A Comparison of Ganglioside Content and Distribution in Normal Murine Neural Tissue and Murine Neural Tumor Tissue

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Abstract

Gangliosides, specialized sialic acid-containing glycolipids found abundantly in the outer leaflet of the neural phospholipid bilayer membrane, have a profound effect on tumor cells which prompts investigation. Mammalian tumors have exhibited drastic abnormalities in ganglioside profile. Gangliosides of normal murine neural tissue and murine neural tumor tissue were extracted through dissolution in both chloroform:methanol- and water-based solutions, isolated using Folch partitioning of polar lipids and non-polar lipids, and purified using ion exchange chromatography and desalting procedures. Ganglioside content was quantified using a Resorcinol assay, yielding a drastic disparity between normal neural tissue and tumor tissue. The distribution of specific gangliosides in the samples were analyzed using high performance thin-layer chromatography, showing a greater proportion of the more structurally complex gangliosides (*e.g.*, GD1a, GT1b) in the healthy murine neural tissue and a greater proportion of the structurally simple gangliosides (*e.g.*, GM3) in the murine neural tumor tissue. These results suggest a role of gangliosides in tumor proliferation and the possible use of the ganglioside profile as a diagnostic tool in cancer therapy.

1 Introduction

The lipid bilayer membrane of neural cells contains specialized glycolipids called gangliosides, which have been shown to play a role general cell surface properties (*i.e.*, membrane transport, cell signaling, and cell adhesion). This family of sialic acid-containing glycosphingolipids constitutes a vital part of the glycoconjugate network extending from the plasma membrane [3]. Recently, gangliosides have been studied as markers of neurological malignancies, including neural gliomas and neuroblastomas. Their apparent association with these diseases motivates the exploration of their properties. Here we examine the chemical structure and composition of gangliosides in relation to their presence in murine tumor cells.

Mammalian tumors have been found to exhibit abnormalities in general ganglioside content and distribution. Current research suggests gangliosides found in murine tumor cells may characteristically have an alternate sialic acid component. The primary sialic acid present in healthy murine neural tissue is N-acetylneuraminic acid (NANA). Murine tumor tissue on the other hand has an abundance of the alternate sialic acid form, N-glycolylneuraminic acid (NGNA). Although NGNA-containing gangliosides have been found in non-neural murine tissue, they are generally absent in healthy murine neural tissue [4]. Until recently, it was not clear whether the NGNA-containing gangliosides in the tumor tissue arose from altered sialic acid caused by malignant transformation or by non-neural cellular components. A recent study has shown that there is an enzymatic conversion from the primary NANA-based ganglioside to the NGNA-based ganglioside. This conversion is catalyzed by the cytosolic enzyme NeuAc-H, which was expressed more heavily in murine tumor tissue than healthy murine tissue. Current research in this field is investigating NeuAC-H regulation of the transformation of NANA-based gangliosides to NGNA-based gangliosides in the hopes of determining new targets for anti-tumor therapy [1].

There are five major gangliosides in the brain: GM1, GD1a, GD1b, GT1b, and GQ1b. These consist of two main components: a hydrophobic ceramide unit, which anchors the

ganglioside to the plasma membrane, and a hydrophilic oligosaccharide chain, to which one or more characteristic sialic acid groups (*e.g.*, N-acetylneuraminic acid, N-glycolylneuraminic acid) are attached (see Figure 1) [3]. We chose to investigate the expression of these various types of gangliosides in murine neural tumor tissue and healthy murine neural tissue. Our research confirms our hypothesis that there is indeed a disparity of content and distribution. By studying the differences in ganglioside content and distribution in healthy murine neural tissue and murine neural tumor tissue, we may be more capable of discovering new diagnostic and preventative measures in cancer therapy.

2 Materials and Methods

2.1 Isolation and Purification of Gangliosides

2.1.1 Extraction of Gangliosides

To obtain an accurate mass measurement before processing for ganglioside content, all moisture was removed from the samples of CBT-1 murine tumor tissue and healthy murine neural tissue by placing the samples in a lyophilizer overnight. An appropriate amount of dried tissue for each sample (20-25 mg brain tissue; 40-45 mg tumor tissue) was weighed and then rehydrated with 0.5 mL water. The samples were stirred for 10 minutes (using magnetic stirrers) in an ice beaker in order to prevent glycolipid degradation.

