroughly dried. The residue was taken up in 2 ml of diisopropyl ether:n-butanol (7:3) and 1 ml of double de-ionized water. After

sufficient vortexing and sonication, the sample was centrifuged and the phospholipid-containing upper organic layer removed with a 9 in. pasteur pipet. The ganglioside-containing aqueous lower layer was reextracted with fresh organic solvent. After removal of the second organic layer, the aqueous layer was freeze-dried. Any remaining phospholipids were destroyed by mild alkaline methanolysis in 0.1 N NaOH in methanol for 2 h at 37 °C. The mixture was neutralized with acetic acid and quickly dried under a stream of nitrogen. A small Sephadex G-50 column was used to remove low molecular weight impurities and salts. Samples to be analyzed by gas-chromatography (GC) were further purified by DEAE-Sephadex A-25 column chromatography and again desalted on a Sephadex G-50 column.

Two-dimensional High-Performance Thin-Layer Chromatography (2-D HPTLC) of Gangliosides.

Reproducible two-dimensional thin-layer chromatography on silica gel was achieved by running multiple plates in separate, identical, wicked chambers containing like amounts of solvent, equilibrated for the same period, and run for the same amount of time at the same time. This type of control over external variables, and the solvent system of chloroform:methanol:0.2% $CaCl_2$ (50:40:10) in the first direction and n-propanol:11.3 N NH_3(aq.) in the second direction (21) provided very useful and reproducible 2-D chromatography. A two dimensional reference map was created of known standards and tumor-associated gangliosides for comparison to the plasma and serum 2-D ganglioside profiles (Fig 1a). Gangliosides from 1 ml of plasma or serum were spotted, along with GM_4 (1.5 nmol) as an internal standard, on

a 10 cm x 10 cm HPTLC plate near the corner and run in two-dimensions. Quantitation was performed on a Bio Image Visage 110 image densitometer and was relative to the GM_4 internal standard. Identification of gangliosides found in the serum was based on co-migration with authentic standards as well as HPTLC data found in the literature concerning serum gangliosides (22-27).

Gas Chromatography of Carbohydrate and Lipid Components of Gangliosides.

Ganglioside mixtures were derivatized for analysis by gas chromatography by the method of Wiesner and Sweeley (28). Gangliosides isolated from serum or plasma were placed in 2.5 mm x 4 cm capillary tubes and dried *in vacuo.* Hydrochloric acid (25 μl, 0.75 N) in dry methanol and methyl acetate (5 μl) was added, the tube sealed, and heated at 110 °C for 24 h. The tube was carefully cracked open and the solvent quickly evaporated under a strong vacuum. Fatty acid methyl esters were extracted with hexane and transferred to separate tubes for fatty acid analysis. Methyl heptadecanate was added as an internal standard. The methanolysis products were derivatized for GC with 5 μl pyridine:acetic anhydride (1:2) for 2 h. Gas chromatography was performed by a Hewlett Packard model 5890A gas chromatograph fitted with a 60 m DB-1 column (0.25 mm I.D. with a 0.1 mm film thickness). The sample (1-3μ) was injected onto the column at 185 °C; after 15 min the column was heated at 4 °C per min to 290 °C and held for 5 min. The sample was co-injected with a series of even hydrocarbons, C12 to C26 except C22, for the use in calculating retention indices.

Results

For analysis of gangliosides by high-performance thin-layer chromatography, sera were obtained from 17 women with ductal cell carcinoma of the breast and plasma from 16 healthy women as a control group. For gas chromatography, 19 and 17 samples, respectively, were obtained. Samples of plasma and serum from the same donor were obtained, isolated and analyzed for sialic acid content using the thiobarbituric acid assay. The sialic acid content of the plasma and serum samples was within 97% of each other. There was no difference found in the ganglioside content between blood plasma and serum and henceforth will be considered equivalent.

High-Performance Thin-Layer Chromatography

Fig. 1a shows a map created of known gangliosides used for identification of ganglioside spots on the 2-D HPTLC. Figs. 1b and 1c show photographs of the 2-D HPTLC plates of gangliosides isolated from cancer and control group samples, respectively. Table 1 list the gangliosides found in serum and their structures. Table 2 shows the quantitation results of the 2-D HPTLC and image densitometry both in terms of lipid-bound sialic acid and total lipid. We found and quantified 12 resorcinol-positive spots isolated from 1 ml of plasma or serum by 2-D HPTLC. The spots were primarily identified by co-migration of authentic standards and comparison with reported plasma gangliosides (22-25). While identification by co-migration is not conclusive, it is suitable for preliminary analysis of the differences in profiles of the control and cancer group.

The 16 human plasma samples comprising the control group contained a level of recovery of 6.1 $\pm$ 1.5 nmol lipid-bound sialic acid/ml of serum. These results are in line with previous reports of between 7 and 20 nmol lipid-bound sialic acid per ml from a single adult male (29, 30), pooled sera (31), or of unknown origin (22, 32), as well as the reported average of 11 individuals (33). This report (33) examined 5 female and 6 male and found 9.9 $\pm$ 3.8 and 11.1 $\pm$ 2.8 nmol lipid-bound sialic acid per ml of serum. In all of the reports as well as our findings, the total concentration of lipid-bound sialic acid varied appreciably among individuals producing significant standard deviations. In all the above studies, larger quantities of starting material were used, and often correction factors were used to adjust the recoveries. In our work, we were limited to only 1 ml of sera or plasma for starting material and made no adjustment for losses during the isolation. To this difference we attribute our lower recoveries. However, in all cases, great care was taken to make sure all isolation steps were identical so that any losses would be reflected evenly in all samples, including the work-up of both cancer patients and control samples in each batch. The profiles of gangliosides determined from our control group were similar to those of previous studies (22, 29, 33). GM_3 ganglioside accounted for 50-70% of the total lipid-bound sialic acid, with the next most abundant ganglioside being GD_3.

When the levels of gangliosides from sera of the control group are compared to those of the cancer group, several observations can be made. Table 2 shows a significant ($P < 0.05$ using student's t) increase in the total and all individual ganglioside species from breast cancer serum over those of the control group. This finding of increased sialic acid content has also been seen in other studies with other

Figure 1. HPTLC of Gangliosides. Figure 1a is a schematic diagram of several gangliosides mapped with two-dimensional high-performance thin-layer chromatography. Gangliosides found in plasma are shaded while other reference gangliosides are white. Figure 1b and c are photographs of actual gangliosides isolated from the serum of breast cancer patients (1b) and the plasma of a control sample (1c).

Figure 1a

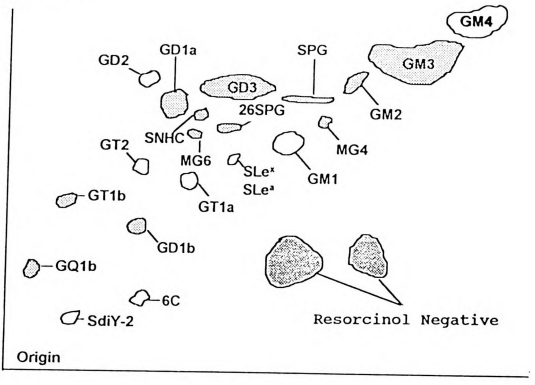


Figure 1b

Figure 1c

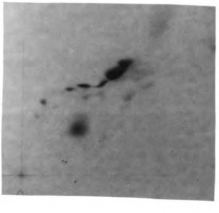


Table 1. Summary of Ganglioside Nomenclature and Structure. Gangliosides found in plasma are marked with an asterisk (*).

Table 1.

Ganglioside	Structure
GM_4	NeuAcα2-3Galβ1-1Cer
precursor	
LacCer	Galβ1-4Glcβ1-1Cer
a-series	
GM_3*	NeuAcα2-3Galβ1-4GlcCer
GM_2*	GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
GM_1	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
GD_{1a}*	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
GT_{1a}	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer
b-series	
GD_3*	(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GD_2	GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GD_{1b}*	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GT_{1b}*	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GQ_{1b}*	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
c-series	
GT_3	NeuAcα2-8NeuAcα2-8NeuAcα2-3Galβ1-4GlcCer
GT_2	GlcNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GT_{1c}	Galβ1-3GlcNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
GQ_{1c}	NeuAcα2-3Galβ1-3GlcNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer
lacto series	
SPG*	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4GlcCer
2-6SPG*	NeuAcα2-6Galβ1-3GlcNAcβ1-3Galβ1-4GlcCer
SLe^a	NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4GlcCer
SLe^x	NeuAcα2-3Galβ1-3(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcCer
neolacto series	
SNHC*	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcCer
Sialyl I	(NeuAcα2-3Galβ1-4GlcNAcβ1-3,6)G lβ1-4GlcNAcβ1-3Galβ1-4GlcCer
6C	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcCer
SdiY-2	NeuAcα2-3(Galβ1-4(Fucα1-3)GlcNAcβ1-3)$_2$Galβ1-4GlcCer

Table 2.

Average ganglioside content of cancer serum and a control group plasma.

Ganglioside	Cancer n=18					Control n=16				
	Total Lipid[a]		Lipid-bound Sialic Acid[a]			Total Lipid[a]		Lipid-Bound Sialic Acid[a]		
	Avg.	S.D.	Avg	S.D.	%LBSA	Avg.	S.D.	Avg.	S.D.	%LBSA
GM_3	3921	1274	3921	1274	52	3628	1024	3628	1024	60
GM_2	341	419	341	419	5	195	79	195	79	3
SPG	317	204	317	204	4	228	119	228	119	4
2-6SPG	188	287	188	287	3	170	177	170	177	3
MG4	121	205	121	205	2	59	54	59	54	1
MG6	47	63	47	63	1	23	32	23	32	<1
SNHC	134	184	134	184	2	57	49	57	49	1
GD_3	530	162	1059	324	14	462	212	924	423	15
GD_{1a}	198	113	396	226	5	159	75	317	150	5
GD_{1b}	102	104	204*	209	3	44	35	88	71	2
GT_{1b}	179	151	536**	452	7	102	50	306	149	5
GQ_{1b}	66	86	264**	347	3	23	17	91	67	1
Total	6144	3252	7516*	2319	100	5150	1923	6088	1490	100
b/a[b]			0.40*	0.14				0.30	0.11	
M3/D3[c]			4.21	3.24				4.60	1.78	
M/DTQ[d]			2.32*	0.90				3.18	1.67	

[a] pmol per ml of serum

[b] Ratio of b-series gangliosides GD_3, GD_{1b}, GT_{1b}, and GQ_{1b} to a-series ganglioside GM_3 and GD_{1a}.

[c] Ratio of GM_3 to GD_3.

[d] Ratio of monosialogangliosides to polysialogangliosides.

*$P < 0.05$ compared to control value using student's t, ** $P < 0.07$ compared to the control values by student's t.

cancers such as retinoblastoma (34) and melanoma (35). An increase in the more complex gangliosides, i.e. more highly glycosylated and sialylated, was also observed. Furthermore, the average ratio of the b-series gangliosides to the a-series gangliosides is increased significantly in the cancer samples over the control. This suggests an altered biosynthetic pathway of serum gangliosides associated with breast cancer. Figures 2a, b and c show range plots of the HPTLC data. These figures show the cancer samples having not only increased averages, but increased ranges and medians over that of the control group.

Gas chromatography

Figures 3a and b show typical gas chromatograms of peracetylated methanolysis products of gangliosides isolated from serum or plasma. Figure 3a shows a chromatogram of the sugar and sphingoid base components while Figure 3b shows the fatty acid species associated with them. Table 3 shows the concentrations found of each peracetylated methanolysis product. The extra purification step was necessary to remove the more polar, neutral glycosphingolipids not removed by the isolation procedure. This extra step decreased the yield, yet much information was still obtainable such as the conspicuous presence or absence of particular sugar residues or ceramide components. Again, the high standard deviations reflect the variability of the ganglioside concentrations between individuals.

