DETECTION OF COLORECTAL CARCINOMA-ASSOCIATED ANTIGENS USING SPECIFIC ANTIBODIES AGAINST HUMAN MILK OLIGOSACCHARIDES

by

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(ABSTRACT)

Rabbit antibodies against human milk sialyltetrasaccharide b Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc) and sialyltetrasaccharide a (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc) were used to detect their homologous haptens as gangliosides in the human colorectal carcinoma cell line SW1116. Sialyltetrasaccharide b-ceramide was detected in the monosialylganglioside fraction from human meconium and a total ganglioside fraction from SW1116 cells on thin layer chromatograms by radioimmune staining using anti-sialyltetrasaccharide b. Sialyltetrasaccharide b-ceramide was not detected in a total lipid extract from normal intestinal mucosa, thus suggesting that it may represent another tumor-associated antigen. Two novel disialylgangliosides recently reported in human colonic adenocarcinoma -- disialyllactotetraosylceramide (NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3 Galβ1-4Glc1-1Cer) and disialyl Le^a (NeuAcα2-3Galβ1-3[NeuAcα2-6 (Fucα1-4)]GlcNAcβ1-3Galβ1-4Glc1-1Cer) -- both contain the

sialyltetrasaccharide a and b structures, either of which may represent the biosynthetic precursor of these disialylgangliosides. The anti-sialyltetrasaccharide a antibody specifically recognizes its reduced homologous hapten and was used in a radioimmmune binding assay to detect sialyltetrasaccharide a as a reduced and tritiated, ganglioside-derived sialyloligosaccharide from SW1116 cells. Sialyltetrasaccharide a-ceramide was recently detected in human embryonal carcinoma cells, and it is the biosynthetic precursor of the sialyl Le^a antigen, a tumor-associated ganglioside in SW1116 cells. This report confirms the existence of sialyltetrasaccharide a-ceramide in SW1116 cells.

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Most of all, to my parents, I express my love and appreciation for their committment to me. Their support was essential in my achieving this goal.

DEDICATION

To my parents

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INTRODUCTION

Cell surface glycoproteins and glycolipids have been associated with a number of biological functions, including physiological receptors for bacterial toxins, hormones and interferon (Kohn, 1978), and their role as blood group antigens is well established (Watkins, 1980). In particular, membrane glycolipids have been implicated in cellular interaction, differentiation and oncogenesis as a result of their changing patterns of expression during these processes (Hakomori, 1981a). For example, a carbohydrate marker found in the eight-cell stage of mouse embryos, termed stage-specific embryonic antigen 1 (SSEA-1) does not occur on cells prior to the morula stage and its expression is restricted to certain tissues upon further development and maturation. SSEA-1 is also expressed in teratocarcinomas and embryonal carcinoma cells in mice, as well as in various human cancers (Koprowski, 1983).

As work in the fields of developmental and cancer biology progresses, the similarity between neoplastic cells and cells under going normal development and differentiation has become increasingly evident. Morphologically, tumor cells resemble embryonic and fetal cells and exhibit some of the same characteristics such as invasion and colonization of tissues at a site distant from their origin (Fukuda, 1985). On a molecular level, tumor cells may express certain fetal antigens, such as carcinoembryonic antigen (CEA), fetal serum proteins, such as α-fetoprotein, and fetal-type isoenzymes, such as placental alkaline phosphatase (Fukuda, 1985).

Some cancer-associated antigens represent the re-expression of structures which initially appeared at a particular stage of fetal or embryonic development, but which are suppressed in the adult stage. Such antigens are designated oncofetal or oncodevelopmental antigens (Feizi, 1985). The advent of monoclonal antibody technology has allowed the detection and characterization of such antigens, many of which are carbohydrates (Feizi, 1985).

The catalog of oncofetal antigens is steadily increasing and each discovery enriches the understanding of developmental processes and neoplastic transformation in living organisms. However, the possibility lies open for diagnosis and treatment of various cancers using immunologic reagents that can detect cancer-associated antigens (Kennel *et. al.*, 1984). Tumor antigens shed by cancer cells, the colorectal carcinoma-associated antigen (CA 19-9 or sialyl Le^a) for example, can be detected in the serum of patients using a sensitive immunoassay (Del Villano *et. al.*, 1983). In this way, the diagnosis of a particular form of cancer or the monitoring of the progress of patients undergoing cancer therapy can be achieved (Kennel *et. al.*, 1984). Radiolabeled monoclonal antibodies can be used to image tumors *in vivo*, as well as ascertain the extent and location of metastases (Kennel *et. al.*, 1984). In the realm of therapy, some monoclonal antibodies can elicit an immune response against the tumor cells to which they bind and host lymphocytes can subsequently destroy the malignancy. Clinical trials in humans have offered promising results in this area (Kennel *et. al*, 1984). Hybridoma antibodies can also be used to deliver cytotoxic agents such as radioisotopes, various toxins and chemotherapeutic

drugs to targeted cancer cells. This would allow a more selective destruction of cancerous growth than is possible with conventional treatments (Kennel *et. al.*, 1984).

The identification of new tumor associated antigens may lead to improved diagnosis and treatment of certain forms of cancer. The description of carbohydrate tumor antigens will certainly enhance the efforts to eradicate cancer in man.

II

LITERATURE REVIEW

2.1 *CELL SURFACE CARBOHYDRATES*

The abundance of glycolipids and glycoproteins on cell surfaces has led many investigators to suspect that these molecules may have important biological functions. The identity of glycoconjugates as the blood group antigens has been established for some time (Watkins, 1980), but only fairly recently have other roles some of these cell membrane components play come to light. Many researchers have devoted particular attention to the carbohydrate portion of these rather enigmatic molecules. Cell surface glycoconjugates have been shown to interact with a number of bioactive factors, including various agents and their toxins, glycoprotein hormones and interferon (Kohn, 1978; Feizi and Childs, 1985). The involvement of glycolipids, in particular, in cell-cell recognition processes (Hakomori, 1981a), as developmental markers (Hakomori, 1984a) and as tumor-associated antigens (Hakomori, 1984b) has been well documented.

Oligosaccharide chains of glycoproteins and glycolipids are secondary gene products; that is, they are assembled as a result of the action of specific enzymes, the glycosyltransferases, encoded for by various genes (Ginsburg, 1972b). The concept of "one allele, one enzyme, one sugar" generally holds true in the biosynthesis of these molecules (Salmon *et. al.*, 1984), and it is a commonly held belief that

changes in cell-surface carbohydrate structures reflect changing gene expression which occurs during ontogenesis and oncogenesis (Ginsburg *et. al.*, 1984).

2.1.1 *Blood Group Antigens*

The most well defined family of carbohydrate antigens are the blood group antigens, whose name arises from their presence on erythrocytes or as soluble macromolecules in serum and other secreted fluids. The blood group antigens are now known to occur on many different cell types, particularly epithelial cells (Russell *et. al.*, 1982). Both glycoproteins and glycolipids carry structures having blood group specificity (Watkins, 1980).

The familiar and clinically important human ABO(H) blood group system, and the closely related Lewis blood group, have structures based on one of either two disaccharide sequences at their nonreducing ends: type 1 (Galβ1-3GlcNAc...) or type 2 (Galβ1-4GlcNAc...) chains (Ginsburg, 1972b). The type 1 and type 2 disaccharides do not occur by themselves in nature, but exist as part of larger oligosaccharide structures. The A, B, H and Lewis antigens (Le^a and Le^b) are built upon these disaccharide cores by the concerted action of glycosyltransferases. The H antigen consists of the structure

Fucα1-2Gal...

and is the precursor for the A, B and Le^b antigens (Ginsburg, 1972b). In secretory tissue, the expression of the H antigen is under the control of the secretor (Sese) locus. Those individuals who are

secretors (SeSe or Sese) will display ABH antigens on soluble blood group specific molecules (Ginsburg, 1972b). The Lewis antigens also are affected by secretor status. The Le^b antigen

Fucα1-2Galβ1-3[Fucα1-4]GlcNAc...

is a result of the combined action of the secretor gene fucosyltransferase and the Lewis gene (Lele) fucosyltransferase, which adds a fucose α1-4 to N-acetylglucosamine. The Le^a structure

Galβ1-3[Fucα1-4]GlcNAc...

is synthesized by the action of the Lewis gene fucosyltransferase alone on the disaccharide core structure. Therefore, nonsecretor individuals (sese) can only produce the Le^a hapten on soluble blood group substances, and then only if they possess the Lewis gene (Ginsburg, 1972b). Due to the linkage of fucose α1-4 to N-acetylglucosamine, the Lewis antigens can only be built on type 1 chains because the 4 position of N-acetylglucosamine is occupied by a galactose residue in type 2 structures (Ginsburg, 1972b). Because the le gene is inactive, individuals belonging to the Lewis negative blood group [Le(a-b-)] cannot express Lewis antigens. Figure 1 illustrates the pathways for the biosynthesis of the Lewis antigens. Table 1 shows the antigenic structures of the Lewis blood group system and the distribution of the Lewis blood types in the U.S. population.

The Ii blood group determinants are also carbohydrate structures which appear to be internal sequences of the ABH blood group antigens. The I antigen, having the following structure

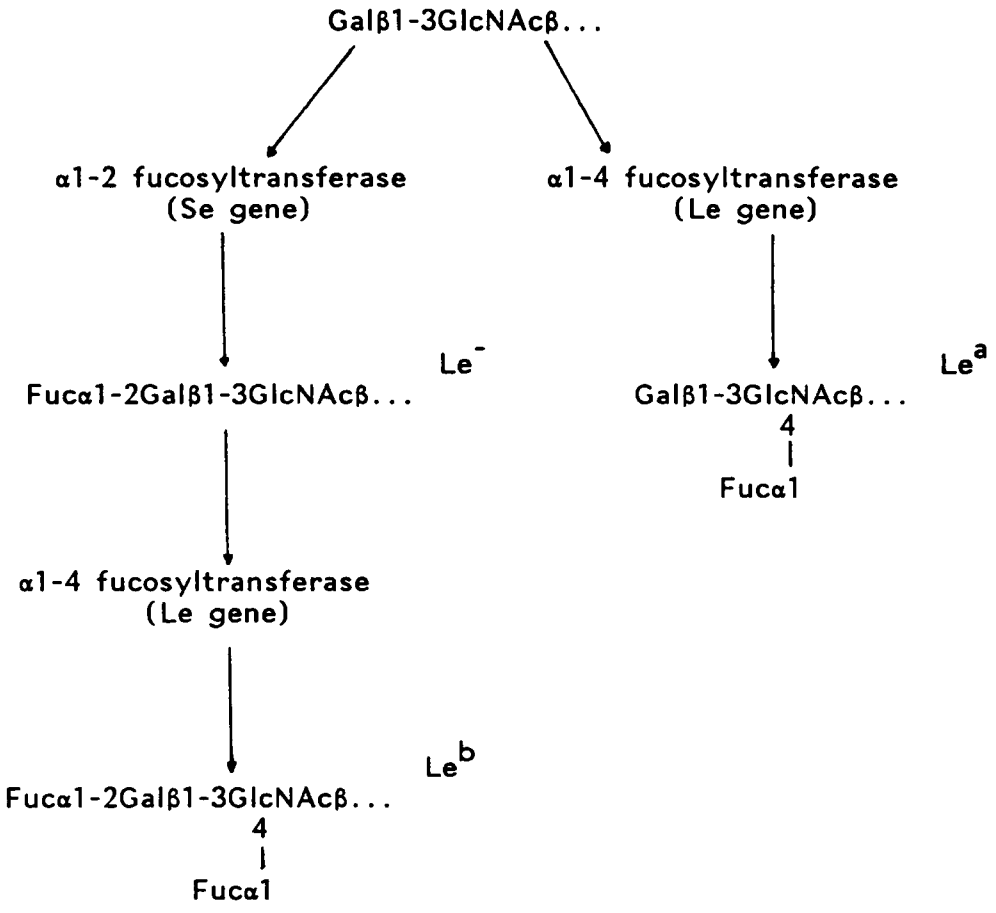


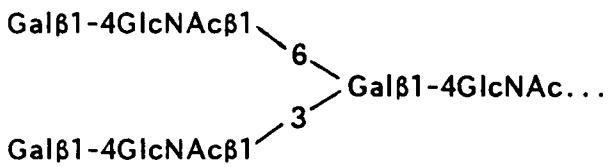
Figure 1: Biosynthesis of the Lewis Blood Group Antigens