Gangliosides are amphipathic and thereby soluble in both chloroform:methanol solutions and aqueous solutions (see Figure 1). Utilizing this attribute of dual solubility, chloroform:methanol- and water-based solutions were added to the tissue samples in order to isolate the gangliosides according to the Folch method. A 1:1 chloroform:methanol solution (5 mL; v:v) was added to each sample, dissolving all lipids and removing any enzymes that could denature the lipids from the sample. The samples were stirred overnight with intermittent vortexing to remove residue along the sample tube walls.

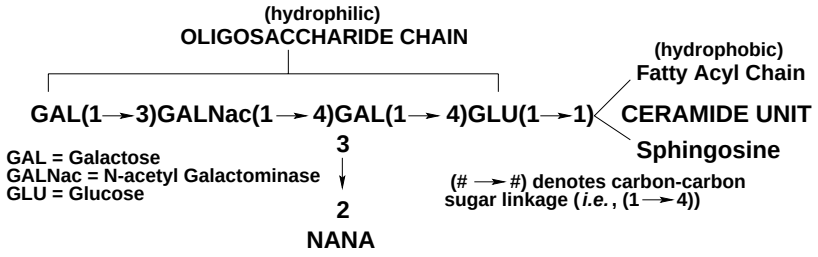

Figure 1: Amphipathic Structure of Ganglioside (GM1)

2.1.2 Isolation of Gangliosides

The samples were centrifuged at 800 g and the supernatant was removed. The remaining solution was washed with 5 ml of chloroform:methanol 1:1 and centrifuged. The resulting supernatant was added to the previously collected supernatant. The supernatant contained neutral lipids, acidic lipids (including gangliosides, due to their sialic acid content), and small insoluble proteins. A 2:1 chloroform:methanol solution (15 mL) and water (a strong polar solvent) were added to the samples and centrifuged at 800 g for 10 minutes. The resulting biphasic solution consisted of an upper phase containing polar lipids and a lower phase containing non-polar lipids. The upper phase was removed and diluted to a chloroform:methanol:water solution (15:30:4).

2.1.3 Purification of Gangliosides

The samples were applied to columns containing DEAE Sephadex A-25, a resin composed of positively charged bead-like particles. A solution of chloroform:methanol:water (15:30:4) was then added. Gangliosides have a negative charge because of the sialic acid group. The samples were then run through the columns, eluting all positively- and neutrally-charged molecules while retaining the negatively-charged gangliosides. A chloroform:methanol:sodium acetate solution (15:30:4) was used to elute the gangliosides. Roto-evaporation was performed on the samples, creating salt crystals and residue in the flasks, thereby evaporating all liquids so that a new chloroform:methanol proportion could be produced. The samples

were collected in chloroform:methanol 1:1 and subjected to nitrogen-evaporation (N-evap) in order to completely remove the chloroform:methanol solution. One mL of 0.1 M NaOH was added to base treat each sample in order to remove all remaining phospholipids.

The samples were then desalted through the use of a Supelco vacuum chamber and Varian bond elution columns, which retained gangliosides and eluted any salts and any other remaining particles. Once the samples were completely desalted, the gangliosides were eluted from the columns by running 2 mL methanol and 3 mL chloroform:methanol (1:1) through the Varian bond columns. The samples were subjected to N-evaporation, then resuspended in 0.5 mL of chloroform:methanol (1:1).

2.2 Analysis

2.2.1 Resorcinol Assay

In order to quantify the ganglioside content of each sample, simple calculations were performed using optical density readings of the sialic acid content of the samples. In this process, the samples were combined with resorcinol reagent, a solution of HCl:$CuSO_4$:water:resorcinol Stock (640:2:80:78). The resorcinol reagent and the sialic acid chemically react when heated to a boiling temperature, creating a translucent blue tint proportional to the content of sialic acid in the sample. The tint was measured using a spectrophotometer, yielding an optical density (OD) reading for the sialic acid content in each sample. In order to determine the experimental error associated with the procedures. OD measurements were taken on standards prepared concurrently with the samples, before and after the desalting process. The assay data were relatively accurate because the curve fitting to the internal standards was within 0.4% of a straight line (Appendix A).

The murine and bovine brain internal standards were used because their sialic acid (SA) content is known to be within 425 - 500 μg per 100 mg sample dry weight. The use of internal standards throughout the isolation and purification procedure as well as the spec-

trophotometer measurements allowed for more reliable data.