When comparing the results from the control group to that of the cancer, the data show a significant increase in the total lipid-bound sialic acid and galactose residues. This supports the HPTLC data which show an increase in total gangliosides

Figure 2a. Box plots of GM_3 and Total Ganglioside Isolated from Cancer Sera and Control Plasma. Values are based on lipid-bound sialic acid concentrations. Definition of boxes is as shown on the right side of the figure.

Figure 2a.

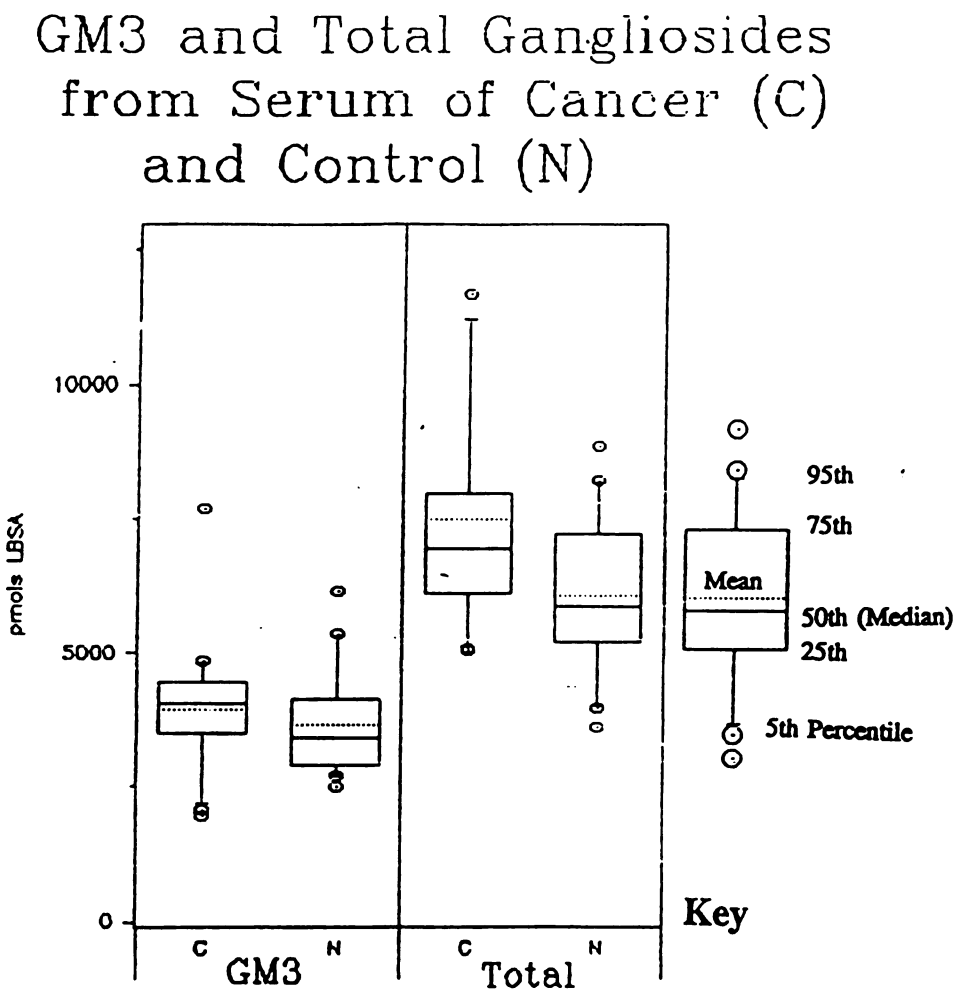

GM3 and Total Gangliosides
from Serum of Cancer (C)
and Control (N)
pmols LBSA
10000
5000
0
C
N
GM3
C
N
Total
95th
75th
Mean
50th (Median)
25th
5th Percentile
Key

Figure 2b. Box plot of Minor Gangliosides Isolated from Cancer Sera and Control Plasma. Values are based on lipid-bound sialic acid concentrations. See Fig. 2a for box definitions and Table 1 for abbreviations..

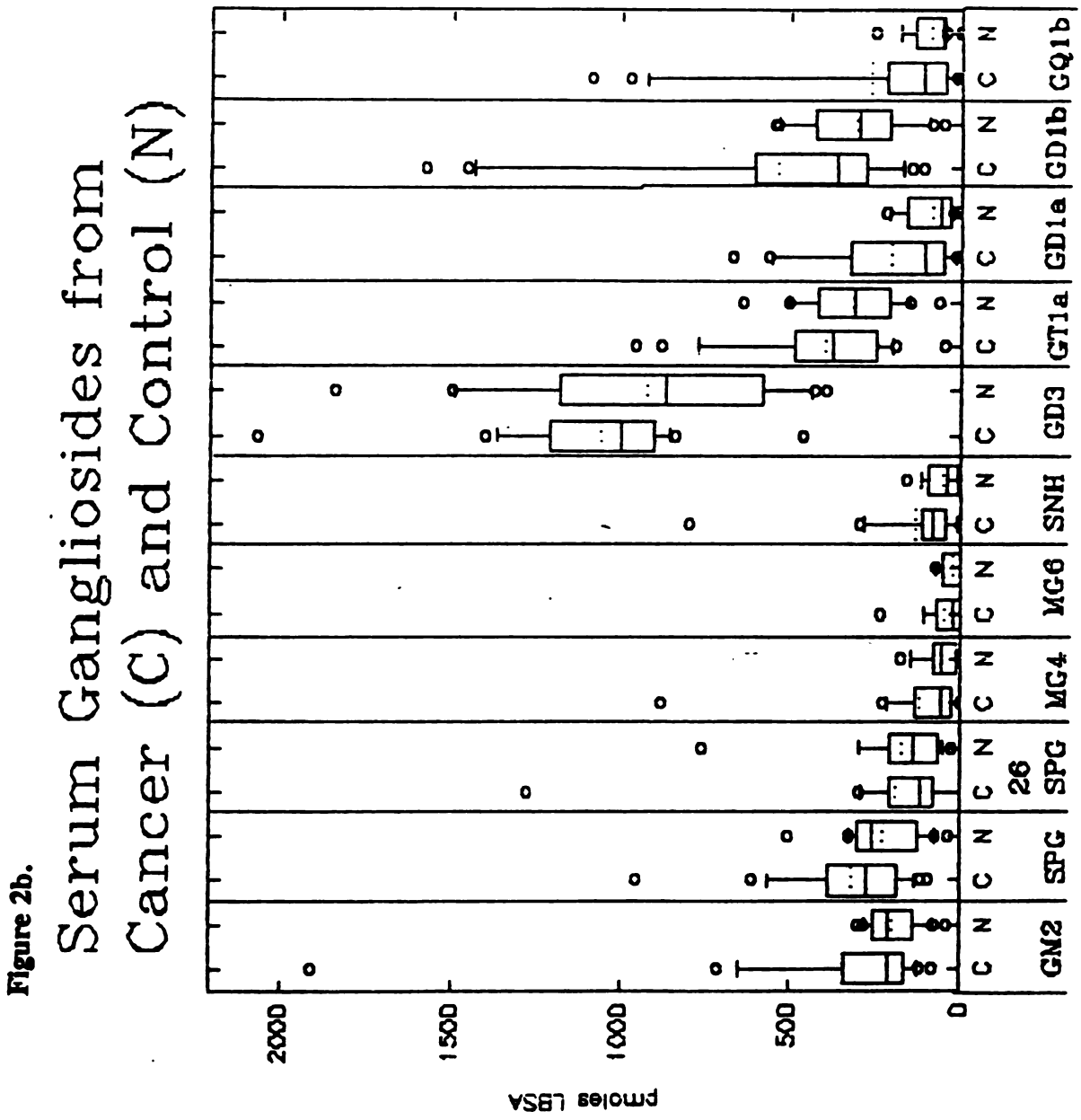
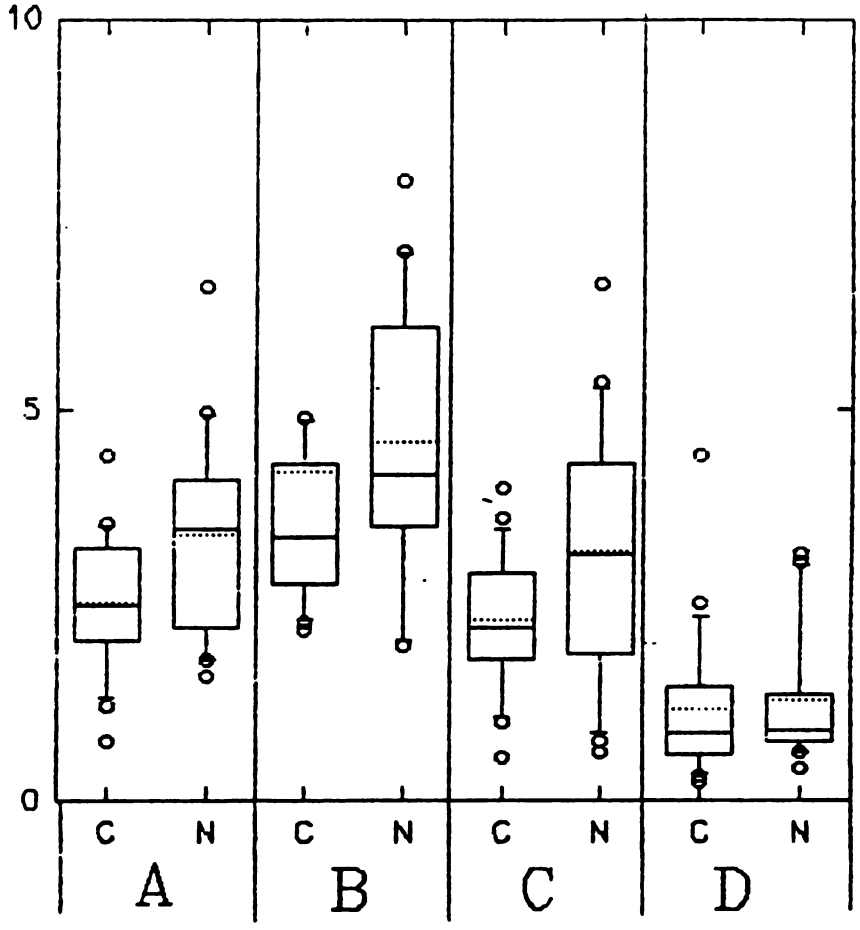

Serum Gangliosides from
Cancer (C) and Control (N)
pmoles LBSA
2000
1500
1000
500
0
C N GM2
C N SPG
C N SPG
C N MG4
C N MG6
C N SNH
C N GD3
C N GT1a
C N GD1a
C N GD1b
C N GQ1b
26

Figure 2b.

Figure 2c. Box plots of Important Ratios of Gangliosides.

Figure 2c.

Box Plot of Various Ratios of Gangliosides in Cancer (C) and Control (N).



A–A Series/B Series
B–GM3/GD3
C–Mono/polysialyl gangliosides
D–GM2/SPG

as well as an increase in the more complex gangliosides. The more complex gangliosides have more sialic acid and galactose residues per molecule than the more simple ones. It was interesting to note that sphingosine was the only long-chain base found in either the cancer or the control group. Yu and Ledeen (29) found sphingosine as the major component with traces of the C_{16} and C_{20} homologs in their analysis of plasma gangliosides of a single male.

The fatty acid composition of the total ganglioside fraction (Table 4) was similar to that reported by Yu and Ledeen (29) and Tao and Sweeley (36) with the major fatty acids being palmitate (C16:0) and stearate (C18:0) with the longer chain fatty acids (C20:0-24:0) having a significant contribution. The fatty acid profile determined by Kundu *et al.* (22) was somewhat different, identifying palmitate and palmitoleate (C16:0 and C16:1), and stearate and oleate (C18:0 and C18:1) as the major species with only trace amounts of the longer-chain species. Of particular interest was our finding of a significant amount of (up to 7%) α-hydroxy fatty acids. Kundu *et al.* (22) reported only traces and Yu and Ledeen (29) and Tao and Sweeley (36) did not find any such compounds. The α-hydroxy fatty acids along with other fatty acids were identified by gas chromatography-mass spectrometry as fatty acid methyl esters. A mass spectrum of α-acetoxytetracosanoic methyl ester, from the FAME fraction from derivatized gangliosides isolated from control plasma, is shown in Figure 5.