TABLE 1

The Lewis Blood Group System

Phenotype --- Genotypes	U.S. population(%)	Structure
Le(a^+,b^-) [Lele,LeLe] [sese]	(15)	Galβ1-3[Fucα1-4]GlcNAc...
Le(a^-,b^+) [Lele,LeLe] [Sese,SeSe]	(80)	Fucα1-2Galβ1-3[Fucα1-4]GlcNAc...
Le(a^-,b^-) [lele] [Sese,SeSe]	(4)	Fucα1-2Galβ1-3GlcNAc...
Le(a^-,b^-) [lele] [sese]	(<1)	Galβ1-3GlcNAc...

This table is from Ginsburg, 1972a.



appears to be a branched variant of the ABH antigenic precursor chains, and the i antigen, having the sequence

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc...

is a linear form of the same. Both I and i are associated with type 2 chains (Salmon *et. al.*, 1984).

The physiological function of the blood group antigens is unknown; however, some associations between these molecules and disease states have been made. Autoantibodies against the blood group I antigen appear in the serum of some patients subsequent to infection by *Mycoplasma pneumoniae* (Feizi, 1985). Similarly, blood group active glycolipids and glycoproteins occuring in seminal fluid have been implicated in the production of autoantibodies that lead to sterility in man (Hannisch, *et. al.*, 1985). The incidence of duodenal ulcer is 40 per cent greater in individuals with blood type O, with the frequency of nonsecretors being even higher. There is also an association of stomach cancer with persons of blood group A, and a progressive loss of A, B and H cell surface antigens occurs in cancers of the gastrointestinal tract, cervix and bladder (Russel *et. al.*, 1982).

2.1.2 *Cell-Cell Recognition, Development and Differentiation*

The complex physiological events that occur in multicellular organisms require that cells be able to communicate with one another, either by direct contact or via some diffusible signal molecule (Kurth, 1983). The ability of cells to recognize adjacent cells is part of this communication process and is attributed partly to specific cell surface carbohydrate-protein interactions between neighboring cells (Hakomori, 1984a). Carbohydrates have been implicated in several biological functions involving cell-cell recognition, including cell adhesion (Harper and Juliano, 1981), clearance of erythrocytes from the blood (Ashwell and Harford, 1982), bacterial attachment to cells (Beachey, 1980), synapse formation (Grunwald *et. al.*, 1985) and formation of the neuromuscular junction (Obata *et. al.*, 1977).

The spatial organization of cells in a specific organ or tissue type is critical to the normal functioning of that tissue. The ordered arrangement of cells achieved during the developmental process is thought to be mediated by the interaction of surface receptors with carbohydrate chains on surrounding cells (Kurth, 1983). That the control of cell growth and differentiation is under the influence of such interactions is implied by marked changes in cell surface oligosaccharide chains known to occur during embryogenesis and during differentiation of certain adult cell lines (Hakomori, 1984a and Fukuda, 1985). For example, the i-antigen and unbranched ABH structures are present in fetal erythrocytes but conversion to I-antigen and branched ABH structures takes place during maturation of the cells to the adult type (Hakomori, 1981b). Also, a shift in glycolipid synthesis from the

ganglio-series to the lacto-series to the globo-series (see Table 2 for structures of the major glycolipid series) occurs during differentiation of the murine myelogenous leukemia cell line M1 into cells with phenotypic characteristics of mature macrophages (Kannagi *et. al.*, 1983).

2.1.3 *Tumor-Associated Antigens*

The alteration of carbohydrate surface antigens during malignant transformation in various tissues has been well documented (Hakomori, 1984b). Specific structures which are unique to tumor cells or appear in elevated levels compared to their normal counterpart cells are defined as cancer-associated antigens. The use of monoclonal antibodies has demonstrated that many cancer-associated antigens are carbohydrate (Hakomori, 1984b and Feizi, 1985). Some examples are the ganglioside G_{D3} (NeuAcα2-8NeuAcα2-3Galβ1-4Glc1-1Cer) in human melanoma tumors (Nudelman *et. al.*, 1982) and lactofucopentaosyl(III)ceramide (Galβ1-4[Fucα1-3] GlcNAcβ1-3Galβ1-4Glc1-1Cer) and related structures of higher molecular weight in human adenocarcinomas (Hakomori *et. al.*, 1982).

Two types of changes have been observed in the pattern of expression of glycolipids in tumor cells: accumulation of precursor structures resulting from blocked synthesis, and appearance of novel structures, "neoglycolipids", due to activation of glycosyltransferases that were previously inactive (Hakomori, 1984b). The former category includes structures resulting from incomplete synthesis of the ABH blood group antigens, which are present on various epithelial cells.

TABLE 2

Major Structures of Glycosphingolipid Series

Series	Structure
Globo	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer
Lacto	Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
Ganglio	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer
Muco	Galβ1-4Galβ1-4Glcβ1-1Cer
Gala	Galα1-4Galβ1-1Cer

Since the majority of human cancers are formed in epithelial tissues, these structures represent important tumor antigens (Hakomori, 1984b).

The structural identification of tumor-associated glycolipids has led to an interesting discovery. In some instances, neosynthesis results in unusual gangliosides, fucolipids and fucogangliosides with type 1 or type 2 chains (Hakomori, 1984b). Some examples of these lacto-series gangliosides (see Table 2 for structures of the major glycolipid series) associated with human cancers are the sialyl Lewis^a antigen (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc1-1Cer) in gastrointestinal cancer (Magnani *et. al.*, 1982) NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc1-1Cer in embryonal carcinoma cells (Fukuda and Dell, 1986) and disialyllactotetraosylceramide (NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc1-1Cer) (Fukushi *et. al.*, 1986) and disialyl Lewis^a (NeuAcα2-3Galβ1-3[NeuAcα2-6(Fucα1-4)]GlcNAcβ1-3Galβ1-4Glc1-1Cer) (Nudelman *et. al.*, 1986) in colonic adenocarcinomas. The growing number of sialyllactotetraosylceramides reported as oncogenic markers strongly implies that the synthesis of or differences in substitution on type 1 carbohydrate chains reflects underlying transforming processes in some cell or tissue types.

One of the most extensively studied tumor antigens, the sialyl Lewis^a antigen, is a lacto-series ganglioside associated with gastrointestinal cancer (Magnani *et. al.*, 1982). The sialyl Lewis^a antigen was defined by a monoclonal antibody (19-9) developed by immunizing mice with the colorectal carcinoma cell line SW1116 (Koprowski *et. al.*, 1979). This antigen is present as a monosialylganglioside in gastrointestinal cancer cells and in human

meconium, which is intestinal material of fetal origin, but is absent in normal intestinal mucosa (Magnani *et. al.*, 1981, 1982). Sialyl Lewis^a was thus designated an oncofetal antigen (Magnani *et. al.*, 1982). Two other sialyl Lewis^a related structures, NeuAcα2-3Galβ1-3 GlcNAcβ1-3Galβ1-4Glc1-1Cer (sialyltetrasaccharide a-ceramide) and Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc1-1Cer (sialyltetrasaccharide b-ceramide) are monosialylgangliosides of human meconium (Prieto and Smith, 1985, 1986). Both of these glycolipids share structural features with the previously mentioned disialylganglioside tumor antigens (see Table 3 for structures). This, coupled with the fact that sialyltetrasaccharide a-ceramide is the biosynthetic precursor of the sialyl Lewis^a antigen in SW1116 cells (Hansson and Zopf, 1985), strongly suggests that these molecules may also be oncofetal antigens.

2.2 *SW1116 CELLS*

The cancer-associated antigen, sialyl Lewis^a, is the major ganglioside component of the human colorectal carcinoma cell line SW1116 (Magnani *et. al.*, 1982). This antigen was defined by a monoclonal antibody (19-9) obtained by immunizing mice with SW1116 cells (Koprowski *et. al.*, 1979). The SW1116 cell line was derived from a colonic tumor of a 73 year old white male patient with O positive blood type (Leibovitz *et. al.*, 1976). Ultrastructurally, colonies of SW1116 cells in culture resemble normal intestinal epithelium and thus appeared to be moderately differentiated. Karyotypically, all cells from this line are hyperdiploid and they synthesize relatively large amounts (7000

TABLE 3

Milk Sialyloligosaccharides That Occur In Gangliosides

Name	Structure
3'-sialyllactose	NeuAcα2-3Galβ1-4Glc
sialyltetrasaccharide a	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
sialyltetrasaccharide b	Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc
sialyltetrasaccharide c	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
sialyl Lewis[a]	NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc
disialyllacto-N-tetraose	NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc

Structures in this Table are from Kobata (1972) with the exception of sialyl Lewis-a which is from Magnani et. al. (1982).

ng/10^5 cells) of carcinoembryonic antigen (CEA), which is a tumor associated glycoprotein (Leibovitz *et. al.*, 1976).