2.2.2 Thin-Layer Chromatography

In order to examine the distribution of specific gangliosides, the samples were subject to high performance thin-layer chromatography (HPTLC). In this manner, the various types of gangliosides in each tissue sample were separated. Through HPTLC, it was determined which type of tissue had a greater concentration of a specific ganglioside. When the sample blots were placed in the solvent, the smaller, structurally simple gangliosides were swept to the higher regions of the plate, while the larger, more structurally complex gangliosides remained in the lower regions, creating a clearly striated column of the various gangliosides. The HPTLC plate was compared to a standard HPTLC ganglioside distribution containing most kinds of gangliosides, providing a method of comparison and identification for the gangliosides columns.

3 Results and Data

3.1 Higher concentration of gangliosides in normal murine neural tissue

Nine samples, including two murine brain (MBI, II) and three bovine brain internal standards (BBI, II, III), and four murine brain tumor tissue samples (CBT-1A, B, C, D) were analyzed. The SA content of the healthy brain tissue samples ranged from 243.5 - 270.7 μg SA per 100 mg dry weight, with one sample retaining higher percentage with 403.8 μg. The SA content of the tumor samples ranged from 52.6 - 74.2 μg per 100 mg dry weight. Also, the healthy murine and bovine brain internal standard samples had comparable SA quantities, indicating a consistent protocol. As illustrated by Figure 3, the SA content of murine tumor tissue was drastically lower than the SA content of the healthy brain tissue, indicating a

lower concentration of gangliosides in murine tumor tissue.

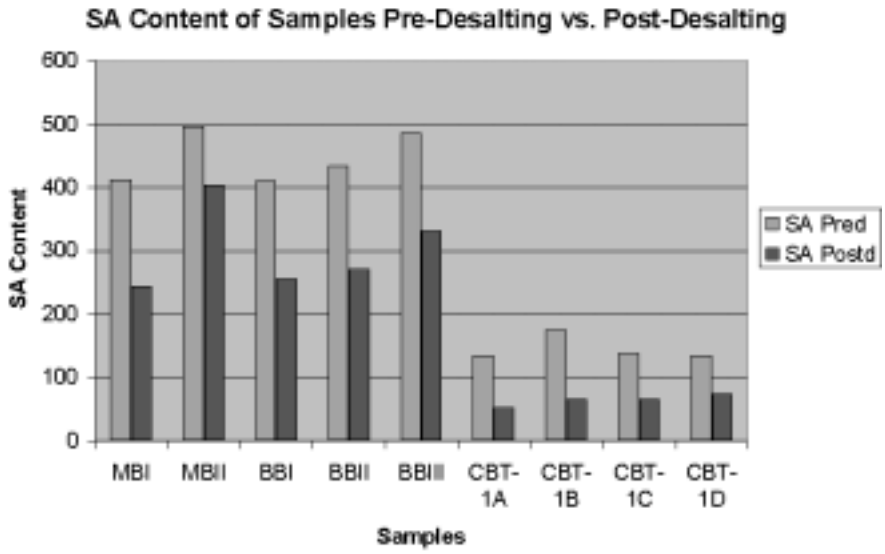

Figure 2: Normal Neural Tissue v. CBT-1 Tumor Tissue SA Content

3.2 High concentration of structurally complex gangliosides in normal murine neural tissue

As illustrated by Figure 4 ,the normal brain tissue from the murine and bovine brain internal standards yielded a heavy concentration of more structurally complex gangliosides such as GD1a, GT1b, and GQ1b (disialoganglioside, trisialoganglioside, quadrasialoganglioside), only faint concentrations of less structurally complex gangliosides such as GM1a, and no expression of GM2 or GM3. The heavy concentration of structurally complex gangliosides indicates neuronal origin of the sample. The absence of less structurally complex gangliosides and NGNA-based gangliosides also corresponds with the ganglioside profiles of healthy murine neural tissue. It was observed that the ganglioside profile of the murine neural tissue and the bovine neural tissue were similar, except for the expression of the LD1 structure in