By comparison of the fatty acid data of the control profile with the cancer, no significant increase could be seen in any singular fatty acid species, yet there appears to be a general increase in the ratio of long-chain fatty acid (22-24 carbon) to

Figure 3. Gas Chromatograms of Peracetylated Methanolysis Products of Gangliosides Isolated from Plasma of Serum. Figure 3a is a typical gas chromatogram of the peracetylated methanolysis products of the sugar residues and long-chain base of gangliosides isolated from plasma or serum. Figure 3b is the gas chromatogram of the fatty acid methyl esters of gangliosides isolated from plasma or serum.

Figure 3a

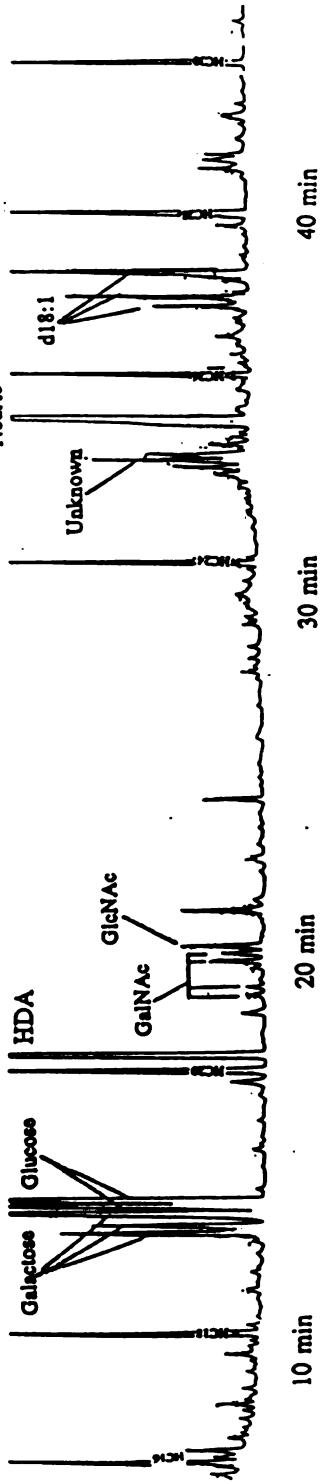


Figure 3b

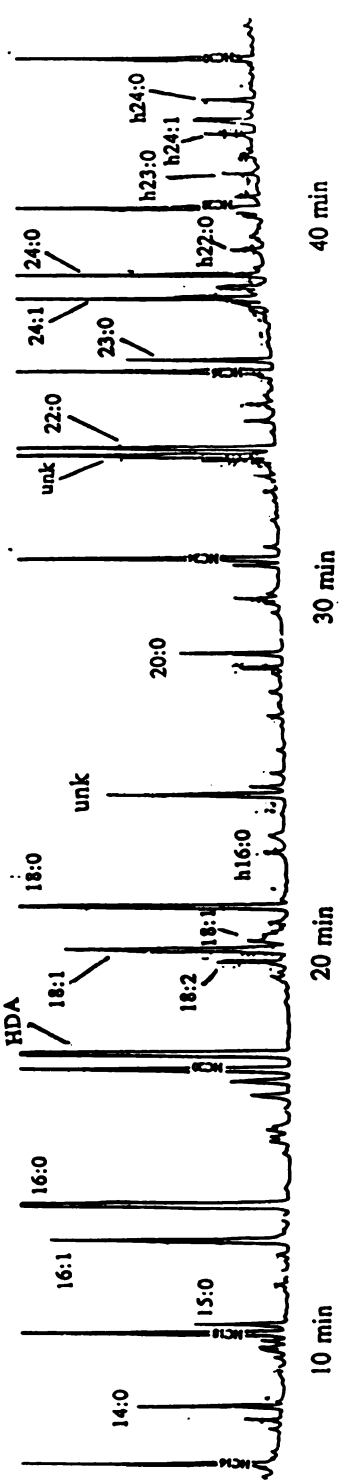


Table 3.

Averages of GC Analysis Peracetylated Methanolysis Products of Plasma Ganglioside Mixture:Carbohydrates and long-chain bases.[a]

Component	Cancer n=19	Control n=17
Galactose	5164 ± 1815*	3745 ± 1477
Glucose	6676 ± 2047	7143 ± 3168
GalNAc	635 ± 338	641 ± 352
GlcNAc	349 ± 128	319 ± 167
Unknown	630 ± 296	532 ± 341
NeuAc	4650 ± 1862*	3899 ± 1338
d18:1	4573 ± 2367*	2803 ± 1283
GlN/GcN[b]	1.85 ±0.86	2.17 ± 0.94
Gal/Glc[c]	0.78 ± 0.20*	0.60 ± 0.26
Hex/HexNAc[d]	7.30 ± 1.86	8.88 ± 6.03

[a]pmole per ml of serum.
[b]Ratio of N-acetylgalactosamine to N-acetylglucosamine.
[c]Ratio of galactose to glucose.
[d]Ratio of galactose and glucose to N-acetylgalactosamine and N-acetylglucosamine
*$P < 0.05$ compared to the control value using students t.

Table 4.

Averages of GC Analysis Peracetylated Methanolysis Products of Plasma Ganglioside Mixture: Fatty acids

Component	Cancer n=19[a]	% of Total	Control n=17[a]	% of Total
14:0[b]	286 ± 142	1.7	229 ± 129	1.8
15:0	182 ± 85	1.1	165 ± 95	1.3
16:1	263 ± 198	1.6	266 ± 246	2.0
16:0	4191 ± 1919	26.0	3879 ± 1325	30.0
18:2	395 ± 317	2.4	419 ± 352	3.3
18:1	609 ± 401	3.7	530 ± 388	4.1
18:1	217± 103	1.3	171 ± 99	1.3
18:0	2952 ± 1494	18.1	2332 ± 1091	18.1
20:0	465 ± 275	2.8	353 ± 219	2.7
unk	1264 ± 679	7.7	990 ± 361	7.7
22:0	1597 ± 991	9.7	1094 ± 566	8.5
23:0	616 ± 365	3.8	495 ± 277	3.8
24:1	1213 ± 856	7.4	940 ± 638	7.3
24:0	1242 ± 750	7.5	898 ± 435	7.0
h16:0[c]	162 ± 113	1.0	121 ± 131	0.9
h22:0[c]	192 ± 116	1.2	157 ± 88	1.2
h23:0[c]	112 ± 75	0.7	80 ± 46	0.6
h24:1[c]	186 ± 163	1.1	151 ± 69	1.1
h24:0[c]	272 ± 200	1.7	247 ± 181	1.9
Total Fatty Acid	16415 ± 6693		12877 ± 5674	

[a]pmol per ml serum.
[b]Number of carbon atoms:number of double bands
[c]α-hydroxy fatty acids

Figure 4. Mass Spectrum of α-acetoxytetracosanoic methyl ester analyzed by GC/MS. Taken from the fatty acid methyl ester of gangliosides isolated from normal plasma.

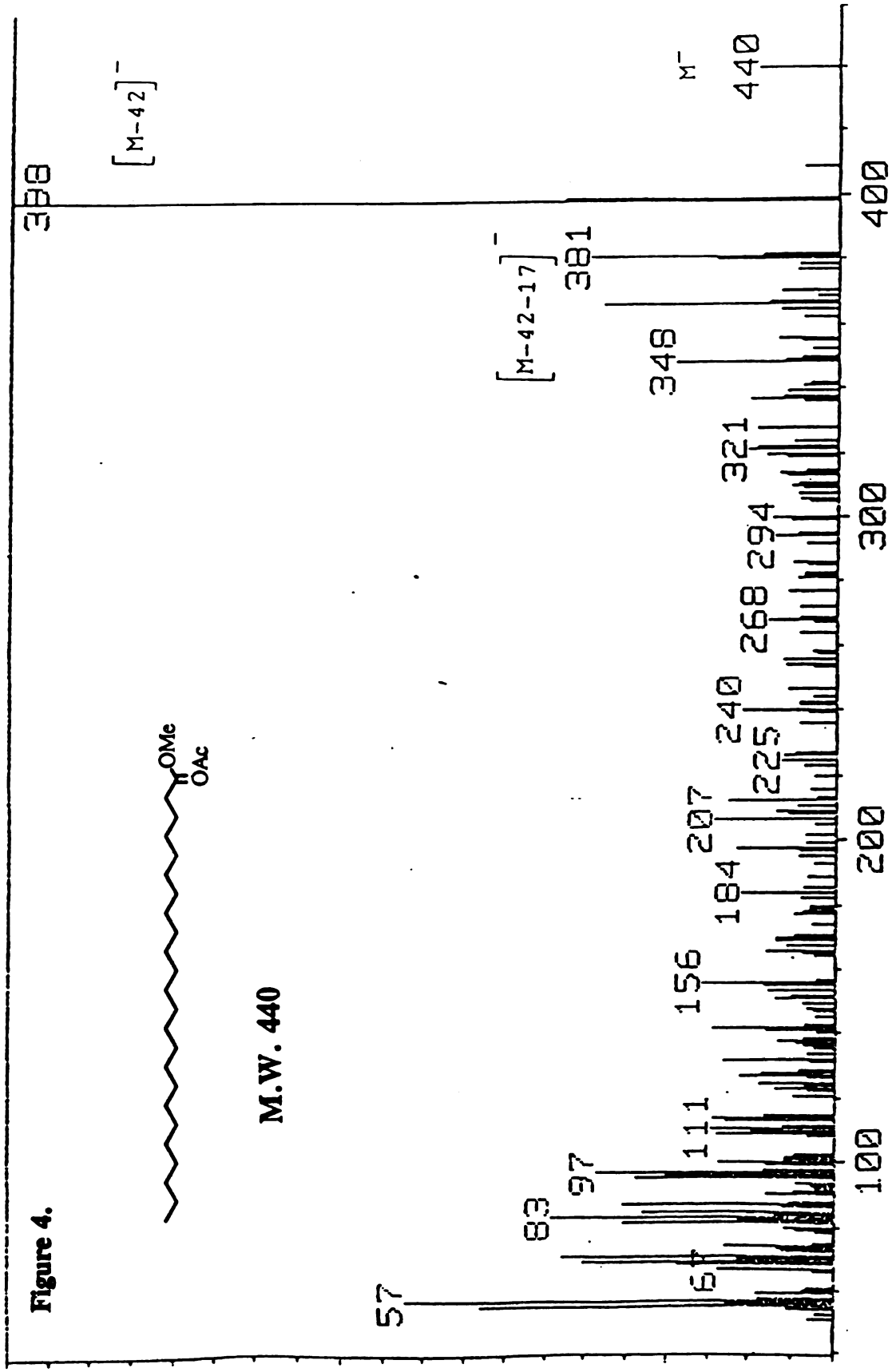

OMe
OAc
M.W. 440
[M-42]⁻
[M-42-17]⁻
M⁻
388
381
348
321
294
268
240
225
207
184
156
111
97
83
57
64
100
200
300
400
440

Figure 4.

short-chain fatty acid (14-20 carbon) moieties in cancer-associated gangliosides over that of the control. Similarly, the ratio of stearate (18 carbon) to palmitate (16 carbon) has decreased in the cancer patients. It should be noted that these were general trends and were not statistically significant. The results, however, along with other results (37, 38), are consistent with the idea that the ceramide portion of the ganglioside may have a greater role in carcinogenesis than previously thought.

Discussion

Since the first observation of changes in glycosphingolipid composition in tumor cells, in 1968-69 (39, 40), extensive studies have been undertaken to elucidate the function of gangliosides in the biology of cancer. From these studies, several tumor-associated ganglioside antigens (see Chapter 1, Table 1) have been identified, primarily with the use of monoclonal antibody technology. After Black (41) noted that several substances were shed from the surfaces of cancer cells into the local environment and eventually in to the plasma, a logical step was to determine if these tumor-associated ganglioside antigens were also found in the plasma and what their possible function might be. This has been studied most extensively in neuroblastoma and melanoma.