2.3 *ANTIBODIES AGAINST MILK OLIGOSACCHARIDES*

The elucidation of glycolipid structures by classical methods is a difficult task due to the small quantities that are obtainable from cell surfaces (Zopf and Ginsburg, 1974). For example, the polylactosaminyl glycolipids of the human erythrocyte cell surface are present at a concentration of 25 x 10^{-18} mol of monosaccharide per cell or 5.0 x 10^5 copies per cell (Viitala and Jarnefelt, 1985). Structures of interest which are minor components would comprise only a very small percentage of the total series and would escape detection by traditional methods unless material from large amounts of blood was analyzed. An immunological approach was developed by Zopf *et. al.* (1975) which takes advantage of the fact that many of the carbohydrate sequences of human milk also appear in cell surface oligosaccharides (Zopf and Ginsburg, 1974). In this approach, antibodies against human milk oligosaccharides are used as sensitive probes for their homologous haptens on related structures (Zopf and Ginsburg, 1974 and Smith *et. al.*, 1981). These antibodies, which are specific for sugar sequences, are prepared by immunizing animals with artificial immunogens consisting of purified human milk oligosaccharides coupled to protein (Zopf and Ginsburg, 1974; Zopf *et. al.*, 1975 and Smith and Ginsburg, 1980).

Most of the major milk oligosaccharides have been structurally characterized by traditional chemical methods (Kobata *et. al.* , 1969

Kobata and Ginsburg, 1972; Ginsburg [et. al.],1976 and Yamashita *et. al.*, 1976). See Table 4 for the structures of some of these oligosaccharides. Structural elucidation of minor components can be carried out on large quantities of milk (Wieruszeski *et. al.*, 1985) or by making use of sensitive probes such as carbohydrate-binding proteins. For example, by employing rabbit antibodes specific for sialyltetrasaccharide b, only 300 nmol of the monosialyloligosaccharide fraction of human milk was necessary to characterize the milk sugar fucosyl-sialyltetrasaccharide b (see Table 4 for structures). Less than 2 pmol of fucosyl-sialyltetrasaccharide b were detected in a complex mixture of oligosaccharides (Prieto and Smith, 1984). The same structure was isolated by Wieruszeski and coworkers (1985) from 10 kg of pooled human milk and characterized using chemical methods. The structural characterization of carbohydrate molecules where sample amounts are limiting does not lend itself to such large scale analytical techniqes. This example clearly demonstrates the sensitivity of immunological methods and their obvious advantage.

Antibodies used to structurally characterize carbohydrate molecules must have carefully defined specificities. This is especially true of polyclonal antibodies, but applies to monoclonal antibodies as well, since there have been reports of hybridoma antibodies with relatively broad specificities (Feizi, 1985). Cross reacitivity of monoclonal antibodies with different antigens is a result of immunoglobulins recognizing common structures within distinct carbohydrate molecules, and not due to the presence of different antibody populations (Feizi, 1985).

TABLE 4

Structures of Some Milk Sialyloligosaccharies

Name	Structure
sialyltetrasaccharide a	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
sialyltetrasaccharide b	Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc
disialyllacto-N-tetraose	NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc
fucosyl-sialyltetrasaccharide b	Fucα1-2Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc
sialyl Lewis[a]	NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc

Structures in this Table are from Kobata (1972) and Wieruszeski (1985).

Data obtained by Smith *et. al.* (1980, 1985) during characterization of the polyclonal rabbit antisera used in the course of the experiments described in this thesis are shown in Table 5 Anti-oligosaccharide antibodies exhibit a high specificity for the reduced forms of their homologous haptens since the oligosaccharides are present as reduced sugars on the synthetic immunogens. However, some antisera are able to recognize the native (i.e. unreduced) structures and thus can be used to detect the carbohydrate sequences of glycolipids and glycoproteins by established immunological procedures. For example, antisera against the milk oligosaccharide sialyltetrasaccharide b was used to detect a meconium ganglioside containing this structure on thin layer chromatograms (Prieto and Smith, 1985). The specificity of the antibody was demonstrated by lack of binding to the asialoglycolipid. This confirms that anti-sialyltetrasaccharide b does not recognize the neutral tetrasaccharide core of this oligosaccharide, which is common to other ganglioside structures. Inhibition of antibody binding to the ganglioside containing the sialyltetrasaccharide b structure on thin layer chromatograms by the addition of the unreduced homologous hapten to the incubation mixture provided further evidence that this antibody is specific for sialyltetrasaccharide b (Prieto and Smith, 1985).

A number of glycolipids have the carbohydrate structures of human milk oligosaccharides (Ginsburg, 1972b). The lacto-series glycolipids, especially, bear a close relationship to milk oligosaccharides since they share the lactose structure as a common precursor. Table 3 lists some sialyloligosaccharides of human milk that correspond to the

TABLE 5

Apparent Binding Constants of Anti-Oligosaccharide Sera

	Antiserum		
	Anti STa	Anti STb	Anti STc
Sugar			
Lac	<100	<100	<100
$Lac_{(OH)}$	---	---	---*
$Glc_{(OH)}$	---	---	7.7×10^2
STa	<660	---	---
$STa_{(OH)}$	2×10^7	---	---
STb	---	4.16×10^6	---
$STb_{(OH)}$	---	6.25×10^5	---
STc	---	---	4.16×10^4
$STc_{(OH)}$	1.6×10^3	---	1×10^8
$LNT_{(OH)}$	5×10^3	<100	---
$LNnT_{(OH)}$	---	---	7.7×10^2

*(---)*not tested. Abbreviations used are*: *STa, STb and STc for Sialyltetrasaccharides a,b and c respectively, Lac for lactose and LNT and LNnT for lacto-N-tetraose and lacto-N-neotetraose respectively. Alditol derivatives are indicated by the subscript (OH). Data for this table was taken from Smith (1980 and 1985)*

carbohydrate moities of gangliosides. The gangliosides NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc1-1Cer, NeuAcα2-3 Galβ1-3GlcNAcβ1-3Galβ1-4Glc1-1Cer and Galβ1-3[NeuAcα2-6]GlcNAc β1-3Galβ1-4Glc1-1Cer are the ceramide derivatives of the milk sialyloligosaccharides disialyllacto-N-tetraose, sialyltetrasaccharide a, and sialyltetrasaccharide b respectively (see Table 4 for structures). The cancer-associated ganglioside in SW1116 cells, the sialyl Lewis^a antigen, has a carbohydrate structure that appears as a free oligosaccharide in human milk as well (Wieruszeski *et. al.*, 1985, and Smith, 1985). Antibodies against human milk oligosaccharides have proven useful in the identification and characterization of gangliosides (Prieto and Smith, 1985, 1986). Sialyltetrasaccharide a-ceramide and sialyltetrasaccharide b-ceramide were both detected as minor monosialylganglioside components of human meconium by the use of antibodies against human milk oligosaccharides to detect their homologous haptens as reduced and tritiated ganglioside-derived sialyloligosaccharides (Prieto and Smith, 1985, 1986). This work, performed by researchers in our laboratory, was possible because antibodies against the human milk oligosaccharide structures contained within these gangliosides, sialyltetrasaccharide a and sialyltetrasaccharide b, were available to us.

Experiments described in this thesis were based on the premise that antibodies directed against human milk sialyloligosaccharides could be used to detect their homologous haptens in the monosialylganglioside fraction of SW1116 cells. The main objectives of this research project were the identification of new tumor associated glycolipids and the

confirmation of the presence of sialyltetrasaccharide a-ceramide, the precursor of the sialyl Lewis^a antigen, in this colorectal carcinoma cell line.

III

EXPERIMENTAL PROCEDURES

3.1 *MATERIALS*

SW1116 gangliosides were a gift from Dr. David Zopf at the NCI, NIH, Bethesda, Maryland. Total lipid extract from normal intestinal mucosa was a gift from Dr. John L. Magnani (NADDKD/NIH, Bethesda, Maryland). Meconium was collected within 24 h of birth from newborns at the infant nursery of Montgomery County Hospital (Blacksburg, Virginia) and stored at -20° C. Structures for the [^{3}H]labeled oligosaccharide alditols used as chromatographic standards are shown in Table 6. Human milk oligosaccharides were isolated (Kobata, 1972 and Smith *et. al.*, 1978) and labeled by NaB[^{3}H]$_4$ reduction as previously described (Smith and Ginsburg, 1980).

DEAE-cellulose (DE53) was from Whatman (Clifton, NJ). Bio-Gel P2 and AG-50W-X8 (H^+ form) were from BioRad (Richmond, CA). N-acetylneuraminic acid, *Clostridium perfringens* neuraminidase (Type X) and DEAE-Sepharose were purchased from Sigma (St. Louis, MO). Affinity purified goat antibody to rabbit IgG (goat anti-rabbit IgG) and beef kidney α-fucosidase were from Boehringer-Mannheim (Indianapolis, IN). Anti-sialyloligosaccharide sera specific for sialyltetrasaccharide a, sialyltetrasaccharide b and sialyltetrasaccharide c have been described (Smith and Ginsburg, 1980 and Smith *et. al.*, 1985).

TABLE 6

Structures of Standard Radiolabeled Oligosaccharide Alditols

Parent Structure	Name or Abbreviation of the Corresponding Reduced Oligosaccharide
Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc	lacto-N-fucopentaitol II
Galβ1-3GlcNAcβ1-3Galβ1-4[Fucα1-3]Glc	lacto-N-fucopentaitol V
NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	STa* alditol**
Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc	STb* alditol**
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	STc* alditol**

** Abbreviations are: STa, STb and STc; sialyltetrasaccharides a, b and c respectively. ** Fucosylated derivatives of these sialyltetrasaccharides compose the human milk sialylpentasaccharide fraction. The structures present in an individual sample are dependent on the Lewis blood type of the donor. The sialylpentasaccharide fraction used as a chromatographic standard in this study was from a Lewis b individual.*

Nitrocellulose filters (No. BH-85, 25mm diameter) were from Schleicher & Scheull (Keene, NH). $NaB[^3H]_4$ (sp. act. 10-20 Ci/mmol) and $Na^{125}I$ (sp. act. 16.5 mCi/μg) were purchased from Amersham (Arlington Heights, IL). Precoated Merck silica gel-60 aluminum backed plates for thin layer chromatography were from Bodman Chemicals (Media, PA).

3.2 *METHODS*

3.2.1 *Analytical Methods*

Sialic acid was quantified by the thiobarbituric acid method (Aminoff, 1961) using N-acetylneuraminic acid as a standard and total hexose was determined by the phenol-sulfuric acid method (Dubois *et. al.*, 1956). Tritium was measured by liquid scintillation counting of aliquots in plastic minivials (Denville Scientific, Denville, NJ) using Ready-Solv EP (Beckman, Fullerton, CA) as cocktail in a Beckman LS-7500 scintillation counter and ^{125}I was measured using a Beckman Gamma 4000 counter.

Ozone concentration in CH_2Cl_2 was determined by the following titrametric procedure (Belew, 1969). An aliquot (1 ml) of O_3 in CH_2Cl_2 was added to a mixture of 2% potassium iodide (15 ml) and a saturated solution of starch in water (15 ml). The resulting mixture was then titrated under constant stirring with 4mM sodium thiosulfate until the blue-black color disappeared. Ozone concentration in the solution was calculated according to the following equation (Belew, 1969):

$$[O_3] \text{ (mM)} = \mu\text{mol } S_2O_3^{-2} / 2 \text{ ml}$$

The molarities of pyridine-acetate buffers are given with respect to pyridine and were prepared by appropriate dilutions of 1.0 M pyridine-acetate buffer, pH 5.4 (80 ml twice distilled pyridine, 30 ml acetic acid and 890 ml H_2O). All chemicals used were reagent grade.

