MONOSIALYLGANGLIOSIDES FROM HUMAN MECONIUM: CHARACTERIZATION USING SPECIFIC ANTI-OLIGOSACCHARIDE ANTIBODIES.

by

Pedro Antonio Prieto-Trejo

Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY AND NUTRITION

APPROVED:

D.F. Smith

R.E. Ebel

R.H. White

T.O. Sitz

T.D. Wilkins

April, 1986
Blacksburg, Virginia

MONOSIALYL GANGLIOSIDES FROM HUMAN MECONIUM: CHARACTERIZATION USING ANTI-OLIGOSACCHARIDE ANTIBODIES

by

Pedro A. Prieto

(ABSTRACT)

Rabbit antisera directed against human milk sialyloligosaccharides were used to detect specific monosialylgangliosides from the lipid fraction of human meconium. Gangliosides of this fraction were detected after thin layer chromatography by immuno-staining with specific anti-oligosaccharide sera. The monosialylganglioside fraction of human meconium was subjected to ozonolysis and alkali-fragmentation and the resulting ganglioside-derived oligosaccharides were reduced with $NaB[^3H]_4$ and partially separated using paper chromatography. The $[^3H]$-oligosaccharide alditols were assayed for binding to specific anti-oligosaccharide sera in a direct-binding radioimmunoassay using nitrocellulose filters to collect immune-complexes. Radiolabeled oligosaccharide alditols which were recognized by specific antisera were affinity purified by eluting nitrocellulose filters containing antibody-oligosaccharide complexes or using columns of immobilized anti-oligosaccharide antibodies. Structural analyses of two sialyl$[^3H]$tetrasaccharide alditols obtained in this way were carried out with sequential enzymatic degradation using specific exoglycosidases.

The products of enzymatic digestions were idenified by cochromatography in paper with known standards. Data obtained from these experiments are consistent with the presence of the following, previously unidentified gangliosides in human meconium:

NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-cer

Galβ1-3[NeuAcα2-6]GlcNAcα1-3Galβ1-4Glc-cer

ACKNOWLEDGMENTS

I would like to express my gratitude an appreciation to Dr. David F. Smith because his generosity and patience were the lights that guided me during my training and academic experience at Virginia Tech. It was a great privilege to work with Dr. Smith.

The members of my comittee were always open to discuss my research and gave valuable advice to me. I gratefully acknowledge their participation in my learning experience as a graduate student.

My wife and my son are the most important collaborators in my work. Their simple presence has kept me strong enough to search for the truth. Their company has been the inspiration that gave me courage and consolation during difficult times. Thank you. I would like to thank my parents for their support, their love, their teachings, their sacrifice to keep me in college and the sense of humor that I learned from their honest smiles. All of these kept me afloat.

and were not only "host family" but real parents and grandparents for us and for my son. I need to thank you from the bottom of my heart.

I am indebted to all the friends that made the road smoother and the sky clearer even during stormy days:

infinitas gracias.

It is important for me to say that I am grateful to the American taxpayers, because ultimately they payed for my research projects. I also need to thank the Mexican people because I received support from the Mexican taxpayers during four and a half years.

DEDICATION

To my wife , to my son

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I

INTRODUCTION

Cell surface glycolipids and glycoproteins have been associated with several biological activities such as binding of interferon, viruses and toxins (Kohn, 1978), and their role as blood group antigens has been clearly defined (Watkins, 1980). With the advent of monoclonal antibodies cell surface carbohydrate structures have been identified as developmentally regulated and cancer associated antigens (Hakomori, 1985). For example, Magnani *et. al.* (1982) isolated and characterized a monosialylganglioside (the sialyl-Le^a antigen) that is present in gastrointestinal cancer cells and absent in normal intestinal cells; therefore, this ganglioside is a cancer associated antigen. Because this glycolipid was also found in human meconium -which is of fetal origin- it has been classified as an oncofetal antigen (Magnani *et. al.*, 1982)