In the case of neuroblastoma, high levels of GD_2 were found in the tissue and in the plasma (11, 12, 15, 16). The levels of GD_2 are typically below the detection limit (<25 pmol) in serum of control samples. Patients with higher amounts of GD_2 at the time of diagnosis had poorer prognosis than those who had lower levels (15). Furthermore, levels of GD_2 in the blood correlated well with the stage and progress

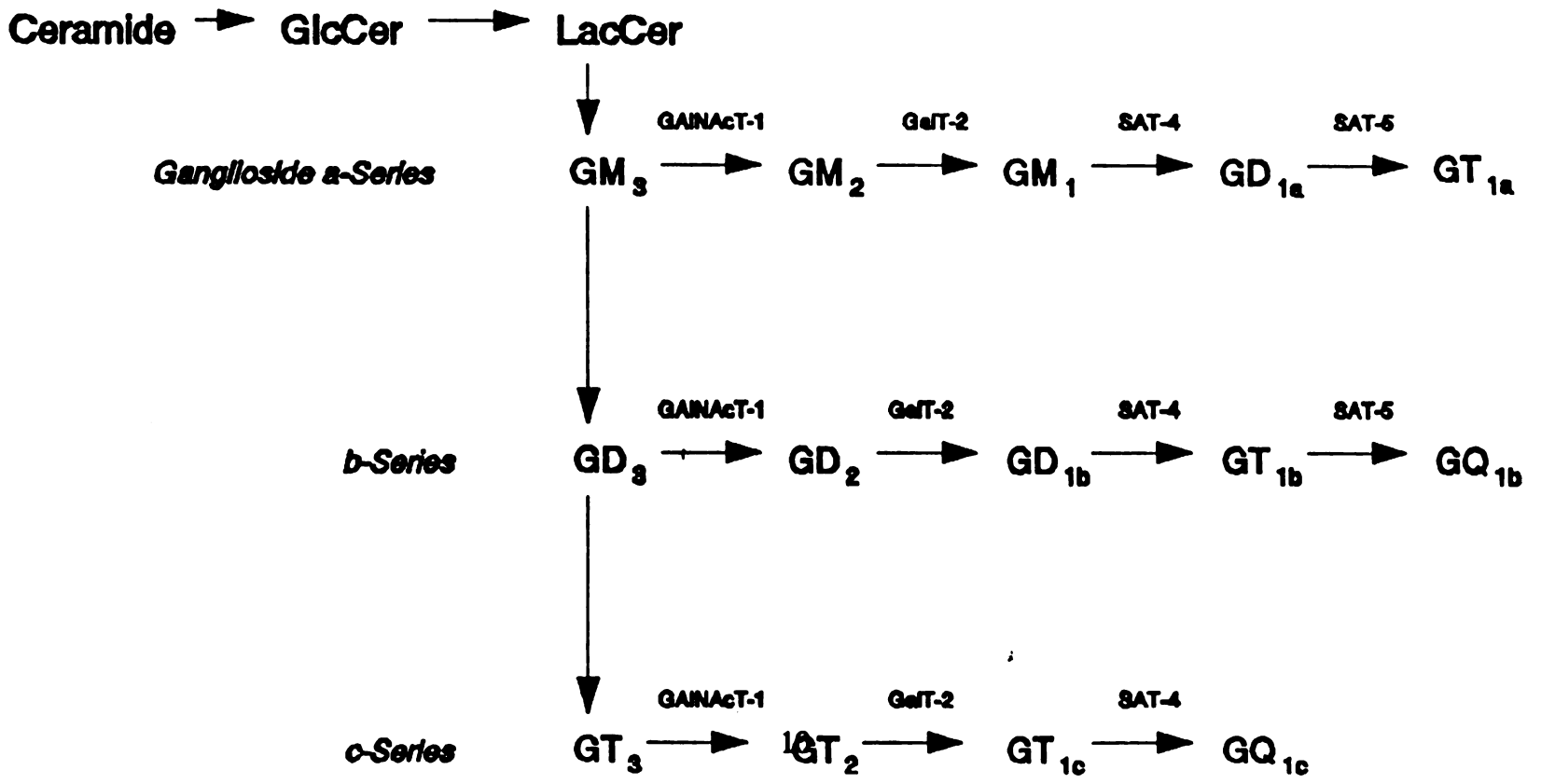
of the disease, the levels dropping off after successful treatment and increasing prior to relapse (15). The authors have suggested the usefulness of GD_2 as a diagnostic tool as well as a tumor marker for monitoring of the disease. GD_3, the precursor of GD_2, has been found in relatively large amounts in the tissue and cells cultured from human melanoma (17, 35, 42, 43). While human melanoma cells in culture have been shown to shed gangliosides, specifically GD_3, into the medium, elevated levels of the gangliosides could not be detected in the plasma or serum of patients (35). The authors suggested that this was a local phenomenon masked by the already high levels of GD_3 found in control plasma.

There has been less extensive work done on the gangliosides in tissue and sera of patients with breast cancer. Some of the first work on breast cancer was reported about twenty years ago by Morré *et al.* (44). They found that the ganglioside profile from rat mammary tumors induced by 7,12-dimethylbenz[a]anthracene was depressed in disialogangliosides with an increase in a ganglioside identified as GM_1. They also noted several alterations in the glycosyltransferases responsible for ganglioside synthesis. Most notably, there was a 7-fold increase in GD_3 synthase (SAT-2; GM_3 + CMP-NAN → GD_3) and a 6-fold increase in GM_2 synthase (GalNAcT-1; GM_3 + UDP-GalNAc → GM_2) with a large decrease in GD_{1a} synthase (SAT-4; GM_1 + UDP-Gal → GD_{1a}) (See Fig 5.). In other studies, various sialyltransferases and glycosyltransferases increased in tissues and cells in culture concomitant with increased levels of glycolipids (31, 45, 46).

In this study, we have examined the ganglioside profile in the serum of patients with ductal cell carcinoma of the breast and the ganglioside profile of plasma

Figure 5. Biosynthetic pathway for ganglio-series gangliosides. See Table 1 for structures.

GANGLIOSIDE BIOSYNTHETIC PATHWAY



from an appropriate control group. We determined that there is no significant difference in the ganglioside content of serum versus plasma, nor is there any significant difference in profiles of gangliosides of plasma with respect to age in our sampling of plasma, which agrees with previous reports (33). There have been many reports in the literature describing elevated levels of "lipid-associated sialic acid" in the sera of breast cancer patients (47-52) using the method of Kapotodis *et al.* (53) in which gangliosides are precipitated with lipoproteins by phosphotungstic acid after which the sialic acid is measured with resorcinol. These reports have been criticized because of their apparent lack of specificity for "lipid-associated" sialic acid due to the amount of acid-α-1-glycoprotein that coprecipitates (54, 55). Reported values of "lipid-associated" sialic acid have ranged from 580 nmol/ml for control to 1.9 μmol/ml in cancer. When gangliosides have specifically been isolated and quantified, the lipid-bound sialic acid content in control samples is around 8 to 15 nmol/ml (29-33). Our results were lower than previously reported values with around 6 nmol/ml LBSA in the control group and 7.5 nmol/ml LBSA in the cancer group. We attribute the low recovery to the small amount of starting material (1 ml). Samples of plasma and serum for the control and cancer were run at the same time in mixed batches, such that any losses incurred during isolation would be reflected similarly in all samples. The results can therefore be compared and meaningful observations made.

When the gangliosides isolated from the cancer patients were compared to those isolated from the control group by thin-layer chromatography, three major observations were made. First, there was a significant increase in the overall content of gangliosides; second, there was an increase in the more complex or

polysialogangliosides over the more common monosialogangliosides. Lastly, and most interestingly, there was an increase in the b-series gangliosides (GD_3, GD_{1b}, GT_{1b}, and GQ_{1b}) relative to the a-series gangliosides (GM_3, GM_2, and GD_{1a}). These have all been noted in Table 2. The increase in sialic acid and galactose, as determined by gas chromatography, supported these observations (Table 3).

Dyatlovitskaya *et al.* (56) have examined the ganglioside profile of breast cancer tissue and reported a large increase in what they called the "more polar gangliosides", presumably the more complex gangliosides. Skipski *et al.* (57) found increases in the polysialogangliosides in methylcholanthrene-induced mammary tumors in rats, contrary to what Morré *et al* (44) observed in tumors induced by 7,12-dimethylbenz[a]anthracene. In the Morré study (44), GM_1 was reported to be a major ganglioside accumulating in the cancer cells. GM_1 was identified by comigration of an authentic GM_1 standard run on a silica gel thin-layer plate in an ammonia-containing solvent. GD_3 will often comigrate with or surpass GM_1 in this type of solvent. The increase in GD_3 synthase (7 fold) suggests that their GM_1 band might have contained some GD_3. Factors in determining the profile of ganglioside expression are relative ratios of glycosyltransferases as well as available substrates (58-60). The ratio between GM_3 synthase (SAT-1) and GD_3 synthase (SAT-2) has been suggested to moderate the expression of the a-series gangliosides and b-series gangliosides (58). Figure 5 shows the biosynthetic pathways to gangliosides in the a-, b-, and c-series (59-61) and Table 1 shows the structures. The enzymes GM_3 synthase (SAT-1) and GD_3 synthase (SAT-2) are the initial step in each of the respective series. In the Morré study (44), GD_3 synthase activity increased almost 7-

fold more than GM_3 synthase, suggesting that there may be a shift from the a-series biosynthetic pathway to the b-series and the increase in GM_1 may actually be an increase in GD_3. All the above experiments support our findings that the sera of patients suffering from breast cancer have an increase in polysialogangliosides with a significant increase in the b-series biosynthetic pathway over the a-series.

The apparent lack of fucose in the ganglioside fraction from either the cancer or the control group was of interest. Fucosylated sialo-antigens have been found in several different cancers. Sialylated Lewis antigens (SLe^a, SLe^x; Table 1) have been associated with several adenocarcinomas, especially colorectal and pancreatic types (62-65), including breast cancer tissue (63, 65). Interestingly, in the tissue, these carbohydrate antigens were localized in sphingolipid fractions (62, 66), but are associated with mucins in the plasma (65, 67, 68). Another fucosylated ganglioside found in cancer is fucosyl-GM_1, which was found in the tissue and plasma of small-cell lung cancer. We did not detect any fucose in gangliosides isolated from either serum of breast cancer or plasma of the control group by gas chromatography, nor was a resorcinol-positive spot found that comigrated with either the SLe^a or SLe^x glycolipid. Our findings suggest that fucosylation of gangliosides is probably not associated with breast cancer and the SLe^x antigen (if present in sera) might be in the form of mucins.

The question about what the impact of the shedding of gangliosides might be in cancer is controversial at best. Is this phenomenon merely a secondary effect that accompanies other more important events or does it play a more direct role? Circulating gangliosides have been proposed to allow tumor cells to escape immune

system surveillance as well as allow tumor cells to metastasize through moderating various cell-binding events (69-77). Of particular interest to this study is the effect of the more highly sialylated gangliosides on the immune system and binding. Skipski *et al.* (57) found that mouse mammary tumors were more metastatic when rich in polysialogangliosides. Exogenous polysialogangliosides have been suggested to aid tumor cells in metastasis through the inhibition of binding to subcellular matrix (9, 78-81). It is possible that breast tumor cells secrete significant amounts of polysialogangliosides into the local environment surrounding the tumor cells. This may be reflected in the slight but significant elevation in the gangliosides found in circulation (35). Tumor cells may be able to disrupt the normal cell-substructure binding by the release of matrix-binding polysialogangliosides which may block binding to the substratum. By this means, tumor cells may become contact-independent, allowing them to leave the local environment and metastasize elsewhere. Whereupon the surface carbohydrates might again be altered in their structure to facilitate binding and infiltration of a region remote to the original site.