3.2.2 *Chromatographic Methods*

Gangliosides and total lipid fractions were applied to thin layer chromatographic plates in 0.5 cm bands at 1 cm from the bottom of the plate. Chromatography was carried out in chloroform-methanol-0.25% aqueous potassium chloride (5/4/1) in glass chambers (30 x 10 x 26 cm) equilibrated for 1 h.

Sialyl[^{3}H]oligosaccharide alditols were separated by DEAE-cellulose (DE53) chromatography according to the method of Smith *et. al.* (1978) for milk sugars. Briefly, radiolabeled oligosaccharide alditols were dissolved in 500 ul of 0.002 M pyridine-acetate buffer and applied to a DEAE-cellulose column (0.9 x 46 cm). The column was washed with starting buffer and sialyl[^{3}H]oligosaccharide alditols were partially separated by elution with 0.02 M pyridine-acetate buffer, pH 5.4. Fractions (2 ml) were collected and aliquots counted to determine radioactivity.

Chromatography on Bio-Gel P2 (-400 mesh) was performed by applying [^{3}H]-labeled oligosaccharide alditols plus 450 µg of glucose and 300 µg of dextran in 150 µl of 0.1 M pyridine-acetate buffer to a

column (0.9 x 95 cm) previously equilibrated in the same buffer. Fractions (0.5 ml) were collected and assayed for total hexose by the phenol-sulfuric acid method to determine the elution volumes of dextran (void volume) and glucose (total volume). Aliquots from fractions were assayed for radioactivity to determine the elution volume of [^{3}H]-labeled oligosaccharide alditols and their glycosidase digestion products.

High performance liquid chromatography (HPLC) was performed using a Beckman Model 332 gradient liquid chromatograph system. The mobile phase consisted of a mixture of acetonitrile and deionized, distilled water containing 15 mM potassium phosphate, pH 5.1. A linear gradient of increasing phosphate buffer content at the rate of 0.2%/min, starting with a mixture of acetonitrile-phosphate buffer [80/20(v/v)], was employed. The flow rate was maintained at 2 ml/min (Bergh *et. al.*, 1981). Sialyl[^{3}H]oligosaccharide alditols were separated on a 10 µ amino phase carbohydrate analysis column (Alltech Associates, Deerfield, IL). Neutral [^{3}H]-labeled oligosaccharide alditols were chromatographed on a Beckman Ultrasil-NH_2 10 µ analytical column (Beckman Instruments, Inc., Columbia, MD) using deionized water as the polar solvent (Blanken *et. al.*, 1985).

3.2.3 *Gangliosides from Human Meconium*

Lipid extracts of meconium were obtained according to the method of Svennerholm and Fredman (1980) for the quantitative isolation of human brain gangliosides. Meconium (100 g) was homogenized in 300 ml of water in a blender at 4^o C for 2 min. The homogenate was poured

into 1.08 l of methanol under constant stirring at room temperature. Chloroform (540 ml) was added and the mixture stirred for 30 min. The homogenate was centrifuged at 5000 x g for 10 min and the supernatant transferred to a separatory funnel. The precipitate was rehomogenized in 160 ml of water, added to 640 ml of chloroform/methanol (2/1), stirred and centrifuged. The combined supernatants and 540 ml of water were mixed in order to give a 1/2/1.4 chloroform/methanol/H_2O-tissue ratio. Phases were allowed to separated overnight and the lower phase was reextracted with 250 ml of methanol and 167 ml of 10 mM KCl. The upper phases were combined and evaporated to dryness under reduced pressure. The dry material was dissolved in 100 ml of chloroform/methanol/H_2O (60/30/4.5) and centrifuged. The supernatant was evaporated to dryness and redissolved in 40 ml of water. Dialysis was carried out against four changes of water (2 l each) for 72 hr in Spectra-Por dialysis membrane tubing, M.W. cutoff 6-8000 (American Scientific Products, McGraw Park, IL). The dialyzed material was lyophilized and a fluffy, green powder was obtained.

The lyophilized material was dissolved in 6 ml of starting solvent and applied to a column of DEAE-Sepharose (1.5 x 24 cm) in the acetate form equilibrated in chloroform/methanol/water (60/30/4.5). The column was washed with 5.6 column volumes of starting solvent, followed by 5.5 column volumes of methanol, and monosialylgangliosides were eluted with 0.01 M potassium acetate in methanol. The monosialylgangliosides detected by sialic acid assay of column fractions were pooled, dialyzed against distilled water and lyophilized. This fraction contained 18 µmol of sialic acid.

3.2.4 *Radioimmune-Staining of Glycolipids*

Goat, anti-rabbit IgG (1 mg/ml in 0.15 M NaCl; 0.1% sodium azide (w/v); pH 6.75) was dialyzed overnight against phosphate buffered saline (PBS, 0.01 M NaH_2PO_4, 0.14 M NaCl) to remove sodium azide and subsequently radiolabeled by a modified iodogen-catalyzed procedure (Fraker *et. al.*, 1978 and Smith, 1983). A glass tube (12 x 75 mm) was coated with 1 ml of Iodo-Gen solution (80 µg/ml, Pierce Chemical Co., Rockford, IL) in chloroform by rotating under vacuum in a 37° C water bath until the solvent was evaporated. The antibody (0.5 ml) and $Na^{125}I$ (1 mCi, 10 µl) were added to the coated tube which was rotated every minute for 5 min at room temperature. The reaction mixture was then applied to a PD-10 column (Pharmacia, Uppsala, Sweden) previously equilibrated in PBS containing 1% polyvinylpyrrolidone (Mr 40,000, Sigma, St. Louis, MO). Pooled fractions (3 ml) were dialyzed exhaustively against PBS containing sodium azide (8.1 mM Na_2HPO_4, 0.14 NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.02% sodium azide). The specific activity of the goat, anti-rabbit IgG was 1.3 µCi/µg.

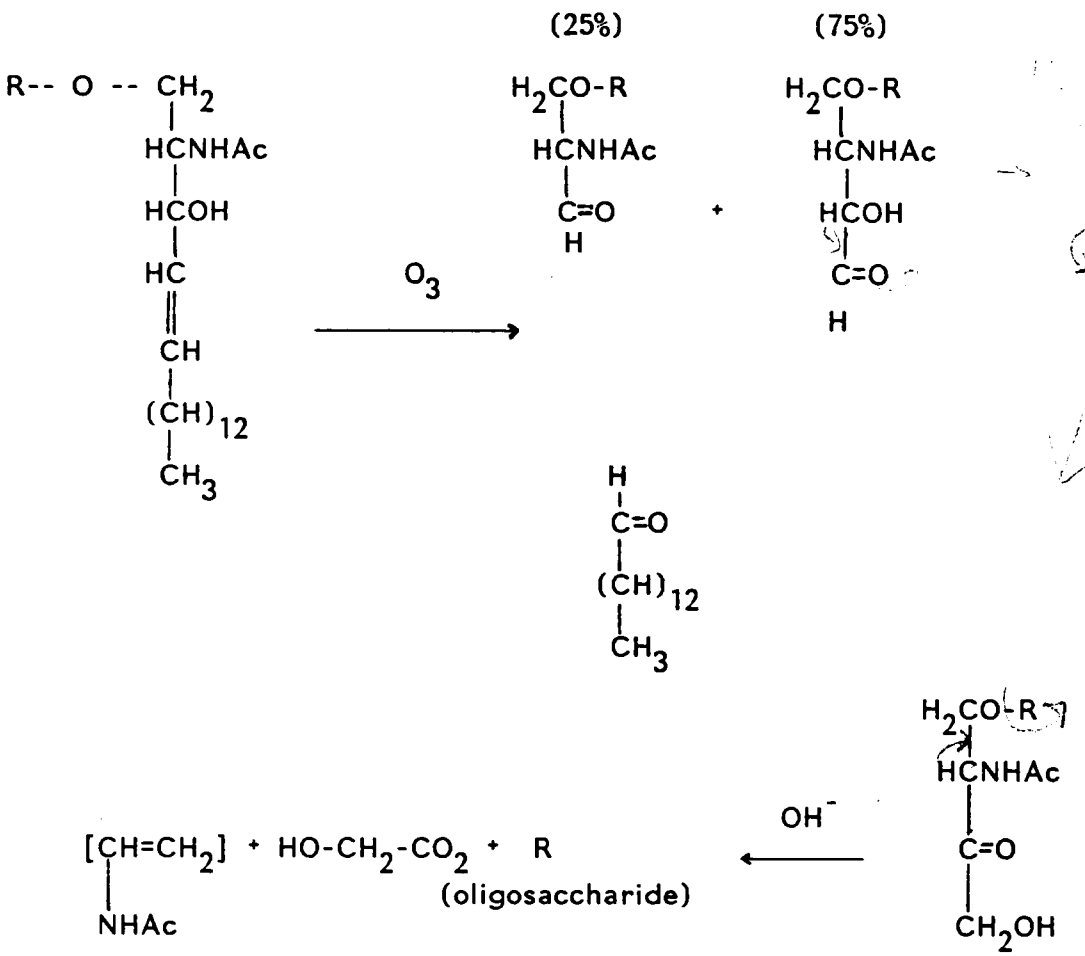
Specific glycolipid antigens were detected by autoradiography of thin layer chromatograms as previously described (Magnani *et. al.*, 1982). Briefly, dried chromatograms were soaked for 1 min in a saturated solution of polyisobutylmethacrylate (Polysciences Inc., Warrington, PA) in hexane in order to cover the plate with a thin film of the polymer and reduce non-specific protein binding. The chromatograms were dried, sprayed with a 2% bovine serum albumin (Sigma, St. Louis, M0) in cold PBS solution (buffer A) and immediately

soaked in the same buffer for 30 min. The plates were then overlaid with a 1/150 dilution of anti-sialyltetrasaccharide b sera in buffer A (50 $\mu l/cm^2$). After incubation for 2 h at 4^o C, the chromatograms were washed by dipping in six successive changes of cold PBS at 1 min intervals and overlaid with ^{125}I-labeled goat, anti-rabbit IgG (10^5 cpm/ml, 50 $\mu l/cm^2$) solution in buffer A. The chromatograms were incubated for 2 h at 4^o C and washed by dipping 11 successive times in cold PBS to remove excess labeled antibody, dried and exposed to XR-5 x-ray film (Eastman-Kodak, Rochester, NY) under an intensifying screen (Cronex, Lighting Plus AH, DuPont, Wilmington, DE) for 18 h at -70^o C.