The sialyl-Le^a antigen is also present in elevated amounts in mucins from the sera of cancer and cystic fibrosis patients (Magnani *et. al.*, 1982 and Roberts *et. al.*, 1986). The monoclonal antibody used to detect the antigen in gastrointestinal cancer cells is now being marketed as part of a diagnostic kit for cancer and cystic fibrosis. However, the diagnostic procedure which involves the use of this antibody, gives false negatives because the synthesis of the sialyl-Le^a antigen depends on the Lewis blood group of the patient. For example, cancer and cystic fibrosis patients who belong to the Lewis *b* and to the Lewis

negative blood groups express the antigen in very small quantities or not at all and test negative in this diagnostic procedure. The biosynthetic pathway of the sialyl-Le^a antigen was elucidated by Mansson and Zopf (1985). In this pathway the sialyl Le^a-antigen is synthesized from a blood group independent precursor which has the carbohydrate structure of a milk sialyloligosaccharide. Many carbohydrate sequences present in milk as free oligosaccharides are also present as components of glycolipids and glycoproteins in cell surfaces (Ginsburg, 1972). For example, the sialyl-Le^a antigen has been described as a free oligoligosaccharide in human milk (Wieruszeski *et. al.*, 1985). Some sialyloligosaccharides of human milk have blood group independent carbohydrate structures that are closely related to the sialyl Le^a-antigen.

In our laboratory we have specific antibodies raised in rabbits against purified human milk sialyloligosaccharides coupled to protein (Smith and Ginsburg, 1980 and Smith *et. al. 1985*). Specific anti-oligosaccharide sera have been used to detect and characterize a minor sialyloligosaccharide of human milk and can be used as probes for specific carbohydrate sequences.

Since the sialyl-Le^a antigen was found in fetal material and since antibodies against the carbohydrate structure of the non blood group dependent precursor of the sialyl-Le^a antigen were available at our laboratory, we decided to look for blood group independent carbohydrate sequences in gangliosides of human meconium. This

dissertation describes the use of rabbit anti-oligosaccharide sera to detect and characterize two previously unidentified monosialylgangliosides of human meconium that are closely related to the sialyl-Le^a antigen. Since the biosynthesis of these gangliosides is blood group independent they may be better diagnostic markers for cancer and cystic fibrosis than the sialyl-Le^a antigen. (Mansson and Zopf, 1985 and Magnani *et. al.*, 1981 and Roberts *et. al.*, 1986).

II

LITERATURE REVIEW

2.1 *CELL SURFACE CARBOHYDRATES*

Cell surface glycolipids and glycoproteins have been associated wih several biological functions, such as receptors for protein toxins, hormones and interferon (Kohn, 1978 and Feizi, 1985). Carbohydrate structures are also important as cell surface antigens or markers (Zopf and Ginsburg, 1974). For example, blood group antigenic determinants are glycolipids and glycoproteins on the cell surfaces of erythrocytes (Ginsburg, 1972). Several carbohydrate structures that are cancer-associated antigens (Hakomori, 1984 and Feizi, 1985) and developmentaly regulated markers (Feizi, 1985) have been described. This section summarizes the proven and proposed roles of cell surface carbohydrates as blood, tumor associated and developmental antigens.

2.1.1 *Blood Group Antigens*

Perhaps the most studied role of cell surface carbohydrates is that as blood group antigens. It has been demonstrated that carbohydrate sequences determine the blood group in the ABO(H) clasification system (Watkins, 1980). The *Ii* blood group antigenic determinants and the antigens of the Lewis bood group classification system are also defined by carbohydrate structures on the cell surfaces of erythrocytes (Shen *et. al*, 1968). For example, two antigens make up the three major

Lewis blood types and the synthesis of these antigens is controlled by two different genetic loci; these are the Secretor (Sese) and the Lewis (Lele) loci (Ginsburg, 1972, see Table 1). The Se gene codes for a fucosyltransferase that transfers fucose from its activated donor (GDP-Fuc) in an α1-2 linkage to a galactose terminal residue of a precursor carbohydrate chain (Ginsburg, 1972) as follows:

GDP-Fuc + Galβ1-3GlcNAc... --> Fucα1-2Galβ1-3GlcNAc...