While the exact mechanisms are not known, in general, a high content of lipid-bound sialic acid either in the tissue or plasma seems to be linked to a poor prognosis while lower lipid-bound sialic acid content indicates a better prognosis and the level is characteristic of the disease state (9, 14-16, 31, 47, 48, 50, 51, 56, 68, 71, 72, 82). Furthermore, alterations in the ceramide portion of the ganglioside molecule (longer or shorter chain length or α-hydroxylation of the fatty acid moiety) can be associated with the shedding of gangliosides from the surface of cells (37, 38, 83, 84). It becomes obvious that more information and additional studies are necessary to better

understand the importance of tissue and circulating gangliosides in the biology of cancer.

Perspectives

It was the intent of this study to determine if a circulating ganglioside(s) isolated from breast cancer patients could be useful as a tumor-associated antigen. This was to be achieved by characterizing the profile of gangliosides found in breast cancer sera and in a control or disease-free group. While much information was obtained about the ganglioside components in serum samples, there was no single ganglioside that could be considered a tumor-associated glycolipid antigen, i.e. a novel or elevated concentration of a ganglioside not ordinary found in healthy individuals' blood. If one had been found, it could perhaps have been useful in diagnosis, prognosis, and monitoring of the disease (15, 16, 85). Furthermore, tumor-associated glycolipid antigens have been examined for use in various therapies. Certain glycolipids, alone, have suspected antitumor properties (9, 86), while antibodies raised against tumor-associated ganglioside antigens have been used in trials and also appear to have anti-tumor activities (9, 87). Furthermore, gangliosides isolated from tumors have been tested as immunogens (88-90), the goal being the replacement of less specific therapies like chemotherapies with an anti-tumor immune response in the host that would be present on a continual basis (91). The use of anti-ganglioside antibodies in therapy can have several approaches. The antibodies themselves can recruit the host immune system for defense against the cancer through macrophage activation or "antibody-dependent cytotoxicity" (92), or antibodies could

have various toxins or radionucleotides attached to them (76). Labeled antibodies could be used for tumor localization by scintillographic photoscanning (93). Hakomori has suggested two new types of therapy he calls anti-adhesion therapy and ortho-signaling therapy (76, 94). In anti-adhesion therapy, glycolipids or antiglycolipid antibodies could be used to disturb the various carbohydrate-initiated tumor cell binding events required for progression and metastasis. Ortho-signaling therapy aims at disturbing the glycolipid-moderated signal transduction events. Problems associated with these therapies are the heterogeneity of different tumor antigens and the shedding of the antigens from the cells (76). Exact knowledge of the antigens found in the tissue as well those shed into the plasma must be known in order to appropriately design a useful therapy.

The results of this study are not of immediate clinical interest but are of biochemical interest. The finding of an altered profile in the breast cancer sera, especially in more complex gangliosides, is suggestive of a possible role of these gangliosides in the biology of breast cancer, possibly in moderating important carbohydrate-initiated binding events. In order to better characterize this possibility, the profiles of gangliosides isolated from serum need to be examined over the course of the disease to determine if the alterations are stage specific. Furthermore, breast cancer tissue needs to be examined at all stages more closely to determine how and if the gangliosides found in the tumors affect those in the circulation. Another interesting experiment would be to determine what effect exogenous gangliosides have on the various receptors found in breast cancer tissue. The loss of the estrogen or progesterone receptor in breast cancer tissue results in a poor prognosis and its

disappearance is a common indicator for the diagnosis and prognosis of breast cancer (95). The presence of the *erb2* gene, which is thought to encode another growth factor receptor, is an unfavorable prognostic factor (95). The effect of several gangliosides on the various growth factor receptors is well known (Reviewed in ref 10). Studies of cell growth in chemically defined media indicate that exogenously added GM_3 and GM_1 alters the binding affinity of cells to platelet-derived growth factor, epidermal growth factor, or fibroblast growth factor. To our knowledge, no one has studied the interactions of gangliosides on growth factor receptors that are important to breast cancer. These experiments would better help define the role of gangliosides in the biology of breast cancer and aid in developing a useful strategies of therapy based on ganglioside interactions with the cell-surface molecules and mechanisms associated with tumorigenesis.

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CHAPTER 7

Sialidase and Free Sialic Acid in the Serum of Patients with Breast Cancer

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Abstract

Assays of free sialic acid and soluble sialidase activity were carried out in with samples of serum from ductal cell breast cancer patients and plasma from a control group of women was determined. Sialidase activity measured by the use of a coupled enzyme assay, sensitive to 200 nU per ml of plasma or serum. The samples were assayed either unmodified, or dialyzed against several buffers at different pH. Fractions of serum or plasma fractionated over a gel filtration column were also assayed. In none of the samples, whether from the cancer or the control group, was any sialidase activity found. Free sialic acid was determined in several samples of cancer sera and control plasma by a modified thiobarbituric acid assay mathematically corrected for any contaminants. The concentrations of free sialic acid in the cancer and control were 1.63 and 1.74 nmol per ml, respectively, and were not found to be significantly different. There was no difference found in plasma versus serum in either case. The activity of sialidase and concentration of free sialic acid were not found to be useful in the diagnosis or prognosis of breast cancer.

Introduction

Sialic acids are generally found on the non-reducing termini of carbohydrate chains of glycoconjugates such as mucins, glycoproteins, gangliosides and milk oligosaccharides (1-5). They have been proposed to have a number of different biological properties such as influencing the structural conformation of oligosaccharides (4-7) as well as involvement in recognition and the masking of binding sites of macromolecules and cells (4, 5). Furthermore, the carboxylic acid of sialic acids has been suggested to give many molecules their antigenicity (6). The sialic acid moiety of gangliosides have been proposed to confer mitogenic properties to the molecule as well as the binding site for various substrates (6, 8). The hydrolysis of terminal sialic acids from their oligosaccharide base is catalyzed by the enzyme sialidase (neuraminidase). Knowing this, it is easy to see how the various activities of different sialidases can be important to many biological processes. It is conceivable that the action of a sialidase on gangliosides involved in cellular adhesion, communication, and transmembrane signaling could have a profound effect on the biology of the cancer state.

Sialidase activities have been associated with the plasma membrane, cytosol, lysosome, and the extracellular aspect of the cell (9, 10). The source of the extracellular sialidase is currently not known; it may be a product of proteolytic cleavage of plasma membrane bound form, it may be exported from the cell as are many of the other lysosomal glycosidases, or it may arise via a unique mechanism. Sialidases typically have a slightly acidic pH optimum of around 4.5-5.5. One of the

extracellular sialidases found in the medium of cultured fibroblasts had a more neutral pH of around 6.5 (11). It is possible that sialidases found outside the cell, in the local environment, could find themselves in the blood stream. The fact that all other glycosidases have been found in the plasma in measurable amounts would suggest that sialidase should also be found in circulation. While sialidases have been found on the plasma membrane of erythrocytes and many lymphocytes (12), there has been only one obscure report of a soluble plasma sialidase, reporting very low traces of activity.

This portion of my study was to determine if there is detectable sialidase activity present in either plasma from the control group or that of patients with breast cancer. The idea was that while apparently healthy individuals (control group) might not have any measurable sialidase activity in their plasma, levels of sialidase in plasma may be elevated in response to the cancerous state. Furthermore, alterations in a plasma sialidase may result in the accumulation or depletion of free sialic acid. Total sialic acid as well as lipid-bound sialic acid have been implicated in many different diseased states and have been suggested as being useful for the diagnosis of certain cancers (13-26). It is possible that the concentration of free sialic acid in plasma may also be an indicator of the tumor burden and shed some light on some mechanisms of tumor biology.

Materials and Methods

Materials

Cholera toxin B subunit-horseradish peroxidase conjugate was purchased form LIST Biological Laboratory (Campbell, CA). All the following were purchased from Sigma Chemicals (St.Louis, MO): free sialic acid, sialyllactose, all gangliosides, *V. claret* sialidase (Type III), *o*-phenylenediamine tablets, bovine serum albumin, and thiobarbituric acid. Hydrogen peroxide was purchased from J.T. Baker Inc. (Phillipsburg, NJ) and Tween 20 as form Bio-Rad Laboratories (Richmond, CA). Polystyrene EIA/RIA plates (96 well) were from GIBCO Laboratories (Grand Island, NY). All water used was distilled and deionized (ddi water).

Coupled Enzyme Assay of Sialidase.

A coupled enzyme assay (27) that measures very small activities of sialidase was utilized for the determination of sialidase in serum or plasma. Ganglioside GD_{1a} was coated onto the bottoms of 96 well polystyrene plates by placing 50 μl (25 pmol) of the ganglioside in ethanol in the well and allowing to dry overnight. After washing 4x with 0.02% Tween 20 in Dulbecco's modified phosphate-buffered saline solution (Tween-PBS) and once with just PBS, the wells were blocked with 50 μl of a 1% bovine serum albumin in PBS (BSA-PBS) for 1 h. The plate was washed as before and 50 μl of an appropriate enzyme solution was added to each well and incubated at 37 °C for 45 min. The plate was again washed as before and 50 μl of the cholera toxin B subunit-horseradish peroxidase conjugate solution (HRP-B, 0.64 mg B subunit/ml of BSA-PBS) was added and allowed to bind to the GM_1 produced by the

action of sialidase for 1 h at 37 °. The substrate solution (10 mg of *o*-phylenediamine and 30 μl of 30% hydrogen peroxide in 10 ml of 0.1 M sodium acetate buffer, pH 5.5, 50 μl) was added after the wells were washed. After a timed interval, typically 2 min, in which the color was allowed to develop, the reaction was stopped with 50 μl of 4 N H_2SO_4. The absorbance at 490 nm was measured with a Bio-Tek Instruments Inc. EIA Reader EL-810 reader. Quantitation of the amount of GM_1 ganglioside produced was based on a standard curve of pure GM_1 that was attached to the plate in the same manner as GD_{1a} and treated with the HRP-B and substrate in the same manner as above. This is easily achieved on the same plate by just omitting the sialidase treatment on the region of the plate containing the GM_1.

4-MU-NeuAc Assay of Sialidase

As an additional method for the determination of sialidase, 4-methylumbelliferyl-α-D-*N*-acetylneuraminate (4-MU-NeuAc) was used as a substrate with the product 4-methylumbelliferyl (4-MU) measured fluorimetrically (28). The assay mixture contained 50 μl of the test sialidase mixture, 25 μl of 32 mM $CaCl_2$, 50μl of the substrate solution, and 75 μl of an appropriate buffer. This mixture was incubated for 2 h at 37 °C. The reaction was halted by the addition of 1 ml of the "stop" solution (0.133 M glycine buffer, pH 10.7, containing 0.06 M sodium chloride). A 100 μl aliquot of this was diluted to 2 ml with additional "stop" solution and the fluorescence was measured in an Aminco fluoro-colorimeter at an excitation wavelength of 365 nm and an emission wavelength of 448 nm. A 25 nmol/ml solution of free 4-MU was determined to have a transmittance of 25% and a 50

nmol/ml a transmittance of 50%. Substrate solutions were either 4.0, 0.4, or 0.04 mM 4-MU-NeuAc.

Thiobarbituric Acid Assay of Free Sialic Acid.

Free sialic acid was measured by a modified version of the colorimetric method of Aminoff (2, 29) and Warren (30). The samples to be analyzed and free sialic acid (0.5 to 10 nmol) were taken up in 250 μl of dd water and thoroughly mixed with 125 μl of 25 mM periodic acid in 0.125 N H_2SO_4. The tubes were capped tightly and incubated for 30 min at 37 ^{0}C. Excess periodate was removed by the addition of 125 μl of 1.6% sodium arsenite in 0.4 N HCl, shaking until the yellow color disappears. A 1 ml solution of 0.9 M thiobarbituric acid (TBA), adjusted to pH 9.0 with NaOH, was added, the tubes capped, and placed in a boiling water bath for 7.5 min. The tubes were quickly cooled in an ice bath and 0.5 ml of n-butanol containing 5% HCl was added with vigorous mixing. After centrifugation, the upper, color-containing organic phase was removed and the color measured at 549 nm and 532 nm. The absorbance at 532 nm was used to correct for interferences of 2-deoxy sugars that might be present.