3.2.5 *Ozonolysis and Alkali Fragmentation*

Oxidation of unsaturated sphingosines in glycosphingolipids using ozone forms an ozonide intermediate which subsequently decomposes to form two aldehydes. Base hydrolysis then catalyzes the elimination of the free oligosaccharide from its lipid fragment as shown in Figure 2. The release of oligosaccharides from glycosphingolipids by O_3-alkali fragmentation was established by Weigandt and Baschang (1965) who bubbled ozone through glycolipid solutions; however, overoxidation of small quantities of glycolipid by ozone may result in degradation of oligosaccharides by oxidation of acetals (Deslongchamps, 1967). Oxidation was controlled by adding aliquots of ozone in methylene chloride instead of bubbling ozone through the reaction mixture (Meyers *et. al.*, 1962).

R = Oligosaccharide



From Weigandt and Baschang 1965.

Figure 2: Ozonolysis and Alkali-Fragmentation of Glycolipids

Since reductive decomposition of ozonides favors aldehyde formation (required for subsequent base-catalyzed elimination of oligosaccharides) dimethylsulfide was used to stop the oxidation (Pappas, 1966). Ozone produced in an OREC 03v5-0 ozone generator (Ozone Research and Equipment Corp., Phoenix, AZ) at 1 amp and an O_2 flow rate of 1 l/min was bubbled through methylene chloride (chilled in an isopropanol-dry ice bath) for 10 min and ozone concentration was calculated as described under Analytical Methods. Total gangliosides from SW1116 cells (60 nmol) were lyophilized in a 1.5 ml polypropylene microcentrifuge tube, dissolved in 100 µl of methanol and placed on ice. An aliquot (500 µl) of O_3/CH_2Cl_2 solution (5.7 mM) was added to the tube containing the gangliosides, and the tube was immediately capped. Ozonolysis was allowed to proceed at 4° C for 15 min and the reaction was stopped by the addition of 50 µl of dimethylsulfide (Aldrich, Milwaukee, WI). The reaction mixture was dried under a stream of N_2 and dissolved in 100 µl of sodium methoxide (0.29 M, 4° C). After 1 h (4° C) the reaction was stopped by neutralizing with 250 µl of 0.1 M acetic acid. The contents were then evaporated using a Speed Vac Concentrator (Savant, Hicksville, NY) and dissolved in 1 ml of chloroform/methanol (2/1) followed by the addition of 100 µl of distilled water. After vigorous mixing, two phases were separated by centrifugation (Eppendorf microcentrifuge). The upper phase was removed, and the lower phase reextracted with another 100 µl of distilled water. The combined upper phases were lyophilized, dissolved in distilled water (200 µl) and applied to a small column (0.5 x 3 cm) of Dowex 50X8 in the H^+ form.

The column was washed with 4 ml of water and the eluate was lyophilized and dissolved in 250 µl of 0.002 M pyridine-acetate buffer. The resulting solution was applied to DEAE-cellulose (DE53, 0.5 x 3 cm column) equilibrated in the same buffer. The column was washed with 4 ml of starting buffer and the sialyloligosaccharides were eluted with 3.5 ml of 0.1 M pyridine-acetate. The ganglioside-derived oligosaccharide alditols were dissolved in 100 µl of 0.05 M pyridine and reduced with $NaB[^3H]_4$ as described below. $[^3H]$-labeled oligosaccharides obtained by this procedure have a specific activity of approximately 0.5 mCi/µmol.

3.2.6 *Reductive Labeling of Oligosaccharides*

Ganglioside-derived sialyloligosaccharides were labeled as previously described (Smith, 1980). In a typical preparation 300 nmol of oligosaccharides were reduced with a 5 fold molar excess of $NaB[^3H]_4$ in 0.2 ml of 0.05 M pyridine, pH 8.6 for 4 h. A 100-fold molar excess of $NaBH_4$ was added to ensure complete reduction. Excess acetone (100 µl) was added to destroy unreacted $NaBH_4$. The radiolabeled products were then transferred to "Y shaped" sublimation vessels (England, 1969), shell frozen and evaporated. The samples were evaporated four times (400 µl each) from distilled water to ensure removal of volatile tritiated 2-propanol and tritiated water. The labeled oligosaccharide alditols were dissolved in 0.5 ml of water and combined with two 0.25 ml washes of the sublimation vessel and applied to an AG-50W (H^+ form) column (0.5 x 3 cm). The column was eluted with 4 ml of deionized distilled water and the eluate evaporated under reduced

pressure. Boric acid was eliminated as its methyl ester by evaporating the sample seven times from methanol. The resulting borate free material conatained the [^{3}H]-labeled oligosaccharide alditols.

3.2.7 *Binding of Radiolabeled Oligosaccharide Alditols*

Direct binding of radiolabeled oligosaccharide alditols to specific antisera was performed as described (Gershman, 1972). Aliquots from HPLC column fractions were added to 12 x 75 mm tubes and dried in a Speed Vac Concentrator. The dried material was dissolved in 0.01 M Tric-HCl buffer, pH 7.5 containing 0.14 M NaCl, 5 x10^{-4} M $MgSO_4$ and 1.5 x 10^{-4} M $CaCl_2$. Specific antiserum (5-20 µl) was added and the mixtures (130 µl) were incubated for 30 min at 37° C and overnight at 4° C. The incubation mixtures were filtered through nitrocellulose filters in a vacuum filter apparatus (Hoffer Scientific Inst., San Francisco, CA) and washed with 10 ml of the incubation buffer. The filters were cut in pieces, added to 1 ml of 0.1 M acetic acid in 12 x 75 mm tubes and sonicated in a bath sonicator for 15 min. Aliquots of the acetic acid eluates containing free [^{3}H]oligosaccharide alditol were assayed for radioactivity.

3.2.8 *Enzyme Digestions*

Radiolabeled oligosaccharide alditols were digested with *Clostridium perfringens* neuraminidase as described (Cassidy *et. al.*, 1965). Briefly, oligosaccharides (4,000-30,000 cpm) were dried in 0.5 ml microcentrifuge tubes and 50 µl of a solution containing 0.2 units of enzyme in 0.1 M potassium acetate buffer (pH 4.9) was added. The

contents of the tube were gently mixed and incubated for 24 h at 37° C and the reaction products applied to Bio-Gel P2 chromatography. Fractions containing oligosaccharide alditols (determined by radioactivity) were pooled and dried in a 12 ml conical pyrex tube using a Rotary Evapo-Mix (HaakeBuchler Instr., Saddle Brook, NJ). The dried material was dissolved in 0.5 ml of 0.002 M pyridine-acetate buffer and applied to a small column (0.5 x 3 cm) of DEAE-cellulose previously equilibrated in the same buffer. The neutral oligosaccharide alditols released during digestion (98%) were eluted in starting buffer and non-digested material remained bound to the column.

Fucosidase digestions were performed as follows: an aliquot (50 µl) of beef kidney α-fucosidase (Levy, 1961) suspended in 3.2 M ammonium sulfate representing 0.2 units of enzyme was centrifuged in a 0.5 ml polypropylene microcentrifuge tube and the supernatant was removed. The pellet was dissolved in 50 µl of 0.05 M sodium acetate buffer (pH 5.4) and the enzyme solution was transferred to another 0.5 ml tube containing dried substrate (10,000-30,000 cpm). The mixtures were incubated for 24 h at 37° C and the reaction products applied to Bio-Gel P2 chromatography.

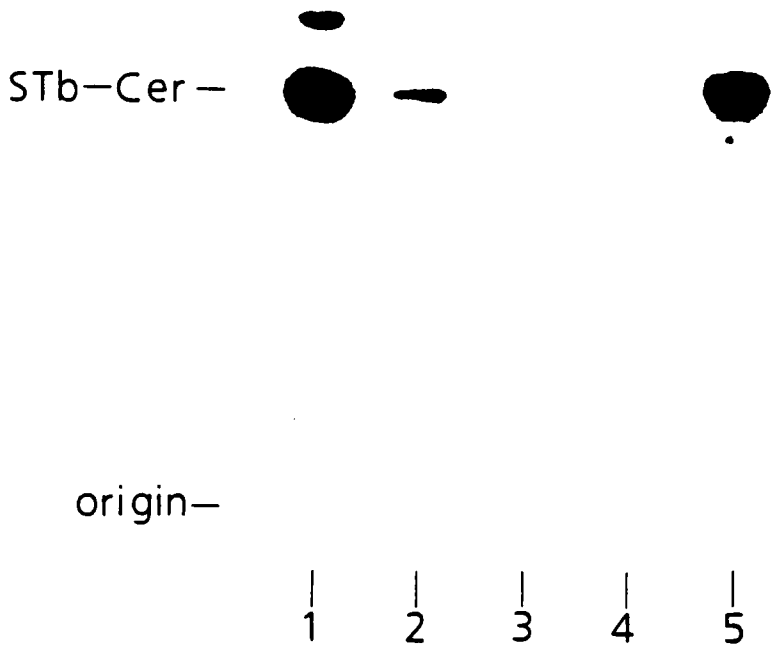
IV

RESULTS AND DISCUSSION

4.1 *DETECTION OF GANGLIOSIDES ON THIN LAYER CHROMATOGRAMS*

Anti-sialyltetrasaccharide b antibodies bound to monosialylgangliosides of human meconium and SW1116 colorectal carcinoma cells separated by thin layer chromatography (Fig. 3). No binding of anti-sialyltetrasaccharide b antibodes to glycolipids from normal intestinal mucosa was observed (Fig. 3). Earlier workers concluded that sialyltetrasaccharide b-ceramide was present as a monosialylganglioside in human meconium and this conclusion was based partially in the binding of anti-sialyltetrasaccharide b antibodies to meconium monosialylgangliosides on thin layer chromatograms (Prieto and Smith, 1985). Anti-sialylterasaccharide b antibodies have a high affinity for their reduced hapten (see Table 5), but bind the unreduced structure with a great enough affinity to allow its detection as a glycolipid on thin layer plates. Previous estimations were that the polyclonal rabbit antisera used in this study bind gangliosides on thin layer chromatograms if the antisera have apparent binding constants equal to or greater than 3.0×10^4 liters mol^{-1} (Prieto, 1986).

Anti-sialyltetrasaccharide b antibodies recognize a monosialylganglioside from SW1116 cells (Fig. 3, lane 2) which comigrates with sialyltetrasaccharide b-ceramide from human meconium (Fig. 3, lanes 1 and 5). Lane 2 represents only 3 nmol of total



Glycolipids were spotted on aluminum-backed thin layer plates and chromatography was carried out in chloroform/methanol/0.25% KCl (5/4/1). The dried chromatograms were overlayed with anti-sialyltetrasaccharide b sera, and the glycolipid-antibody complexes visualized by autoradiography after treating the chromatogram with 125-I labeled goat, anti-rabbit IgG as described under Experimental Procedures. Lanes 1 and 5, human meconium monosialylgangliosides (12 and 6 nmol per lane, respectively); lane 2, total gangliosides from 5 x 10^7 SW1116 cells; and lanes 3 and 4, lipid extract from normal intestinal mucosa representing 12.5 and 25 mg of tissue, respectively.