The se gene is inactive; therefore individuals with sese genotype will not add the Fucose in α1-2 linkage to the terminal galactose. Similarly, the Le gene codes for a fucosyltransferase that transfers fucose from GDP-Fucose in an α1-4 linkage to GlcNAc residues of the precursor chain. There are two immediate precursors for the Lewis antigens; the structure synthesized by persons with a Se gene in their genotypes (Fucα1-2Galβ1-3GlcNAc...) and the non fucosylated structure of persons that have a sese. genotype (Galβ1-3GlcNAc...)(Ginsburg, 1972). Therefore there are two possible structures that are synthesized by persons that express the Le gene.

Fucα1-2Galβ1-3[Fucα1-4]GlcNAc...

which is the Le^b antigen synthesized by individuals who belong to the $Le(a^-,b^+)$ blood group, and

Galβ1-3[Fucα1-4]GlcNAc...

which is the Le^a antigen synthesized by individuals who belong to the $Le(a^+,b^-)$. The le gene is inactive; therefore, persons who lack a Le gene belong to the the Lewis negative $Le(a^-,b^-)$ blood group (Ginsburg,1972). 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 biological significance of blood group antigenic determinants is not known; however, it is known that blood group antigens are involved in some autoimmune diseases. For example, autoantibodies against the blood group antigen *I* (Galβ1-4GlcNAcβ1-6Gal/GlcNAc) appear in the blood of some patients after they have suffered *Mycoplasma pneumoniae* infections (Feizi, 1985). Similarly, blood group active glycolipids and glycoproteins in seminal fluid have been implicated in the production of autoantibodies that lead to sterility in man (Hanisch *et. al.*, 1985). Blood group antigens and related structures in red blood cells change quantitatively and qualitatively through ontogenesis (Hakomori, 1984) therefore, changes in cell surface glycolipids and glycoproteins have also been associated with development and cell differentiation.

2.1.2 *Development and Cell-Cell Recognition*

Carbohydrates on cell surfaces are changed or modified during embryogenesis or during differentiation of certain cell lineages in adults (Hakomori, 1981 and 1985). For example, Buehler *et. al.* (1985)

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, 1972.

demonstrated that undifferentiated bone marrow blast cells and myeloblast cells synthesize galactosyl-ceramide; however, more differentiated promyelocytes and fully mature granulocytes synthesize lactosyl-ceramide instead.

Changes in cell surface glycolipids and glycoproteins may be related to the abillity of cells to recognize adjacent cells by means of mutual carbohydrate-protein interactions (Feizi, 1985). Carbohydrates have been associated with several biological activities involving cell-cell recognition. Examples of these activities are: cell adhesion (Harper and Juliano, 1981), platelet aggregation (Helenquist *et. al.*, 1985), bacterial attachment to cells (Korhonen et. al.,1984), synapse formation (Grundbald *et. al.*, 1985) and malignant transformation of cells (Hakomori, 1985).

2.1.3 *Tumor Associated Antigens*

Changes in cell surface carbohydrates have been detected during cancer expression in several tissues (Hakomori, 1981 Feizi, 1985). It has been proposed that malignant transformation is a process in which cells undergo "reverse differentiation" (Openheimer, 1976). Some carbohydrate and protein structures that are immunogenic for laboratory animals are present in human malignant tissues but are absent from the corresponding normal tissues; these antigenic determinants are called cancer-associated antigens. With the advent of monoclonal antibody technology many cancer-associated carbohydrate antigens have been

described (Hakomori, 1984). Furthermore, some cancer-associated antigens have also been found in fetal tissues but are not present in the corresponding adult tissues; these antigenic determinants are called oncofetal antigens (Feizi, 1985). For example, Magnani *et. al.* (1982) described the structure of a cell surface glycolipid associated with gasgtrointestinal cancer, the sialyl-Le^a antigen.

NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc.