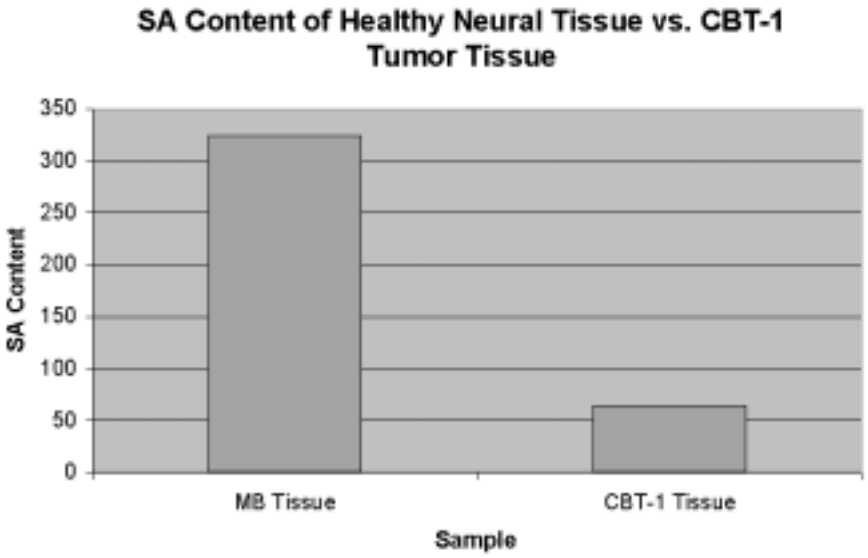

Figure 3: Normal Neural Tissue v. CBT-1 Tumor Tissue SA Content

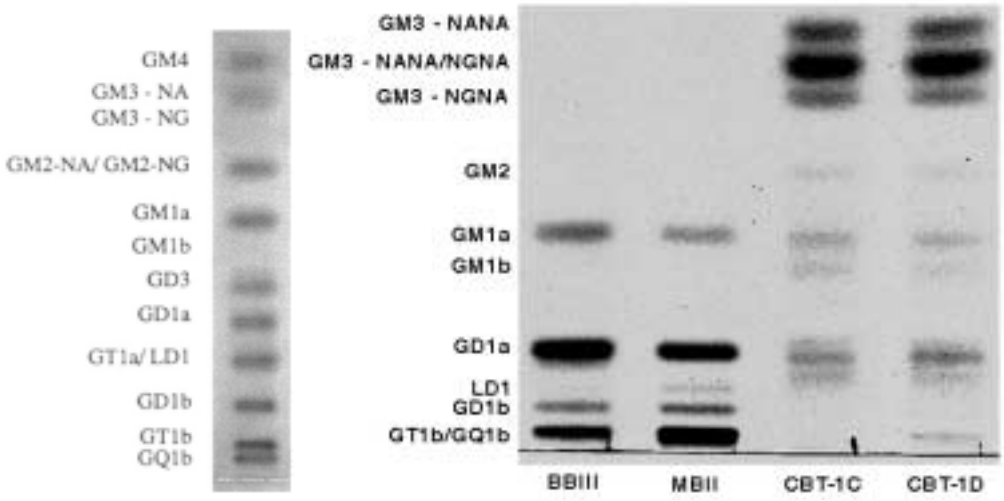

Figure 4: The TLC plate for healthy murine neural tissue and CBT-1 murine tumor tissue shows high concentration of structurally complex gangliosides in murine neural tissue and of structurally basic gangliosides in CBT-1 murine tumor. Lane I: bovine brain; Lane 2: murine brain; Lanes 3 and 4: CBT-1 murine tumor tissue

the murine sample (MBII), but not in the bovine sample (BBIII).[1] The gangliosides with the highest concentrations were GD1a, GT1b, GQ1b, GD1b, and GM1a (refer to Figure 4).

3.3 High concentrations of structurally basic gangliosides in CBT-1 Murine Tumor Tissue

Figure 4 shows that the CBT-1 murine tumor tissue yielded a greater concentration of structurally simple gangliosides, especially the GM3 ganglioside. There were also concentrations of GM1a, GD1a, and faint concentrations of GM2, GM1b, and GT1b/GQ1b in the tumor tissue. The expression of only faint concentrations of structurally complex gangliosides compared to heavy concentrations of GM3 suggests that the complex gangliosides did not originate from the tumor cells of the sample and may have originated from other cellular elements of the CBT-1 tumor. The GM3 ganglioside was divided into three portions, with the middle having the highest concentration of gangliosides. This division indicated a separation of the GM3 ganglioside into both NGNA-based and NANA-based GM3. Comparing the tumor samples with the normal samples, significant concentrations of NGNA-based gangliosides were expressed only in the tumor samples. It was also observed that the chromatography pattern of the two CBT-1 tumor patterns were very similar, affirming the precision of the experimental procedures (refer to Figure 4).