Figure 3: Radioimmune Staining of Glycolipids on Thin Layer Chromatograms

ganglioside sialic acid from approximately 4 x 10^8 SW1116 cells. The binding of anti-sialyltetrasaccharide b antiserum to such minute quantities of material is consistent with its relatively high apparent binding constant for the unreduced hapten (6.26 x 10^5 liters mol^{-1}.

Limited amounts of material precluded experiments designed to detect SW1116 gangliosides by chemical staining or to inhibit anti-sialyltetrasaccharide b binding. For the same reason binding of other anti-sialyltetrasaccharide antisera was not tested; however, it was not anticipated that antibodies against sialyltetrasaccharide a, the other structure of main interest, would bind its ceramide derivative on thin layer chromatograms, if present, due to the low affinity of these antibodies for the unreduced homologous hapten (6.66 x 10^2 liters mol^{-1}).

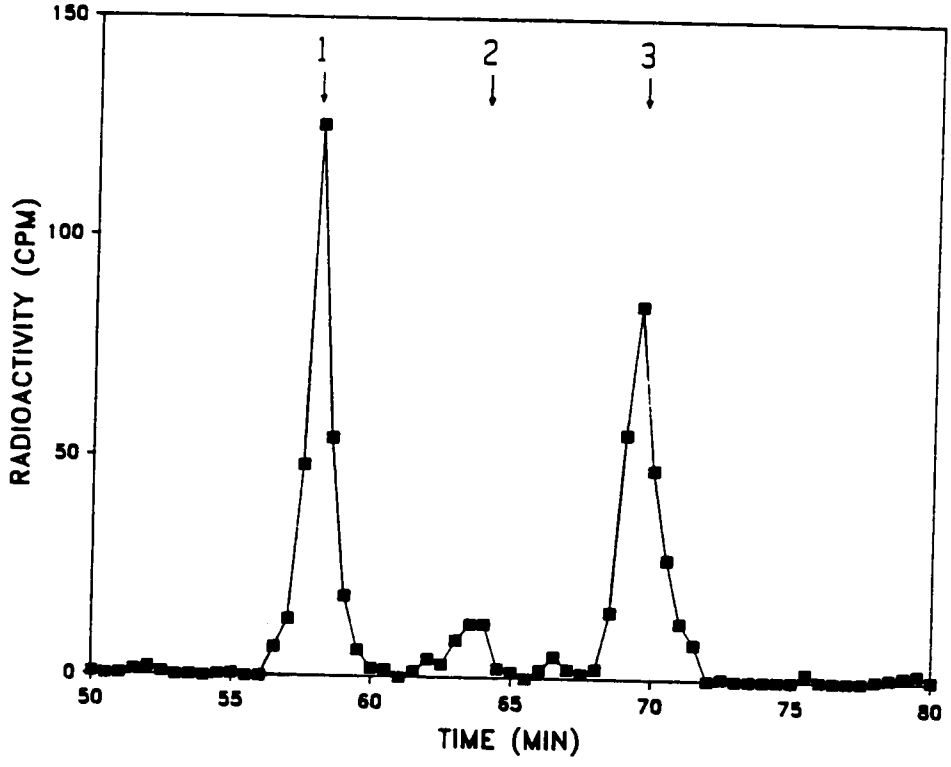
Negative results obtained with anti-sialyltetrasaccharide b antibodies when attempting to detect sialyltetrasaccharide b-ceramide in a total lipid extract from normal intestinal mucosa (Fig. 3, lanes 3 and 4, representing 12.5 and 25 mg of tissue respectively) suggested that the sialyltetrasaccharide b structure is only present in undifferentiated or transformed intestinal tissue.

4.2 *SW1116 GANGLIOSIDE-DERIVED SIALYLOLIGOSACCHARIDE ALDITOLS*

Although anti-sialyltetrasaccharide b serum binds its homologous hapten on thin layer chromatograms, anti-sialyltetrasaccharide a serum only binds its reduced homologous hapten with great enough affinity to

allow detection of the sialyltetrasaccharide a structure. In order to analyze the carbohydrate structures of SW1116 gangliosides, oligosaccharides were released from their parent glycolipids by ozonolysis-alkali fragmentation and reduced with $NaB[^3H]_4$ as described under Experimental Procedures. The resulting ganglioside-derived [^{3}H]-labeled oligosaccharide alditols were applied to a column of DEAE-cellulose (DE53) (Fig. 4). Fractions containing [^{3}H]-labeled monosialyloligosaccharides were eluted with 0.02 M pyridine-acetate buffer (pH 5.4). Peak 2 (Fig. 4) is the sialyltetrasaccharide and sialylpentasaccharide (monofucosyl sialyltetrasaccharide) region that contains sialyl[^{3}H]tetrasaccharide c alditol determined by anti-sialyltetrasaccharide c antibody binding of material under this peak (data not shown). The sialyl[^{3}H]tetrasaccharide and sialyl[^{3}H]pentasaccharide alditol fractions (Fig. 4, peak 2) were pooled, lyophilized and applied to HPLC as described under Experimental Procedures.

Four peaks (Fig. 5, panel A, 1-4) were observed which had elution times matching those of authentic human milk sialyl[^{3}H]oligosaccharide alditols used as standards (Fig. 5, panels B and C): sialyl[^{3}H]tetrasaccharide a, sialyl[^{3}H]tetrasaccharide b, sialyl[^{3}H]tetrasaccharide c and total human milk sialyl[^{3}H] pentasaccharides (see Table 6 for structures). The identity of the three SW1116 ganglioside-derived sialyl[^{3}H]tetrasaccharide alditols (Fig. 5, panel A, 1-3) was confirmed by using aliquots of the HPLC separated fractions in a direct-binding radioimmunoassay as described under Experimental Procedures. The comparison of the



[^{3}H]-labeled oligosaccharide alditols from the ganglioside fraction of SW1116 cells (60 nmol of sialic acid) were dissolved in 1.0 ml of 0.002 M pyridine-acetate buffer, pH 5.4 and applied to a column of DEAE-cellulose (0.9 x 46 cm) equilibrated in the same buffer. The monosialyl[^{3}H]oligosaccharides were partially separated by elution with 0.02 M pyridine-acetate, pH 5.4 (indicated by the arrow). Aliquots (100 µl) of 2.0 ml fractions were assayed for radioactivity by liquid scintillation counting. Peak 2 contains the sialyl[^{3}H]tetrasaccharides and sialyl[^{3}H]pentasaccharides.

Figure 4: DEAE-cellulose Chromatography of SW1116 Ganglioside-derived Oligosaccharide Alditols

anti-sialyltetrasaccharide binding profile with the HPLC elution profile is shown in Fig. 6.

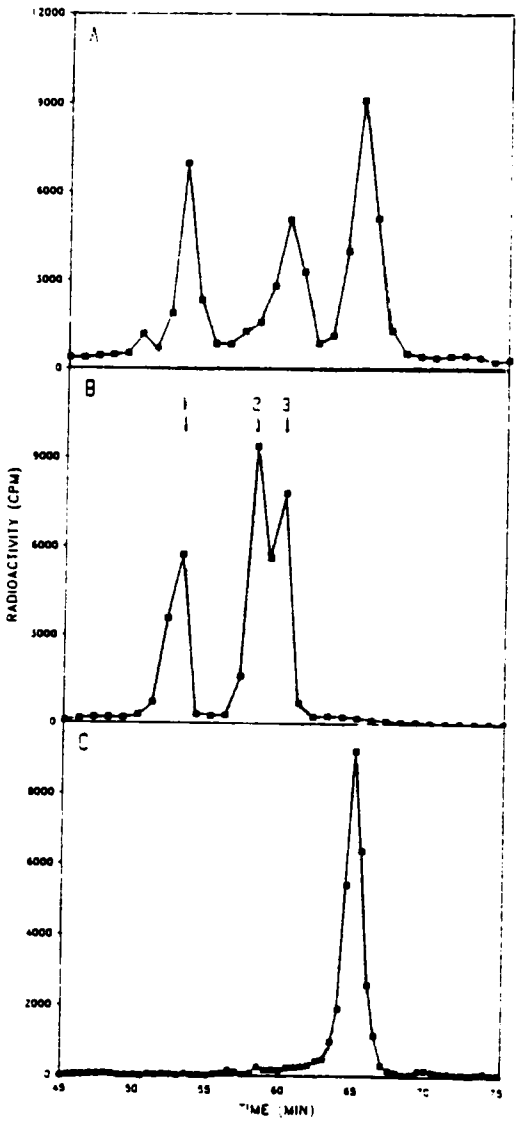
4.3 *GANGLIOSIDE-DERIVED SIALYLPENTASACCHARIDES*

The sialyl[^{3}H]pentasaccharide fraction from SW1116 gangliosides (Fig. 5, panel A, peak 4) was pooled, lyophilized and desalted using Bio-Gel P2. A peak of radioactivity (20,000 cpm) eluted with a K_D matching that of authentic human milk sialyl[^{3}H]pentasaccharides (see Table 7).

4.4 *THE MAJOR AFUCOSYL DERIVATIVE OF SW1116 SIALYLPENTASACCHARIDES*

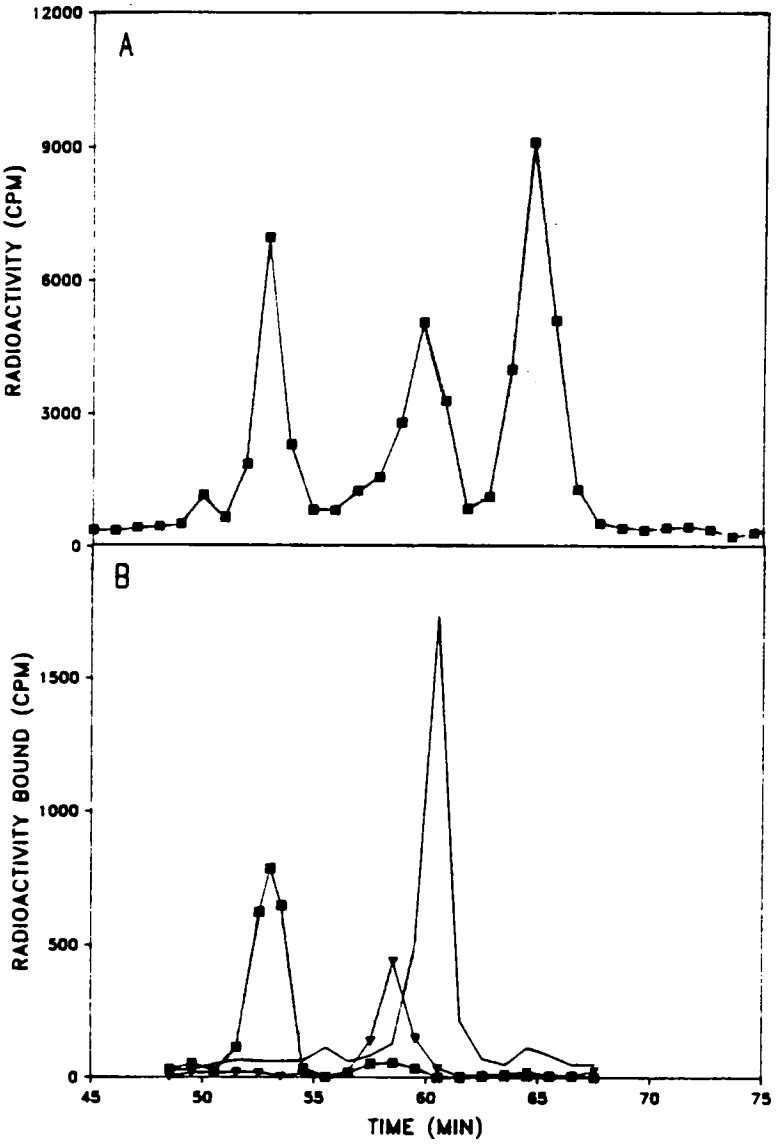
An aliquot of the desalted SW1116 sialyl[^{3}H]pentasaccharides (11,000 cpm) was incubated with beef kidney α-fucosidase as described under Experimental Procedures. The resulting material was desalted on a P2 column.