This glycolipid was detected in colorectal carcinoma cells (Magnani *et. al.*, 1982) using a monoclonal antibody produced by a hybridoma obtained from a mouse immunized with the gastrointestinal cancer cell line SW1116 (Herlyn *et. al.*, 1979). Because this ganglioside was also detected in human meconium it was defined as an oncofetal antigen (Magnani *et. al.*, 1982). The synthesis of this glycolipid is Lewis blood group dependent. For example, patients with colorectal carcinoma who belong to the $Le(a^-b^-)$ blood group can not synthesize the sialyl-Le^a antigen because they lack the fucosyltransferase necessary to transfer a fucose residue from GDP-fucose in an α1-4 linkage to the

GlcNAc residue. This transferase is present in individuals who belong to the Le(a^+b^-) and Le(a^-b^+) blood types. Fortunaltely, these groups comprise more than 90% of the population (Ginsburg, 1972) and the sialyl-Le^a antigen can be detected in the serum of gastrointestinal cancer patients who belong to these blood groups. This favored the application of the monoclonal antibody against the sialyl-Le^a antigen as a diagnostic tool for early detection of cancer (Magnani *et. al.*, 1982).

Mansson and Zopf (1985), described the biosynthetic pathway for the synthesis of this antigen. In this pathway sialic acid is transferred to a neutral glycolipid core of lacto-N-tetraosyl-ceramide as follows:

CMP-NeuAc + Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer

The product of this reaction is sialyltetrasaccharide *a*-ceramide:

NueAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer

which is the substrate for the fucosyl transferase encoded by the Lewis gene which in turn is the enzyme responsible for the final step in the synthesis of the sialyl-Le^a antigen;

GDP-fuc + NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer →

NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-Cer

As discussed above, the ability to synthesize this glycolipid depends on the blood group of the patient. Since the synthesis of sialyltetrasaccharide *a*-ceramide is blood group independent, it is possible that sialyltetrasaccharide *a* (the precursor of the sialyl-Le^a antigen) would be a better diagnostic marker for colorectal cancer.

2.2 *HUMAN MECONIUM*

2.2.1 *Definition and Description*

Human meconium is the first stool passed by the newborn infant (Harris, 1978 and Antonowicz, 1979) It is a viscous, greenish or brownish-black substance which begins to accumulate in the fetal intestine during the fourth month of gestation and is generally excreted during the first 24 h of life. (Harris, 1978). Meconium is composed of gastrointestinal mucus and secretions, bile, amniotic fluid, lanugo hair and cellular debris. The cellular debris is mainly derived from desquamated cells from mouth, skin and alimentary tract (Antonowicz, 1979); hence, meconium is -at the molecular level- a record of compounds synthesized during fetal development.

2.2.2 *Chemical Composition*

Dry weight accounts for 20 to 30% of the wet mass of human meconium, and it includes inorganic materials such as sodium, potassium, calcium, magnesium, phosphorus, copper, zinc, iron and manganese (Kopito *et. al.* , 1966). About 75% of the dry weight of meconium is mucin that is highly resistant to the action of proteolytic enzymes; blood group substances and influenza virus hemagglutination inhibitors are associated with these mucins. (Harris, 1978). Antonowicz and Shawman (1979) found that blood group specific substances in meconium corresponded with the blood group specific substances in amniotic fluid and with the blood group of the newborn and not that of the mother. Other proteins -mainly plasma proteins and enzymes- account for approximately 10% of the dry weight of meconium. Albumin, acid proteases, chymotrypsin, renin, dipeptidases and lysosomal enzymes are examples of these proteins (Harris, 1978). Free oligosaccharides can be found in meconium and it has been postulated that their presence is due to accumulation of catabolic fragments from glycoproteins (Harris, 1978). Fifteen of these oligosaccharides have been described by Herlant-Peers *et. al.* (1981).

The lipid fraction of meconium accounts for less than 10% of its dry weight. Steroids, sterols, free fatty acids, fatty acid esters and glycosphingolipids are constituents of this fraction. Some of the glycolipids found in meconium have also been found associated with specific biological functions in different tissues. For example, some

meconium glycolipids are blood group antigenic determinants (Buchanan *et. al.*,1951), ganglioside GM_1 is a receptor for cholera toxin (Holmgren *et. al.*, 1975), and at least one meconium ganglioside has been defined as an oncofetal antigen associated with gastrointestinal cancer (Magnani *et. al.*, 1982). Hence, meconium is not only a source of fetal glycolipids but it is also a potential source of important glycolipids found in cell surfaces of certain tissues.