4 Discussion

The reduced number of total gangliosides in the tumor tissue and the difference in types of gangliosides found in the healthy neural and tumor tissues demonstrate a possible relationship between specific ganglioside occurrence and tumor growth (*i.e.*, GM3). This relationship may suggest a process that transforms more complex gangliosides into simpler gangliosides.

[1] LD1 is a lacto- series glycolipid (compared to a ganglio- series), and has a slightly different chemical structure than the ganglio- series.

Simpler gangliosides may greatly facilitate the transport of certain extracellular elements which aid cellular proliferation, such as Ca^{2+}, across the cell membrane.

While the CBT-1 tumor samples expressed a significant amount of NGNA-based gangliosides (GM3-NGNA), the healthy murine neural tissue did not express any NGNA-based ganglioside. This result may suggest a role for NGNA or NGNA-based gangliosides in the tumor proliferation process or a conversion process of NANA to NGNA (*e.g.*, the aforementioned conversion enzyme, NeuAc-H) or of NANA-based gangliosides to NGNA-based gangliosides directly involved in the tumor proliferation process.

The faint concentrations of structurally complex gangliosides found in the CBT-1 samples may not have been from the tumor itself. Every tumor is a cellular society, composed of tumor cells, blood vessels, macrophages, transport cells, and other invasive and supportive cells. These specialized cells also express specific gangliosides in their phospholipid cell membranes. The gangliosides from these cells may account for the other gangliosides found in the tumor samples. This hypothesis is supported by findings that cell cultures of CBT-1 grown *in vitro* express only the GM3 ganglioside [2], and findings that macrophages in the cellular society of tumors express more complex gangliosides [2].

The GM3 ganglioside of the tumor tissue had three divisions because two types of GM3 ganglioside, NANA-based GM3 and NGNA-based GM3, were separated (refer to Figure 4). The highest division was only NANA-based GM3, the lowest division was only NGNA-based GM3, and the middle division was a combination of the two gangliosides, making it much higher in ganglioside concentration. This NANA-NGNA separation occurred only in the murine tumor samples, indicating little to no NGNA-based ganglioside content in the healthy murine neural tissue. To study the proportion of NANA-based gangliosides to NGNA-based gangliosides, a portion of the three divisions could be removed and subjected to gas chromatography (GC).

Similar information such as TLC and GC data from gangliosides of tumors from various cancers, can be used for identification and comparison purposes. Certain tumors that have

higher concentrations of specific gangliosides, such as GM3 in neural gliomas, or structurally different version of similar gangliosides (*i.e.*, NGNA-based/NANA-based ganglioside content and proportion), can be identified by their ganglioside profile, thus making ganglioside profile analysis a potential tool in cancer research. This diagnostic measure is applicable to the CBT-1 tumor line, which has a distinctively high concentration of GM3 and a significant concentration of NGNA-based ganglioside. Such identification information could be used to determine methods of interrupting the transformation of benign ganglioside profiles to malignant ones. Identification information could also aid in targeting individual cells known to be cancerous because of a specific ganglioside profile.

The ganglioside composition of various tissue may also help to identify the source of the tissue sample. In neural tissue, there are two main categories of tissue: glial and neuronal. Glial cells are the supportive cells in the brain; neuronal tissue are comprised of neurons. After development at birth, neurons are post-mitotic. For this reason, tumors of the neuronal cells (*e.g.*, adolescent adrenal neuroblastoma) are rare. However, glial cells, retain the abilities to grow and reproduce. Glial cells consist of less complex structures and express less complex gangliosides, while neurons consist of relatively more complex structures and express more complex gangliosides. Considering the structurally less complex gangliosides found in the CBT-1 tumor, it is likely that the original tumor was glial in nature.

5 Conclusion

Dramatic differences in ganglioside content and distribution were found between normal murine neural tissue and murine neural tumor tissue. Normal murine neural tissue were observed to express higher concentrations of structurally complex gangliosides, while murine neural tumor tissue were observed to express higher concentrations of structurally simple gangliosides. This data suggests that gangliosides may have a significant role in tumor proliferation and the determination of its attributes. The data also suggests that content

and distribution of gangliosides in murine tumors could be utilized as a diagnostic tool in cancer research.