Pooled radioactivity from P2 chromatography was applied to HPLC and results are shown in Fig. 7. The major peak (peak 1) comigrated with authentic sialyltetrasaccharide a alditol, which is the afucosyl derivative of the sialyl Lewis^a hapten. Another peak (Fig. 7, peak 3) eluted in the same position as reduced sialylpentasaccharides and may represent undigested sialyl[^{3}H]pentasaccharides. A minor component (Fig. 7, peak 2) eluted close to the positon of sialyltetrasaccharide c alditol, but due to the low number of counts available the identity of this fraction could not be confirmed.



The sialyl[^{3}H]tetrasaccharide and sialyl[^{3}H]pentasaccharide fraction from DEAE-cellulose chromatography (Peak 2, Fig. 4) were applied to HPLC as described under Experimental Procedures. Aliquots (100 µl) of 1.0 ml fractions were assayed for radioactivity (panel A). Authentic [^{3}H]-labeled sialyltetrasaccharide a, sialyltetrasaccharide b and sialyltetrasaccharide c (peaks 1-3 respectively, panel B) and sialyl[^{3}H]pentasaccharides (panel C) from human milk were chromatographed on HPLC in the same manner.

Figure 5: HPLC of Ganglioside-derived Sialyltetrasaccharides and Sialylpentasaccharides



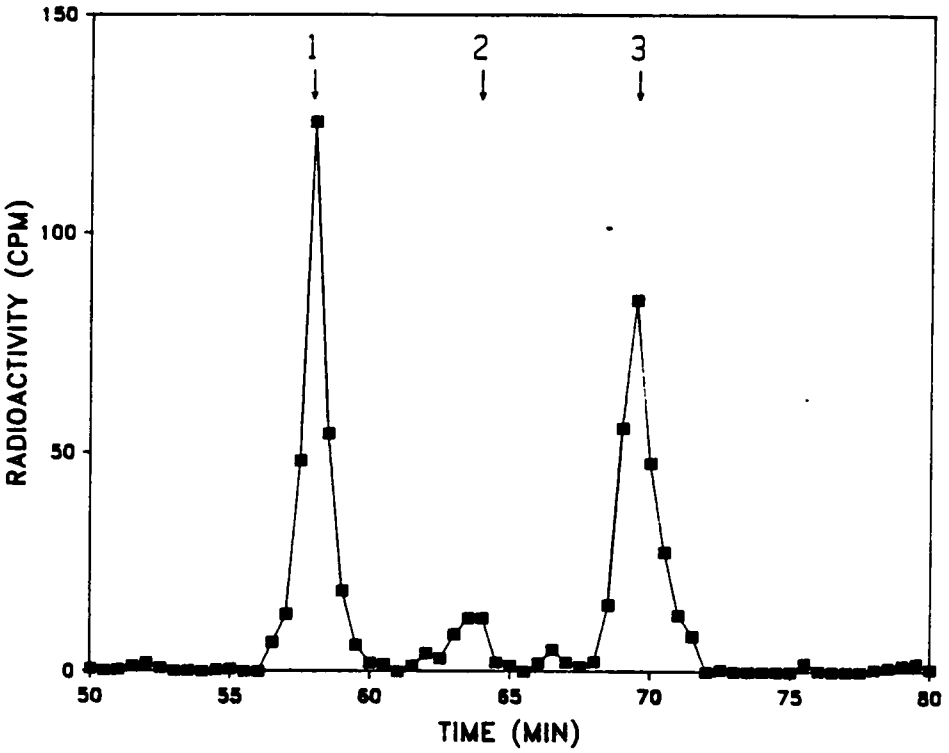
Aliquots (50 µl) from HPLC fractions of SW1116 sialyl[^{3}H]tetrasaccharide and sialyl[^{3}H]pentasaccharide (panel A) were assayed for binding to anti-sialyltetrasaccharide a (—■—), anti-sialyltetrasaccharide b (—▼—), and anti-sialyltetrasaccharide c (——) sera as described under Experimental Procedures (panel B).

Figure 6: Identification of Specific Sialyloligosaccharide Alditols

TABLE 7

Distribution Coefficients of Oligosaccharide Alditols Separated by Bio-Gel P2 Chromatography

Oligosaccharide	Treatment	K_D
Milk sialyl[^{3}H]pentasaccharides	None	0.24
	Fucosidase	0.27
	Neuraminidase	0.38
SW1116 sialyl[^{3}H]pentasaccharides	None	0.25
	Fucosidase	0.27
	Neuramindase	0.39



An aliquot (11,000 cpm) of ganglioside-derived sialyl [^{3}H]pentasaccharides (Fig. 5, panel A, peak 4) was incubated with 50 µl of 4 Units/ml of beef kidney α-fucosidase at 37° C for 24 hr. Digestion products were applied to HPLC as described under Experimental Procedures.

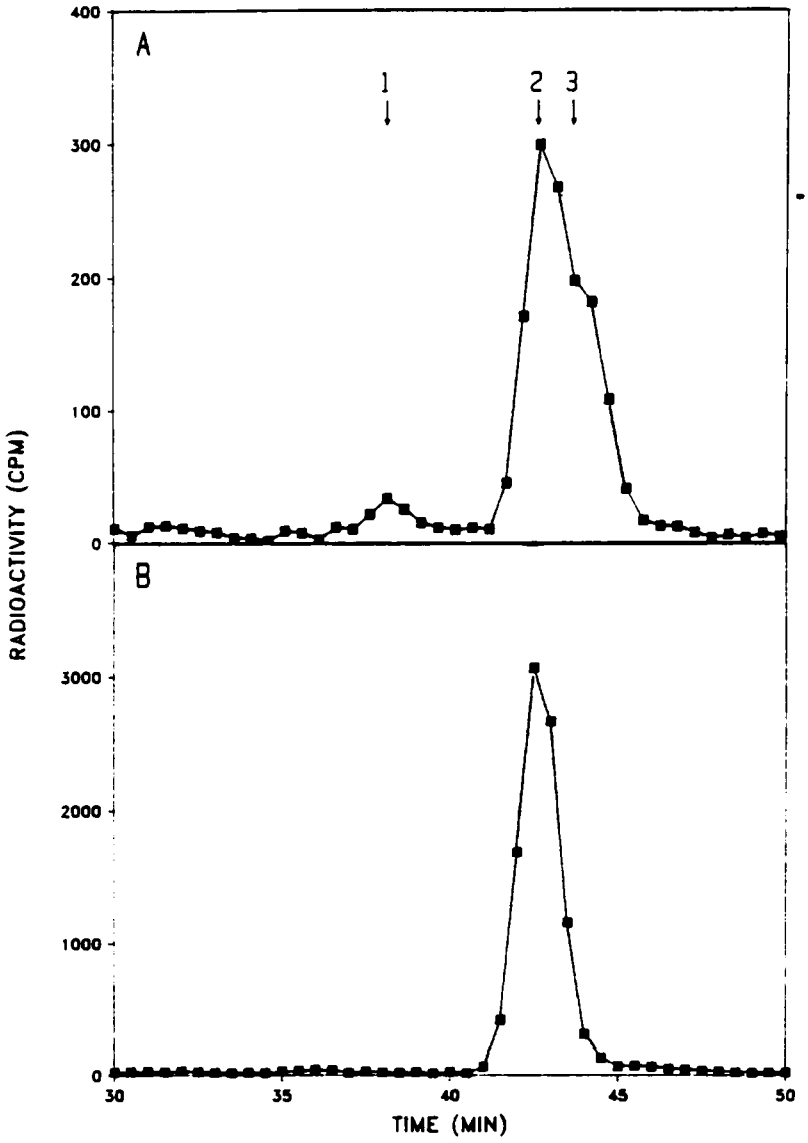
Figure 7: Identification of the Major Afucosyl Derivative of SW1116 Sialylpentasaccharides

4.5 *THE MAJOR ASIALO DERIVATIVE OF SW1116 SIALYLPENTASACCHARIDES*

An aliquot of the desalted SW1116 sialyl[^{3}H]pentasaccharides (5,000 cpm) was incubated with *Clostridium perfringens* neuraminidase as described under Experimental Procedures. The resulting neutral oligosaccharides were applied to P2 chromatography and eluted with a K_D matching that of neutral oligosaccharides obtained from human milk sialy[^{3}H]pentasaccharides which were subjected to the same treatment (Table 7). The distribution coefficient was similar to that of a neutral pentasaccharide.

Pooled material from the P2 column was applied to HPLC and results are shown in Fig. 8, panel A. The major peak (peak 2) comigrated with authentic lacto-N-fucopentaose II alditol (Fig. 8, panel B), which is the asialo derivative of the reduced sialyl Lewis^a hapten (see Tables 3 4 for structures). The reason for the presence of a shoulder region (peak 3) is not clear but may be due to the existence of lacto-N-fucopentaose III alditol, which would elute at this position. Lacto-N-fucopentaose III (Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc) is the asialo derivative of another sialylpentasaccharide structure reported in SW1116 cells, the sialyl Lewis^x antigen (NeuAcα2-6Galβ1-4[Fucα1-3] GlcNAcβ1-3Galβ1-4Glc). The afucosyl derivative of sialyl Lewis^x, sialyltetrasaccharide c, was not present in any significant amount after fucosidase treatment of the SW1116 sialylpentasaccharides (see Figure 7), but this could be due to the resistance of an α1-3 linked fucose as compared to an α1-4 linked fucose in sialylated lacto-series structures.

No data is yet available to support this hypothesis. If the sialyl Lewis^x structure is present and is resistant to fucosidase treatment, then peak 3 of Figure 7 may represent primarily the sialyl Lewis^x hapten. A minor constituent (Fig. 8, panel A, peak 1) eluted in the same position as authentic lacto-N-fucopentaose V alditol. Results of the glycosidase digestions of SW1116 sialyl[^{3}H]pentasaccharides were consistent with the presence of the sialyl Lewis^a antigen in SW1116 gangliosides.