Preparation and Analysis of Serum and Plasma

Heparinized plasma was collected from 8 adult women who were considered to be in good health. The plasma was taken by venipuncture after an overnight fast and the women were asked not to be menstruating. This was considered the control

group. Serum was taken from 9 women who had recently been diagnosed with ductile cell carcinoma of the breast and had undergone no treatment as of that point. Samples were used as soon as possible, and stored at -70 °C if necessary.

The coupled enzyme assay was used to determine the sialidase activity of plasma prepared in several different ways. The different methods of preparation included dialysis against buffers of various pHs and dd water. The activity of unmodified plasma was also measured. In an attempt to isolate any potential sialidase activity in both the cancer and control groups, 1 ml of each was run over a Sephacryl S-200 HR gel filtration column (1 x 65 cm) with 1 ml fractions collected during elution with 20 mM phosphate buffer, pH 6.5 containing 0.2 M sodium chloride. Protein was determined for each fraction (absorbance at 280 nm) and sialidase activity was determined by the coupled enzyme assay.

The TBA assay was used to determine the free sialic acid concentration of the serum and plasma following a few brief clean-up steps. First, proteins were precipitated from 1 ml aliquots of plasma or serum with 20 vol of cold ethanol. After thorough mixing, the proteins were pelleted by centrifugation and the supernatants removed and dried under a stream of nitrogen. The residues, after drying, were taken up in a small volume of water (0.5 ml) and extracted twice with equal volumes of hexane, lyophilized and analyzed by TBA analysis.

Results

Sialidase

Several different approaches were used to ascertain the presence of sialidase in plasma or serum. Initially, 50 μl of heparinized plasma was assayed by the coupled enzyme assay of Ogura *et al.* (27). The reported detection limit for this method was 3 nU per well with a well volume of 50 μl. Units were defined as μmols of product formed (GM_1) per minute. In our hands, 10 nU of commercial bacterial sialidase per well was reproducibly detectable while 3 nmol was detectable, but was not reliable. With this detection limit of 10 nU per 50 μl, as little as 200 nU of sialidase activity per ml from plasma or serum was capable of being detected. Other glycosidase found in plasma, such as β-galactosidase or β-glucosaminidase, have reported activities around 20 and 600 mU per ml of plasma (31) and sialidase from sonicated human fibroblasts was 1.2 mU/mg protein (31). While the ability to detect the activity of sialidase with a sensitivity two orders of magnitude greater than necessary for other plasma glycosidases, still no sialidase activity was found in the untreated plasma. Recognizing that most sialidases have pH optima at a more acidic pH, aliquots of plasma were dialyzed against 0.1 M sodium acetate buffers of either pH 4.2 or 5.5. In addition, plasma was dialyzed against pure water for analysis of the possibility of small molecular weight inhibitors. These were assayed with both the coupled enzyme assay as well as using 4-MU-NeuAc as a substrate and measuring the fluorescence. These assays showed little more than a trace of potential activity in plasma with either no activity or results just barely over the detection limit. Where traces of sialidase activity were found, these results were not reliably repeatable.

Another strategy employed in an attempt to find sialidase activity in plasma, was fractionation of plasma or serum by size exclusion chromatography. Aliquots of plasma or serum (1 ml) were applied to the top of a Sephacryl S-200 HR gel filtration column. Each fraction was measured for protein (A_{280}) and sialidase activity by the coupled enzyme assay of a 50 μl aliquot. Figures 1 and 2 show the elution profile and sialidase activity from control plasma and cancer serum. Protein came off in a large peak spanning 30 fractions. Sialidase was not detected in any of the fractions in the profile of either the control or the cancer sample. It is possible that an inhibitor may have interfered with the assay. To try to test for this, protein-containing fractions were spiked with varying amounts of bacterial sialidase and assayed with the coupled enzyme assay. The activity of the bacterial sialidase was highly inhibited in these mixed fractions but this was later attributed to the absence of calcium ions in the buffer or desorbtion of GD_{1a} from the plate through the action of plasma lipoproteins. When the bacterial sialidase was assayed in the elution buffer (20 mM phosphate, pH 6.5, containing 0.2 M sodium chloride) with and without 4 mM $CaCl_2$, the enzyme functioned as expected in the calcium containing buffer and was extremely low in the calcium-free buffer. Due to multiple possibilities of inhibitory factors, this approach was abandoned.

Sialic Acid

Plasma from 8 women comprising the control group and serum from 9 women with ductal cell cancer of the breast were analyzed for their free sialic acid content. The sialic acid concentration was computed using the equation derived by

Figure 1. Elution profile of 1 ml of plasma from a control subject on Sephacryl S-200 HR column. Each fraction was assayed for protein (left axis, A_{280}) and sialidase activity (right axis, A_{490}).

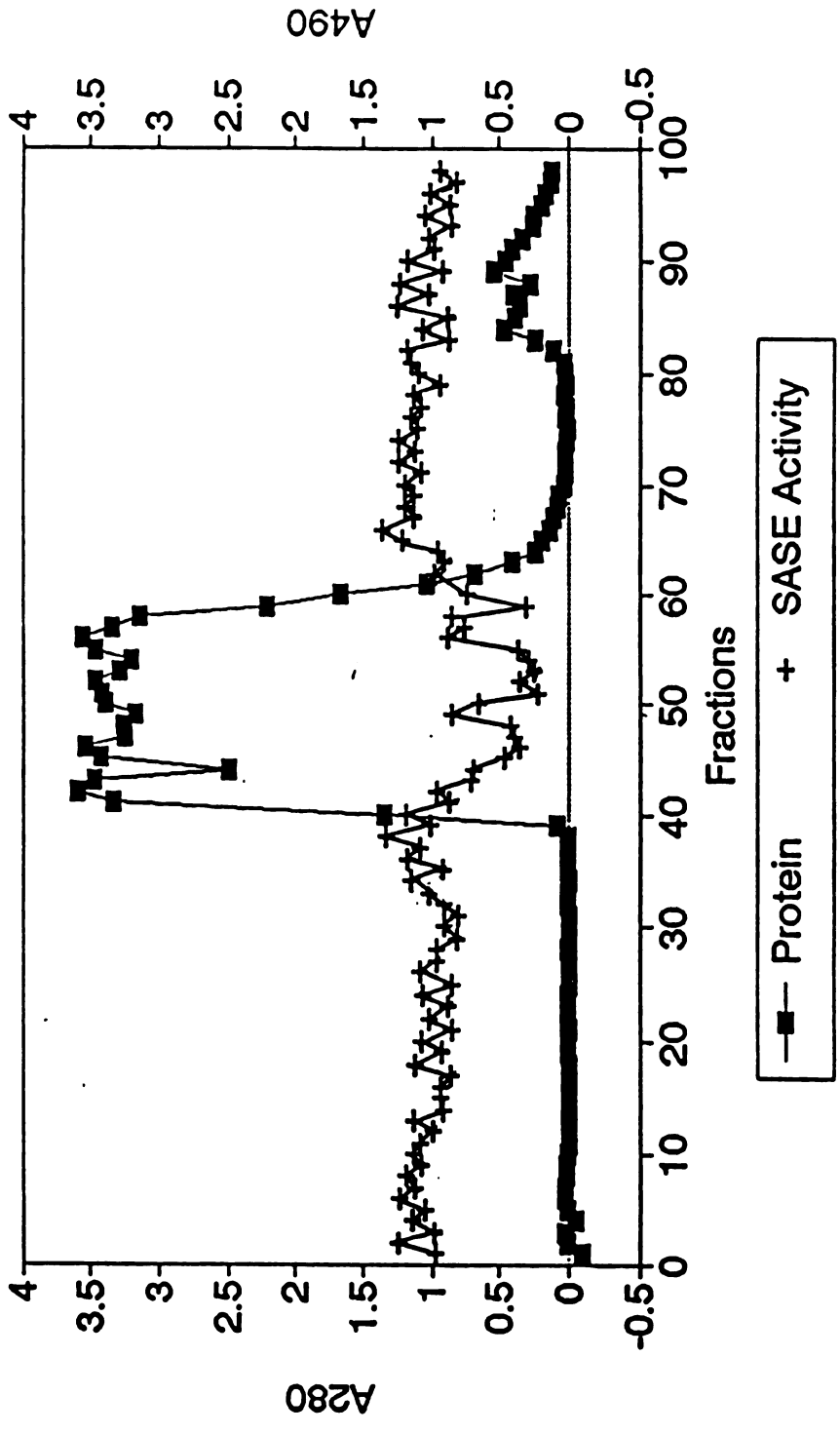

Figure 1.

Figure 2. Elution profile of 1 ml of serum from a cancer subject on Sephacryl S-200 HR column. Each fraction was assayed for protein (left axis, A_{280}) and sialidase activity (right axis, A_{490}).

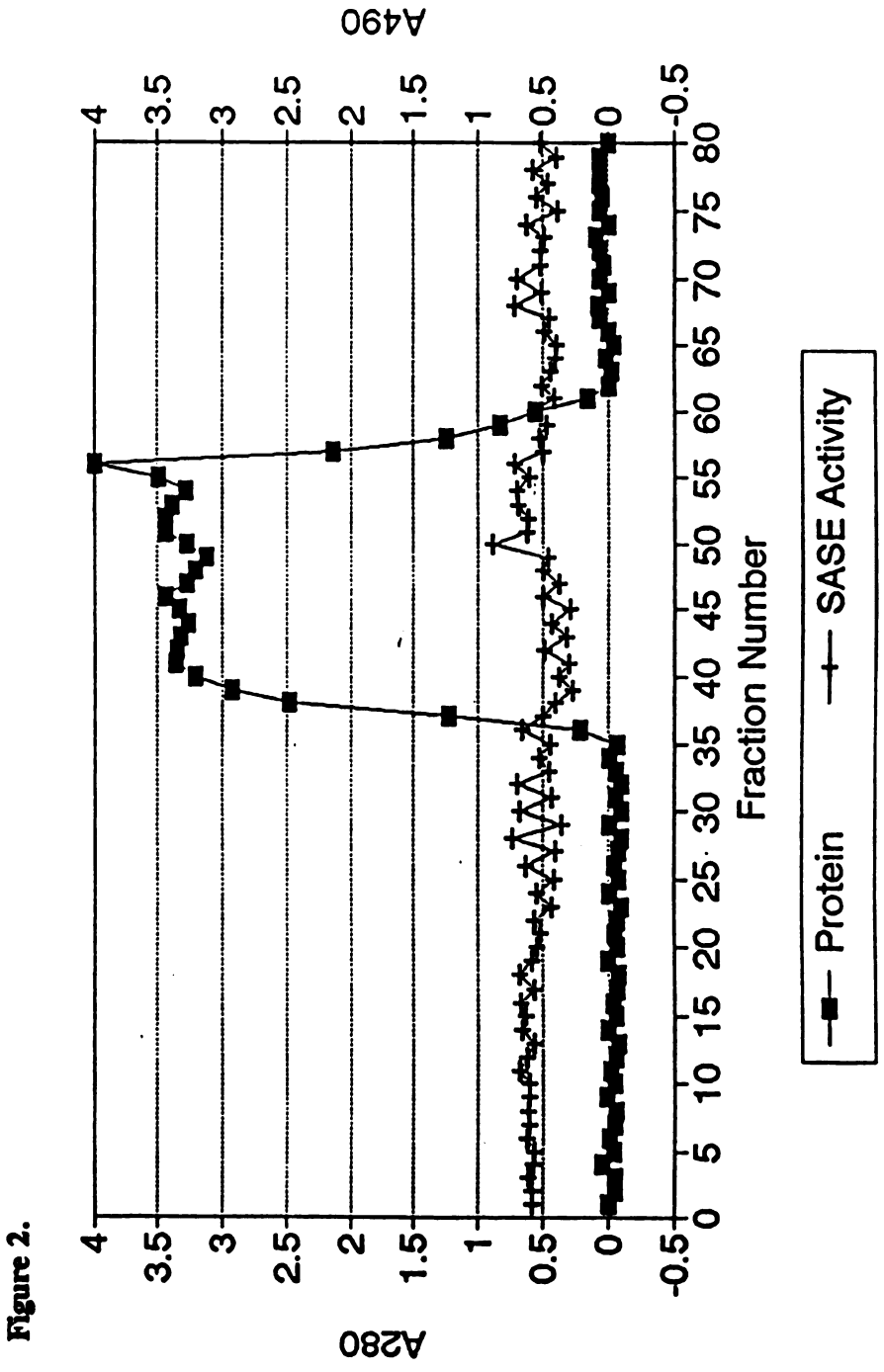

Figure 2.