6 Acknowledgments

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A Resorcinol Assay Standards Verification Data

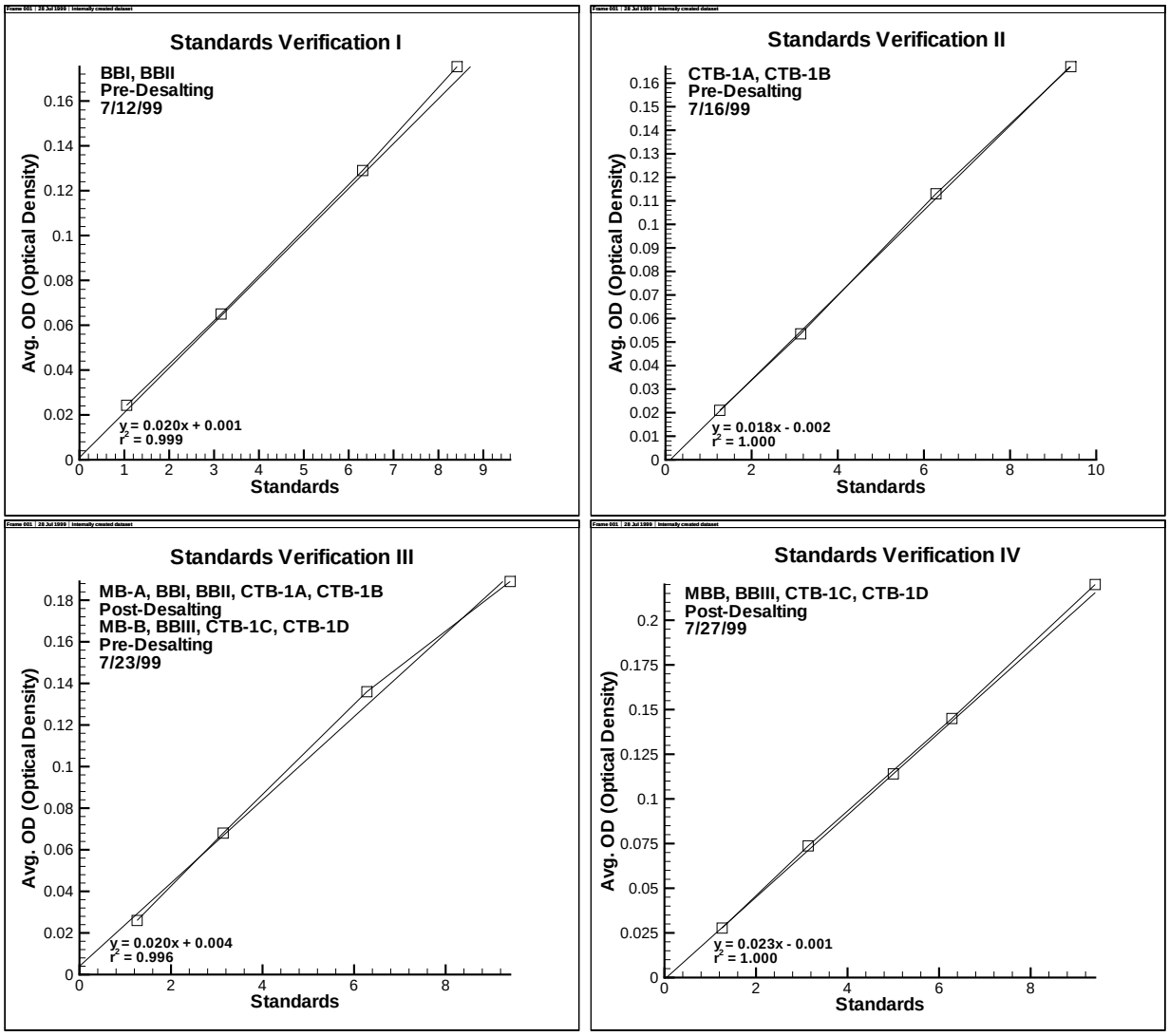

Figure 5: Resorcinol Assay Standards Verification Data

B Abbreviations Used

- NANA - N-acetylneuraminic acid
- NGNA - N-glycolyneuraminic acid

- SA - sialic acid
- GM - monosialoganglioside (only 1 sialic acid branch (NANA or NGNA) off oligosaccharide chain)
- GD - disialoganglioside (2 sialic acid branches of oligosaccharide chain)
- GT - trisialoganglioside (3 sialic acid brances of oligosaccharide chain)
- GQ - quadrasialoganglioside (4 sialic acid branches of oligosaccharide chain)
- HPTLC - high performance thin layer chromatography
- GC - gas chromatography