Several glycolipid structures of human meconium -both, neutral glycolipids and gangliosides- have been determined by classical chemical methods such as methylation analysis and mass spectrometry. For example, Karlsson and Larson (1981) described several neutral glycolipids of human meconium some of which are shown in Table 2 . The carbohydrate structures of most of these glycolipids are identical to oligosaccharide structures that are found as free oligosaccharides in human milk.

The structures of some meconium gangliosides have also been elucidated as shown in Table 3 (Nilsson *et. al.*, 1981 and Magnani *et. al.*,1982). N-acetylneuraminic acid is the only sialic acid present in characterized meconium gangliosides, and glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine are the monosaccharides present in these glycolipids. However, one meconium ganglioside (the sialyl-Le^a antigen) also contains a fucose residue. This ganglioside is the oncofetal antigen described above (Magnani *et. al.*, 1982).

TABLE 2

Some Neutral Glycolipids of Human Meconium

Structure	Series
Glcβ1-1Cer	--
Galβ1-1Cer	gala
Galβ1-4Glcβ1-1Cer	lacto
Galα1-4Galβ1-1Cer	gala
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	gala
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	lacto
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	"
Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	"

Structures in this table are from Karlson (1982).

TABLE 3

Gangliosides of Human Meconium

Structure	Abbreviation (% of the ganglioside fraction)
Galβ1-4[NeuAcα2-3]Glc-Cer	GM_3(94)
NeuAcα2-6Galβ1-4GlcNacβ1-3Galβ1-4Glc-Cer	6'LM_1(9.0)
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer	3'LM_1(0.8)
Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer 3 NeuAcα2	GM_1(<0.5)
Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-Cer 3 NeuAcα2	Sialyl-Le^a

All structures are from Nilson (1981) with exception of Sialyl-Le^a described by Magnani (1982).

2.2.3 *Meconium Ileus and Cystic Fibrosis*

Meconium ileus is a pathological condition defined as the intestinal obstruction in the newborn due to inability to pass meconium (Harris, 1978). Newborns with meconium ileus are usually diagnosed as having cystic fibrosis (Ryley *e. al.*, 1979). Cystic fibrosis is a disease which is the most common lethal genetic trait of caucasians; however, most cystic fibrosis patients do not present meconium ileus and the disease may not be diagnosed until irreversible lung damage has occurred (Antonowicz, 1979). The carbohydrate composition of glycoproteins obtained from meconium of cystic fibrosis patients is different from that of carbohydrates present in glycoproteins of meconium from normal newborns (Clamp *et. al., 1979*). For example, glycoproteins from cystic fibrosis meconium have greater amounts of fucose and lesser amounts of sialic acid than those from normal meconium (Clamp *et. al.*, 1979). Amounts of total fucose, albumin, proteases and other components of meconium have been used as diagnostic parameters for the disease (Ryley *et. al.*, 1979; Kolberg *et. al.*, 1975; Kholsclaw, *et. al.*, 1978 and Clamp *et. al.*, 1979). Fucose content also varies with the blood group of the donor and other diagnostic procedures based on chemical diferences between meconium samples are unreliable and often not sensitive enough (Antonowicz, 1979). Recently Roberts *et. al.* (1986) reported that the sialyl-Le^a antigen (Table 3) is present in serum proteins of cystic fibrosis patients. The use of carbohydrate binding proteins for the detection of specific sugar sequences as a

basis for diagnosis represents a promising approach for the early detection of cystic fibrosis. Prior to the completion of the work described in this dissertation, the sialyl-Le^a ganglioside was the only meconium glycolipid detected by immunological methods. This ganglioside is a minor component of the monosialylganglioside fraction of meconium and its detection was not possible using traditional chemical methods were applied to the study of meconium glycolipids. The importance of the sialyl-Le^a antigen resides not only in its use as a diagnostic basis for cystic fibrosis and for cancer. The monoclonal antibody (called 19-9) used for the detection of the antigen is now marketed as part of a kit for an earlier detection of colorectal carcinoma. Unfortunately (as discussed above) the presence of the sialyl-Le^a structure is blood group dependent, and about 10% of cystic fibrosis and colorectal carcinoma patients can not synthesize this antigen. Furthermore, although patients who belong to the $Le(a^-b^+)$ blood group have at least one Le gene, only low amounts of sialyl-Le^a antigen can be detected in their sera. This is due to the fact that the enzyme encoded by the Se gene -which is present in $Le(a^-b^+)$ individuals- is responsible for the transfer of fucose in an α1-2 linkage to the galactose residue of a sialated-precursor as follows:

GDP-Fuc + NeuAcα2-3Galβ1-3GlcNAc... ←

Neuα2-3[Fucα1-2]Galβ1-3GlcNAc...