Warren (30) which corrects for possible interference from 2-deoxyribose. The amount of sialic acid present in a given sample can be determined from equation 1:

μmols Sialic acid =

$$\left[\frac{\epsilon_3}{\epsilon_2\epsilon_3-\epsilon_1\epsilon_4} \text{O.D.}_{549} - \frac{\epsilon_4}{\epsilon_2\epsilon_3-\epsilon_1\epsilon_4} \text{O.D.}_{532} \right] \text{X} \quad \text{V} \qquad (1)$$

where ϵ_1 and ϵ_2 are the molecular extinction coefficients X 10^{-3} cm/nmol of sialic acid at 532 and 549 nm, respectively, and ϵ_3 and ϵ_4 are the molar extinction coefficients X 10^{-3} cm/nmol of 2-deoxyribose at 532 and 549 nm. The values for ϵ_1, ϵ_2, ϵ_3, and ϵ_4 were calculated by measuring the absorbance of either free sialic acid or 2-deoxyribose at the specific wavelength and using Beer's law. They were determined to be ϵ_1 = 19, ϵ_2 = 48, ϵ_3 = 133, and ϵ_4 = 48 cm/nmol which is in agreement of reported values (30).

No difference was found in sialic acid concentrations when serum and plasma obtained from the same individual obtained at the same time were analyzed. Table 1 lists the values obtained for free sialic acid concentrations found in the 9 breast cancer patients plasma and 8 control women samples. The averages were very similar and showed no statistically significant difference. The cancer plasma was slightly lower than that of the control group and had a greater range.

Discussion

Sialidase activity has been found in a wide range of mammalian tissues as well as several subcellular locations. Activity has been found in the lysosome (9, 12, 32, 33), on the lysosomal membrane (9), on the plasma membrane (9, 12), in the cytosol

Table 1. Sialic acid concentrations found in 9 breast cancer patient plasma samples and 8 women control sera. Averages shown ± S.D. along with the median values. concentrations were determined by the TBA method. See text for conditions and calculations.

Table 1.

Free Sialic Acid Concentrations in Plasma and Serum

	Cancer (n=9)	Control (n=8)
	1.000	1.387
	0.698	2.215
	0.723	2.024
	1.139	1.132
	1.566	1.922
	1.907	1.138
	3.681	1.564
	2.347	2.392
	1.613	
Average	1.63 ± 0.94 pmol/ml	1.72 ± 0.48 pmol/ml
Median	1.56	1.74

(9, 34), and more recently, a secreted extracellular sialidase has been found (11, 35, 36). However, sialidase has rarely been reported to be present in measurable amounts in plasma or serum (37-39), which is unexpected because most other glycosidases can be found in measurable amounts. However, in 1976, Schauer *et al.* (39) used radioactive sialic acid analogs in α_1-glycoprotein to measure sialidase activity in plasma and human milk. They found minute amounts of sialidase activity of 10 fU/ml. Their brief studies on this activity found a pH maximum of 5.5 and norequirement for metal ions. They also found it to be stable in a frozen state for up to three months. While the authors could not verify what the source of this sialidase was, their experiments suggested that it did not come from autolytic blood cells or in any particulate form. This paper is very intriguing yet suspicious at the same time. The report of 10 fU/ml of activity is extraordinary when considering the sensitivity of the techniques of 1976. Even more strange is a total lack of citations of this report in the bulk of the later literature. Even one of the authors fails to cite it in further manuscripts in which it would be relevant.

When sialidase activity is reported to be found in plasma, it is typically associated with some type of bacterial infection (37, 38). In this case, the sialidase has been attributed to the bacteria, and not from the host. One of these reports found no sialidase activity in plasma (37), while the other reported a value of 100 nU/ml based on a personal communication that was never published (38). This trace amount would require detection methods more sensitive than the most current methods. The method of Ogura *et al.* (27) is capable of detecting 200 nU/ml under typical conditions and is considered to be one of the most sensitive.

The inability to find measurable sialidase activity in plasma or serum may be due to one of several reasons. One possibility is the actual absence of sialidase in plasma. This would be unusual because of the presence of the other glycosidases found in plasma. The finding of sialidase activity in the medium of cultured fibroblasts suggests that a mechanism, albeit unknown, is in place for the secretion of sialidase into the extracellular space. Since the many other glycosidases are found in plasma, a selective mechanism for the secretion of specific enzymes must be involved.

If a sialidase were secreted into the plasma or serum, it is highly likely that it would be very tightly regulated. Studies have shown that sialidase in plasma can be very harmful to the surrounding environment. Sialic acid acts as a biological mask in many ways and the action of sialidase, i.e. the removal of sialic acid from glycoproteins, oligosaccharides, and sphingolipids, has many biological effects (6). When free sialidase was injected into the blood of rats several effects were noted including the removal of much of the serum glycoproteins and tissue dammage. The removal of the masking effect of sialic acid can cause the removal of glycoproteins (40) and erythrocytes (41) from the blood. Sialic acid can mask the antigenicity of carbohydrates, thus when the sialic acid moiety is removed, a possible anti-immune response can occur (7). One group of investigators found that desialylation of plasminogen, the plasma zymogen of the fibrinolytic enzyme, fibrin, alters the zymogen's kinetics and binding without structure changes (1). Desialylation can also activate the zymogen to 10 % of the fibrinolytic activity of the processed enzyme. The fact that several bacterial infections are accompanied by the secreting of large amounts of sialidase into the plasma and infected tissue suggests that sialidase aids in

the infection process by any of the above-mentioned processes (37, 38). Extracellular sialidases have been shown to be involved in the control of the cell cycle, acting through GM_3 and epidermal growth factor receptor (35, 36). It becomes obvious that biological consequences of an unrestricted sialidase activity in plasma could be hazardous and this strongly suggests that if there is indeed some enzyme activity, it would be tightly regulated. Plasma may have some indigenous inhibitor against sialidases such as salts or specific cations which have been shown to be unnecessary or inhibitory to various plasma sialidases (34, 39, 42, 43). It is possible that a plasma sialidase is associated with a protective protein such as β-galactosidase as found in lysosomal sialidase in human placenta (33, 44-46). This protective protein may closely regulate the activity of sialidase in a tightly controlled manner.

The presence of sialidase in plasma or serum has yet to be proved in a convincing way. We found no activity of sialidase in either breast cancer sera or control plasma along with no alterations in the amount of free sialic acid, which tends to agree with these findings. Our data show that a circulating sialidase may not have a role in the progression of breast cancer, yet the possibility of an extracellular sialidase working in the local environment of the tumor cells has not been determined.

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CHAPTER 8

Discussion and Perspectives

The goal of this study was to determine if the ganglioside profile in plasma or serum from breast cancer patients was different than that which could normally be found in plasma or serum of healthy individuals. A unique circulating ganglioside profile could aid in the assessment of the onset of breast cancer and might be useful for diagnostic and/or monitoring purposes. Furthermore, by determining the components of circulating ganglioside profiles, more information would be at hand to try to understand the mechanism of breast tumor growth and the propensity for these tumors to metastasize. If a particular ganglioside were a tumor-associated antigen and unique to breast cancer, a possible next step would be the development of antibodies against it. Once these antibodies have been raised, assays could be developed for the simplification of the detection of the ganglioside antigen with less complicated isolation procedures. This ganglioside-antigen might also be a candidate for immune therapy as discussed previously. The end result would be a quick and simple assay that could provide the physician with potentially very useful information for diagnosis, prognosis and the possibility for another choice in therapeutic strategies.

It was stated in the statement of the problem that in order to properly assess this potential, the current technology and protocols would have to be reassessed and

optimized where possible. Limited amounts of plasma or serum obtainable from patients, often less than 3 ml, requires that current isolation protocols be able to maximize the recovery from such small volumes. We devised modifications to the method of Ladisch *et al.* (1) that allow for both qualitative and quantitative analysis. Our development of a system of 2-D HPTLC and image densitometry analysis has aided in increasing the resolution and detection of gangliosides mixtures isolated from plasma or serum. By employing these techniques and modifications, gangliosides can be well-characterized from as little as 1 ml of plasma or serum.

Gas chromatography was originally employed to quantify ganglioside standards for use on HPTLC. It was quickly recognized for its potential to characterize mixtures of gangliosides by component analysis. In component analysis, unusual sugars such as fucose could easily identify the presence of potentially interesting gangliosides, as well as characterize other alterations in profiles by changes in the ratio of certain sugars. The concomitant analysis of the fatty acids and long-chain bases added to our information concerning the nature of the hydrophobic tail of gangliosides found in the circulation. Our modifications to the current protocol for analysis by GC made the method much faster, simpler to use, and capable of dealing with smaller sample sizes.

After having developed a solid means of isolating and characterizing gangliosides isolated from plasma or serum, we profiled the circulating gangliosides of cancer patients and an appropriate control group. The methods and protocols we developed were well suited for this type of analysis. We were able to successfully characterize 12 different resorcinol-positive spots on 2-D HPTLC corresponding to

gangliosides isolated from 1 ml of plasma. Other studies required larger starting volumes of plasma in order to achieve the degree of analysis we observed (2, 3). The GC analysis was supportive of the HPTLC work and we were able to describe 5 different α-hydroxy fatty acids associated with circulating gangliosides not previously reported.

The concentration of free sialic acid was observed to be the same in the circulation of both the control individuals or cancer patients. Also, we were unable to find and sialidase activity in plasma or serum of either group. Consequently, neither of these can be considered useful for diagnostic purposes. The apparent lack of involvement of extracellular free sialic acid and sialidase in the alteration of gangliosides and possibly other glycoconjugates suggests that any alterations in the degree of sialylation of gangliosides most likely occurs within the cell and is modulated by intracellular reactions.