An aliquot (5,000 cpm) of ganglioside-derived sialyl[^{3}H]pentasaccharides (Fig. 5, panel A, peak 4) was incubated with 50 µl of 4 Units/ml *Clostridium perfringens* neuraminidase at 37° C for 24 h. Digestion products were applied to HPLC for neutral oligosaccharides as described under Experimental Procedures. Panel A: Elution profile of neuraminidase-treated SW1116 sialyl[^{3}H]pentasaccharides. Panel B: Elution profile of [^{3}H] lacto-N-fucopentaitol II (LNF II) from human milk.

Figure 8: Identification of the Major Asialo Derivative of SW1116 Sialylpentasaccharides

V

CONCLUSIONS

Some limitations of the work presented in this thesis must be recognized. For example, chemical compositional analyses were not performed on the gangliosides or the ganglioside-derived oligosaccharide alditols because these molecules exist in such minute quantities in SW1116 cells. Although specific anti-oligosaccharide antibodies can be used to characterize carbohydrate structures, corroborating evidence from other methods is generally assumed necessary for positive identification. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are considered the methods of choice for unequivocal assignment of carbohydrate structures, but immunological techniques involving the use of anti-oligosaccharide antibodies for the isolation and characterization of oligosaccharides have proven to be a valid approach. For example, a minor sialyloligosaccharide (fucosyl-sialyltetrasaccharide b) from human milk was detected using anti-sialyltetrasaccharide b sera (Prieto and Smith, 1984). The same sugar sequence was subsequently described by Wieruszeski and coworkers (1985) using chemical methods on large pooled samples of milk. In the case of work presented here, sialyltetrasaccharide a-ceramide and sialyltetrasaccharide b-ceramide were detected in only 60 nmol of total ganglioside sialic acid obtained from approximately 4×10^8 SW1116 cells. This demonstrates the usefulness of anti-oligosaccharide antibodies in detection of minor components from cell surfaces.

Two monosialylgangliosides of SW1116 colorectal carcinoma cells, sialyltetrasaccharide a-ceramide (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4 Glc1-1Cer) and sialyltetrasaccharide b-ceramide (Galβ1-3[NeuAcα2-6] GlcNAcβ1-3 Galβ1-4Glc1-1Cer), were detected using rabbit antibodies against human milk sialyloligosaccharides. The structural relationship of sialyltetrasaccharide a-ceramide and sialyltetrasaccharide b-ceramide to two tumor-associated disialylgangliosides, disialyllactotetraosylceramide (NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc1-1Cer) and disialyl Lewis^a (NeuAcα2-3Galβ1-3[NeuAcα2-6(Fucα1-4)]GlcNAcβ1-3Galβ1-4 Glc1-1Cer) lend support to the hypothesis that these two monosialyl-gangliosides are tumor antigens.

Based on the fact that the sialyltetrasaccharide a structure is contained within disialyllactotetraosylceramide and disialyl Lewis^a, and that sialyltetrasaccharide a-ceramide is the biosynthetic precursor of the sialyl Lewis^a antigen in SW1116 cells (Hansson and Zopf, 1985), one can speculate that sialyltetrasaccharide a-ceramide may be the biosynthetic precursor of these two disialylgangliosides. In this proposed scheme (Fig. 9, pathway 1), a sialic acid residue is first joined to a neutral glycolipid core in an α2-3 linkage to the terminal galactose forming sialyltetrasaccharide a-ceramide. Another sialyltransferase would add sialic acid α2-6 to N-acetylglucosamine, producing disialyllactotetraosylceramide. Conceivably, a fucosyl-transferase could subsequently place a fucose α1-4 to N-acetylglucosamine to give disialyl Lewis^a. Evidence for the order of the steps in the synthesis of these molecules comes from studies on the biosynthesis of the sialyl Lewis^a antigen (Hansson and Zopf, 1985)

and a related structure, the sialyl Lewis^x hapten [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc...] (Holmes *et. al.*, 1986), which demonstrated that addition of sialic acid to the neutral oligosaccharide core precedes addition of fucose. The fucosylation reaction would presumably be an unfavorable one due to steric hindrance from sialic acid already present on N-acetylglucosamine. In fact, based on results of chemical staining of gangliosides on thin layer chromatograms, the amount of disialyl Lewis^a appears to be considerably less than that of disialyllactotetraosylceramide in human colonic adenocarcinoma (Fukushi *et. al.*, 1986).

Recent studies of de Heij and coworkers (1986) show that the milk oligosaccharide disialyllacto-N-tetraose (see Table 4 for structure), which is the carbohydrate structure found within disialyl Lewis^a and disialyllactotetraosylceramide, is preferentially formed by the addition of sialic acid to N-acetylglucosamine of siayltetrasaccharide a (Fig. 9, pathway 1). This is consistent with the scheme just proposed. It is not known whether disialyllactotetraosylceramide or disialyl Lewis^a exist as gangliosides in SW1116 cells, but if they should, it is reasonable to assume that sialyltetrasaccharide a is their biosynthetic precursor. This speculation also implies that sialyltetrasaccharide b-ceramide is most likely a terminal structure in SW1116 cells.

Using immunostaining procedures, sialyltetrasaccharide b-ceramide was detected in human meconium monosialylgangliosides, but not in normal intestinal mucosa. These results suggest that sialyltetrasaccharide b-ceramide may be an oncofetal antigen. Even though sialyltetrasaccharide a-ceramide was reported as a ganglioside

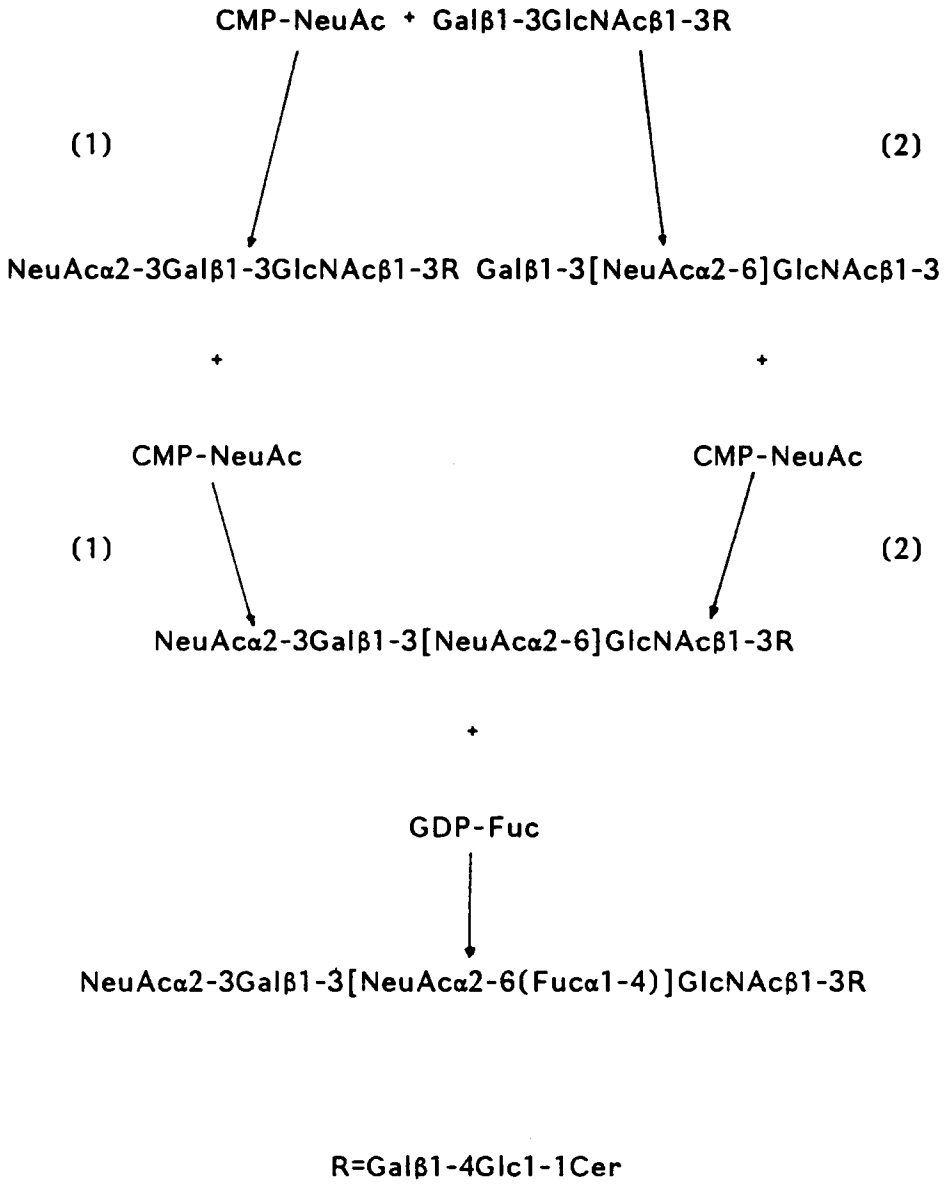


Figure 9: Proposed Biosynthetic Pathway for Disialyllactotetraosylceramide and Disialyl Lewis-a

component of human embryonal carcinoma cells (Fukuda and Dell, 1986), its existence in the SW1116 cell line, where it is the biosynthetic precursor of the sialyl Lewis^a antigen, was not confirmed until the work reported in this thesis was carried out.

Results obtained from glycosidase digestions of ganglioside-derived siayl[^{3}H]pentasaccharides from SW1116 cells are consonant with the presence of the sialyl Lewis^a antigen. These results may also indicate the presence of the sialyl Lewis^x antigen, which has been reported in this cell line (Blaszczyk *et. al.*, 1984).

The sialyl Lewis^a hapen can be detected in the serum of patients with colorectal and pancreatic cancer (Magnani *et. al.*, 1983 and Koprowski *et. al.*, 1981), cystic fibrosis (Roberts *et. al.*, 1986) and, in some instances, ulcerative colitis (Olding *et. al.*, 1986). In all these cases, the antigen appears on a mucin (Magnani *et. al.*, 1983). The sialyl Lewis^a antigen only occurs in individuals who express the Le^a and/or Le^b blood group antigens (Koprowski *et. al.*, 1982) and thus results in some false negatives in a diagnostic test for cancer using the 19-9 (anti-sialyl Lewis^a) antibody. Sialyltetrasaccharide a and sialyltetrasaccharide b are both Lewis blood group independent structures, and if found as circulating glycoprotein antigens associated with these same disease states, would be more reliable indicators than sialyl Lewis^a.

The continued use of rabbit polyclonal antibodies available in our laboratory to probe cell surface components of other existing tumor cell lines, as well as normal tissue, can be expected to uncover additional tumor antigens. The accumlulation of structural data on tumor antigens

will hopefully lead to an understanding of their biosynthesis and their possible physiological roles.

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