As mentioned above the enzyme encoded by the Le gene is responsible for the transfer of a fucose residue in an α1-4 linkage to N-acetylglucosamine, but if this transfer is made to NeuAcα2-3[Fucα1-2]Galβ1-3GlcNAc... the product would be the sialyl-Le^b antigen (NeuAcα2-3[Fucα1-2]Galβ1-3[Fucα1-4]GlcNAc...) and not the sialyl-Le^a antigen (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAc...). Therefore, the fucosyl transferase encoded by the Se gene uses precursor chains which are then no longer available for the synthesis of the sialyl-Le^a antigen. However, it is possible that those cystic fibrosis patients that can not synthesize the sialyl-Le^a antigen synthesize its immediate metabolic precursor sialyltetrasaccharide *a*-ceramide (Mansson and Zopf, 1985). Detection of this molecule may have significance as a cystic fibrosis and cancer diagnostic procedure; however, sialyltetrasaccharide *a* has never been described as a ganglioside in any tissue. One of the objectives of this dissertation was to look for sialyltetrasaccharide *a*-ceramide in human meconium using specific anti-oligosaccharide antibodies in sensitive radioimmunoassays.

2.3 *ANTIBODIES AGAINST MILK OLIGOSACCHARIDES*

Glycolipids are difficult to purify and characterize using classical techniques since there are limiting amounts of samples (Zopf and Ginsburg, 1979 and Vitala and Juliano, 1985). For example, a whole glycolipid series -the polylactosaminyl series- of human erythrocyte cell

surfaces is present at a concentration of 164 x 10^{-18} mol of monosaccharide per cell or 1.91 x 10^{6} copies per cell (Vitala, and Juliano, 1985) Minor components would represent only a small percentage of the whole series and would require large amounts of blood to be detected and characterized by chemical methods. Ginsburg *et. al.* developed an immunological approach which takes advantage of the fact that many sugar sequences of human milk are also present in cell surface oligosaccharides (Zopf and Ginsburg, 1974). In this approach antibodies directed against milk oligosaccharide structures are used as sensitive probes for their homologous haptens or related structures (Zopf and Ginsburg, 1974 and Smith *et. al.*, 1981). Purified milk oligosaccharides are coupled to protein. The resulting carbohydrate-protein conjugates are used as immunogens in laboratory animals and antisera specific for sugar sequences can be obtained from the blood of the immunized animals (Zopf and Ginsburg, 1974; Jeffry *et. al.*, 1975; Zopf. *et. al.*, 1975, and Smith and Ginsburg, 1980). Detailed procedures for the production and characterization of the antisera used in the experiments described in this dissertation are described in Smith and Ginsburg (1980) and Smith *et. al.* (*1985*).

There are two types of human milk oligosaccharides; neutral and sialyloligosaccharides (structures of some of these oligosaccharides can be found in Tables 4 and 5). Most of the major carbohydrate components of human milk have been characterized using traditional chemical methods (Kobata and Ginsburg, 1972; Kobata *et. al.*, 1979;

Ginsburg *et. al.*, 1976 and Yamashita *et. al.*, 1976) Characterization of minor components can be accomplished using large amounts of milk (Wieruszeski *et. al.*, 1985) or applying sensitive techniques involving the use of specific carbohydrate-binding proteins. For example fucosyl-sialyltetrasaccharide *b* was detected using a specific rabbit antiserum against sialyltetrasacchride *b* (Prieto and Smith, 1984) and its characterization required only 300 nmol of the monosialyloligosaccharide fraction of human milk. Less than 2 pmol of fucosyl-sialyltetrasaccharide *b* were needed to detect the structure in a complex mixture of sialyloligosaccharides (Prieto and Smith, 1984). Studies to determine the blood group dependence of carbohydrate structures must be performed in single-donor milk samples and this is not possible if pooled samples from different blood group donors are used. Thanks to the sensitivity of the methods involving the use of specific anti-oligosacchaide antibodies it was determined that fucosyl-sialyltetrasaccharide *b* was a Lewis blood group dependent structure which was only found in Le(a^-b^+) and Le(a^-b^-) individuals that had at least one copy of the Se gene. Wieruszeski and coworkers (1985) isolated fucosyl-sialyltetrasaccharide *b* from 10 Kg of pooled human milk and characterized it by chemical methods. This exemplifies the sensititvity of immunological methods for the detection of specific sugar sequences.