We determined that the profiles of circulating gangliosides were altered in the sera of breast cancer patients. There was not found a serum ganglioside that was unique to breast cancer that could be characterized as a circulating tumor-associated ganglioside antigen. We were, however, able to report some interesting biochemical findings about the nature of circulating gangliosides associated with breast cancer. A potential reason for the lack of finding a tumor-associated ganglioside in breast cancer patients may be related to the nature of breast cancer. The two types of cancer that have well-characterized tumor-associated ganglioside antigens are neuroblastoma and melanoma. Both cancers are very fast growing, aggressive and metastatic. Breast cancer, while highly metastatic, is not as aggressive and rapidly growing, and the

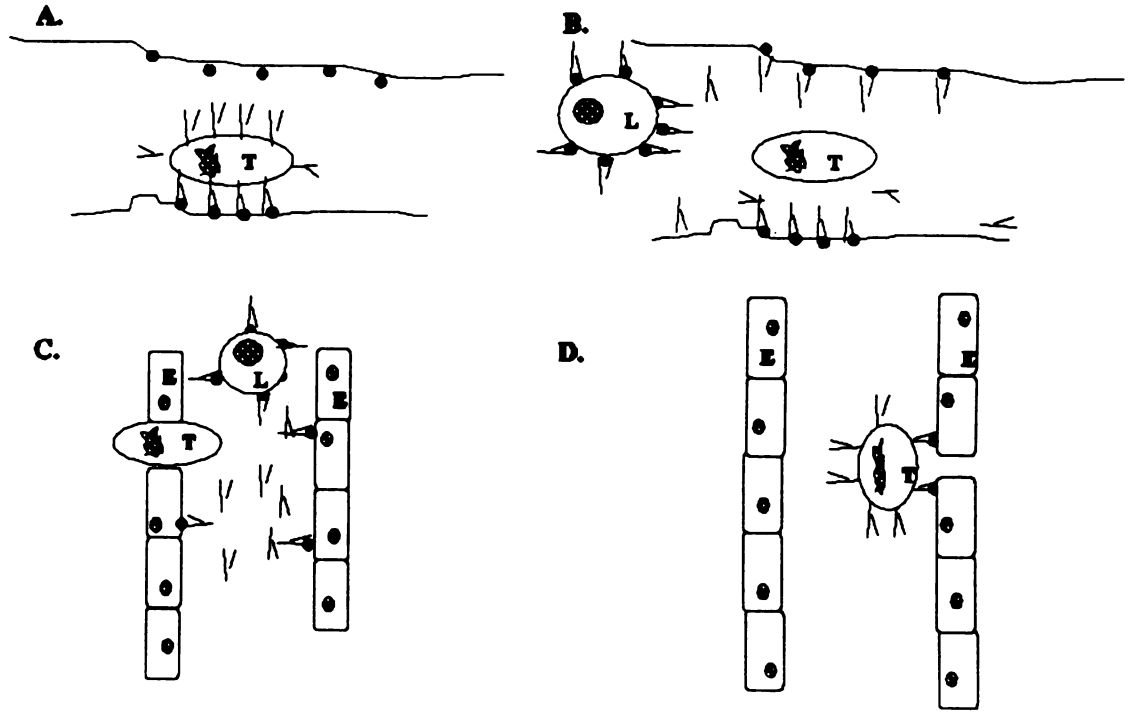
tumors are usually quite small at the time of diagnosis . The continuous cellular division of more aggressive cancers such as neuroblastoma and melanoma may have some causal effect on the shedding of gangliosides into the circulation. Furthermore, this high degree of shedding may aid these aggressive cancers in their ability to grow and metastasize by inhibiting the immune system.

Our findings of an increase in the relative proportions of the more complex gangliosides, and in particular, b-series gangliosides, are consistent with the theory that polysialogangliosides are involved in metastasis. The polysialogangliosides have been shown to bind to various extracellular matrices, and one can speculate that free gangliosides could compete for and occupy cellular binding sites, freeing tumor cells from substrata anchoring and associated mechanisms. A possible model for the involvement of gangliosides in metastasis is described in Figure 1: A) After a tumor cell population becomes established in the breast tissue, alterations in the profile of gangliosides found on the tumor cell surface reflect a move to the more complex ganglioside. B) These gangliosides are subsequently shed from the cell surface. As a result, current anchors may be released and potential binding cites of the extracellular matrix are blocked. The cells are now freed of potential contact inhibitory signals and continue to grow. The gangliosides shed from the now rapidly growing tumor cells protect the cells from immune surveillance and allow the cells to escape the local environment and enter the circulation. C) Once in circulation, the cellular surface again alters its surface gangliosides and other glycoconjugates. D) This new surface allows the tumor cells to bind to the blood cell walls and subsequently invade new tissue and establish new regions of growth.

Further experiments will be necessary to support this hypothesis. Ganglioside profiles from the circulation and the tissue would be monitored through the progression of the disease, noting any profile changes and attempting to correlate them with stage-specific tumorigenic events. This way, the role of circulating and tissue gangliosides might be better understood in slow-growing yet metastatic tumors. By better understanding of these tumorigenic processes, we become more able to develop treatments which may eventually lead toa cure.

Figure 1. Possible role of gangliosides in metastasis. A)Tumor cell (T) in tissue bound to matrix through ganglioside mediated interactions. B) Shedding of gangliosides frees tumor cell from matrix and blocks matrix binding sites as well as blocking detection by lymphocytes (L). C) Tumor cells translocate through the blood cell wall epithelial cells (E), continually shedding gangliosides to block binding and detection. D) Specific gangliosides reappear on tumor cell surface to initiate binding to blood cell walls, and infiltration of new tissue.

Figure 1.



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APPENDIX

Table 1. Patient Bio Data and HPTLC Data
All data ordered based on a/b ratio

					pmol LBSA					
Exp#	Age	Diff.	E.R.	Stage	GM3	GM2	SPG	2-6s	mg4	mg6
3	60	WD	P	II	4218	164	118	26	52	0
29	62	WD	P	II	3512	711	281	289	71	
27	59	WD	P	I	7678	1910	451	0	157	0
26	44	PD	N	I	4672	226	952	127	117	0
31	60	MWD	P	II	4023	121	164	111	28	13
23	56	MWD	P	I	4439	163	385	79	57	57
2	52	MWD	P	II	3626	191	96	133	129	73
28	54	MWD	N	I	4118	497	610	103	149	0
9	53	PD	N	III M	3289	145	227	12	14	33
1	60	MWD	P	IV M	2601	263	154	52	121	16
35	54	PD	N	IV M	4087	221	784	214	215	158
4	56	MWD	P	II	4118	335	271	269	229	23
21	44	PD	N	I	2598	362	193	88	53	65
33	53	MWD	P	I	3590	166	186	129	22	16
24	57	MWD	P	II	3897	84	276	204	69	109
25	49	MWD	N	I	4481	237	163	294	879	77
30.19	61	MWD	P	I	2522	203	221	112	11	41
34	54	PD	N	II	2084	214	341	144	0	
20	70	MWD	P	IV	1981	266	406	0	0	0
22	50	PD	N	I						
7	51	PD	N	I						
11	52	WD	P	I						
15	42	PD	N	III M						
36	55	PD	N	IV						
38	49	PD	N	II						
39	60	MWD	P	II						

Differentiation (Diff): PD-Poorly differentiated, MWD- Medium well
Estrogen Receptors (E.R.): P- Postitive, N- Negitive

Table :. Patient Bio Data and HPTLC Data (cont'd)
All data ordered based on a/b ratio

							pmol LBSA				
Exp#	snhc	GD3	GD1a	GD1b	GT1b	GQ1b	Total	a/b	M/D	M/DTQ	m2/sp
3	45	892	229	20	114	19	5897	4.41	4.73	3.63	1.39
29	89	1008	312	23	241	3	6540	3.56	3.48	3.12	2.53
27	114	459	418	326	1579	609	13701	3.37	16.73	3.04	4.42
26	796	955	382	162	416	103	10060	3.23	4.89	3.99	0.24
31	31	1241	192	13	146	27	6110	3.04	3.24	2.77	0.74
23	215	1031	247	116	338	109	7235	3.04	4.31	2.93	0.42
2	65	1210	470	61	231	95	6380	2.68	3.00	2.09	1.99
28	89	991	487	167	616	140	7967	2.67	4.16	2.32	0.81
9	15	933	222	60	322	64	5336	2.65	3.53	2.33	0.64
1	10	912	117	21	185	40	4492	2.57	2.85	2.52	1.71
35	323	1224	706	172	448	220	8772	2.43	3.34	2.17	0.28
4	64	1284	49	59	418	140	7259	2.37	3.21	2.72	1.24
21	0	1020	278	53	319	61	5090	2.23	2.55	1.94	1.88
33	293	894	365	533	387	48	6629	2.21	4.02	1.98	0.89
24	90	1402	505	80	481	148	7345	2.13	2.78	1.81	0.30
25	75	925	419	299	327	972	9148	2.04	4.84	2.11	1.45
30.19	36	837	218	408	323	118	5050	1.75	3.01	1.65	0.92
34	101	908	488	98	606	217	5201	1.52	2.30	1.24	0.63
20	0	902	880	667	1386	1086	7574	0.77	2.20	0.54	0.66
22											
7											
11											
15											
36											
38											
39											

a/b – Ratio of a series/ b – series gangliosides
M/D – Ratio of GM3 to GD3
M/DTQ – Ratio of mono– to polysialogangliosides
M2/ spg – Ratio of GM2 to sialoparagloboside

Table 2. Gas Chromatography Data: Sugars and Long-Chain Base
Order is based on a/b ratio (See Table 1)

Number	Gal	Glc	GalNAc	GlcNAc	Unk	NeuAc	d18:1
3	4847	4975	697	265	860	4785	1835
29	4307	6124	539	356	556	4624	4011
27	5907	7397	480	316	361	4825	6255
26	5804	4717	386	236	1308	3406	8748
31	7314	8378	722	655	734	6724	6692
23	8053	9319	502	353	817	6812	6553
2	4627	5281	704	214	742	4967	1576
28	8153	10362	781	522	1014	7762	4910
9	3126	4141	262	109	360	2618	5128
1	3645	5288	862	418	849	4869	2898
35							
4	6458	7896	1022	256	61	6984	3613
21	5578	8498	710	383	892	3898	8843
33	5233	9344	1455	541	807	3695	4341
24	1298	2934	210	122	607	1080	3682
25	1265	3568	204	290	631	877	1922
30,19	3462	4861	305	277	285	3850	3630
34	4744	7982	1283	442	586	3592	1605
20							
22	2777	3964	220	142	471	2165	2501
7							
11							
15							
36							
38							
39							

Table 3. Patient Data – Gas Chromatography Data: Fatty Acids
Order is based on a/b ratio (See Table 1)

	pmol										
Exp #	14:0	15:0	16:1	16:0	18:2	18:1	18:1	18:0	20:0	u3	22:0
3	236	131	188	1849	407	458	134	2097	282	604	544
29	493	389	577	4800	924	1099	365	4084	456	958	1405
27	355	202	292	4889	997	774	325	4730	605	740	1697
26	398	194	196	6826	107	582	172	2063	470	2537	1597
31	224	204	271	3515	593	570	233	3037	370	549	1286
23	421	263	234	6021	117	288	138	2518	511	1871	1394
2	188	122	184	1498	375	375	97	1417	205	830	527
28	436	360	949	6727	747	959	444	5855	981	950	2865
9	100	340	547	2518	756	823	136	2028	271	2079	611
1	368	180	283	343	44	647	193	3060	421	390	1441
35											
4	487	247	235	4200	876	988	273	4392	461	2349	1333
21	65	115	150	5586	306	552	210	2729	946	2496	4084
33	102	156	473	5855	50	79	318	5288	1040	933	3477
24	147	80	73	2259	70	216	144	701	134	774	569
25	409	205	175	7908	122	1902	300	2047	0	1852	474
30.19	68	29	11	684	57	117	34	384	60	229	167
34	69	130	163	4551	654	577	212	5385	620	928	2049
20	457	203	214	4078	33	482	180	1876	354	2045	1186
22	296	121	81	1650	69	152	39	978	199	1059	590
7											
11											
15											
36											
38											
39											

Table 3. Patient Data – Gas Chromatography Data: Fatty Acids (cont'd)
Order is based on a/b ratio (See Table 1)

Exp #	23:0	24:1	24:0	h16:0	h22:0	h23:0	h24:1	h24:0
3	189	513	395	44	91	0	60	75
29	687	1227	1302	106	259	158	333	511
27	930	1604	1176	152	325	186	377	505
26	580	1624	1100	473	117	97	176	195
31	611	1075	1093	94	201	125	228	428
23	432	1327	1057	164	177	113	113	190
2	288	430	511	46	120	102	57	94
28	1474	3852	2394	205	434	277	770	727
9	221	584	557	58	76	40	70	98
1	634	111	1046	69	198	99	9	297
35								
4	530	670	1817	148	164	0	119	157
21	1330	2189	2848	210	309	240	159	410
33	1434	2298	2793	127	267	154	219	477
24	167	336	445	62	32	22	57	43
25	224	403	355	348	76	90	134	51
30.19	55	180	126	47	0	0	137	59
34	815	1036	1420	164	334	198	178	520
20	557	969	947	95	141	109	154	181
22	235	436	417	53	91	75	66	125
7								
11								
15								
36								
38								
39								