Before polyclonal antibodies (such as the antibodies used in the experiments described in this dissertation) are used for detection of specific carbohydrate structures their specificities have to be carefully

TABLE 4

Some Neutral Oligosaccharides from Human Milk

Trivial Name	Structure
lactose	Galβ1-4Glc
2'Fucosyllactose	Fucα1-2Galβ1-4Glc
3'Fucosyllactose	Fucα1-3Galβ1-4Glc
Lacto-N-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-N-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose II	Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc

All the structures in this table are from Kobata (1972).

TABLE 5

Some Sialyloligosaccharides from Human Milk

Name	stucture
3'Sialyllactose	NeuAcα2-3Galβ1-4Glc
6'Sialyllactose	NeuAcα2-6Galβ1-4Glc
sialyltetrasaccharide a	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Sialyltetrasaccharide b	NeuAcα2 (6) Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Sialyltetrasaccharide c	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Fucosyl-Sialyltetrasaccharide b	Fucα1-2Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc
Fucosyl-Sialyltetrasaccharide a (Sialyl-Le^a antigen)	NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc

Structures in this Table are from Kobata (1972).

characterized. Characterization studies are also important in the case of monoclonal antibodies since there are reports of monoclonal antibodies with relatively broad specificities (Feizi, 1986); in the latter case this is due to recognition of parts of structures that are common to several carbohydrate structures and not to the presence of different antibody populations (Feizi, 1986).

Data obtained by Smith *et. al.* (1980, 1985) during the characterization of the antisera used in the course of the experiments described here are shown in Table 6 . Anti-oligosaccharide antibodies have high specificity for the reduced forms of their homologous haptens. This is due to the fact that oligosaccharides are reduced during the immunogen synthesis. However, some antisera are able to recognize native oligosaccharide structures and are therefore suitable for direct probing of glycoconjugates by established immunological methods.

A rabbit antisera raised against the keyhole limpet hemocyanin derivative of Lacto-N-fucopentaose I (see Table 4 for structures), has the ability to recognize oligosaccharide alditols with a lacto-N-tetraitol core, that is:

Galβ1-3GlcNAcβ1-3Galβ1-4Glcol

TABLE 6

Apparent Binding Constants of Antioligosaccharide sera

	Antiserum			
Sugar	Anti3'Sl	Anti ST*a*	Anti ST*b*	Anti STc
3'Sl	$2.2x10^3$	---	---	---*
$3'Sl_{(OH)}$	$1.2x10^7$	---	---	---
Lac	<100	<100	<100	<100
$Lac_{(OH)}$	<100	---	---	---
$Glc_{(OH)}$	<100	---	---	$7.7x10^2$
STa	---	<660	---	---
$STa_{(OH)}$	---	$2x10^7$	---	---
STb	---	---	$4.16x10^6$	---
$STb_{(OH)}$	---	---	$6.25x10^5$	---
STc	---	---	---	$4.16x10^4$
$STc_{(OH)}$	---	$1.6x10^3$	---	$1x10^8$
$LNT_{(OH)}$	---	$5x10^3$	<100	---
$LNnT_{(OH)}$	---	---	---	$7.7x10^2$

*(---)*not tested. Abbreviations used are: STa, STb and STc for Sialyltetrasaccharides a,b and c respectively, 3'SL for 3'sialyllactose, 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)*

This antiserum will not bind oligosaccharides with a lacto-N-*neo*tetraitol stucture (Galβ1-4GlcNAcβ1-3Galβ1-4Glcol) in which the only difference from lacto-N-tetraitol is the linkage of the terminal galactose residue. This antiserum is especially useful because it is not possible to separate these positional isomers by paper chromatography. The specificity of this antiserum allowed the characterization of the lacto-N tetraose neutral core of a previously unidentified sialyloligosaccharide of human milk (Prieto and Smith, 1984).

As mentioned above most of the glycolipids of human meconium have carbohydrate structures that are also present in free oligosaccharides of human milk. For example, the two most abundant gangliosides of human meconium GM_3 and $6'LM_1$ have the carbohydrate structures of 3'sialyllactose and sialyltetrasaccharide *c*, respectively. Although sialyltetrasaccharide *a*-ceramide may be an important precursor of a cancer-associated antigen, this glycolipid had never been reported as a ganglioside in any tissue. Similarly, a ganglioside with the sialyltetrasaccharide *b* structure had not been previously described. Fortunately, antibodies against the milk oligosaccharides sialyltetrasaccharide *a* and sialyltetrasaccharide *b* were available at our laboratory; therefore, we were able to use these antibodies as sensitive probes for specific carbohydrate structures.

The rationale for the experiments described in this dissertation was that anti-milk oligosaccharide antibodies could be used to detect and characterize their homologous haptens and related structures in the

monosialylganglioside fraction of human meconium. The identification of previously unidentified fetal glycolipids in meconium was the central objective of the present research project.

III

EXPERIMENTAL PROCEDURES

3.1 *MATERIALS*

Structures for the [^{3}H]-labeled oligosaccharide alditols used as chromatographic standards are shown in Table 7. Human milk sialyloligosaccharides were purified and reductively labeled with NaB[^{3}H]$_4$ as previously described (Smith and Ginsburg, 1980). [^{3}H]-lacto-N-tetraitol and [^{3}H]-lacto-N-*neo*tetraitol were obtained after neuraminidase digestions of pure sialyl[^{3}H]tetrasaccharide *a* and sialyltetrasaccharide *c* alditols, respectively. [^{3}H]-Lacto-N-triose alditol, [^{3}H]-lactitol and [^{3}H]-glucitol were obtained from [^{3}H]-lacto-N-tetraitol by sequential enzymatic degradation with β-galactosidase (1 U/ml) and β-N-acetylhexosaminidase (0.7 U/ml). [^{3}H]-Labeled oligosaccharide alditol of ganglioside GM$_1$ was obtained by ozonolysis and alkai fragmentation of the parent ganglioside (Supelco Inc, Bellefonte, PA) and reduction with NaB[^{3}H]$_4$. The tetraose alditol of the ganglio series (Galβ1-3GalNAcβ1-4Galβ1-4Glc) was obtained by hydrolysis of [^{3}H]-labeled GM$_1$ oligosaccharide alditol in 0.01 M of HCl for 1 h at 100°C, and the triose alditol (GalNAcβ1-4Galβ1-4Glc) was obtained after β-galactosidase digestion (1 U/ml, 72 h, 37°C) of the [^{3}H]-tetraitol of the ganglio series. Enzyme digests and purification of digestion products are described below. Gangliosides from human brain used as standards for thin layer chromatography were

a gift of Dr. Pam Fredman (Gottenborg University, Sweden), and their structures are shown in Table 8 . Human meconium was collected within 24 h of birth from newborns at the Montgomery County Hospital (Blacksburg, VA, Dr. John C. Ougburn director of newborn nursery) and stored at -20°C.

Nitrocellulose filters (No. BH-85, 25 mm diameter) were from Sceicher & Scheull (Keene, NH). $NaB[^3H]_4$ (sp. act. 10-20 Ci/mmol) and $Na^{125}I$ (sp. act. 15.7 mCi/ μg) were purchased from Amersham (Arlington Heights, IL). Precoated Merck High Performance silica gel-60 aluminum backed plates for thin layer chromatography were from Applied Analytical Services (Wilmington, NC).

DEAE-cellulose (DE-53) and Whatman No. 1 chromatographic paper were from Whatman (Clifton, NJ). Bio-Gel P2, AG-50W-X8 (H^+ form) resin and Affi-Gel 10 were from Bio Rad (Richmond, CA). N-acetylneuraminic acid, mannose, glucose, fucose, N-acetylglucosamine, DEAE-sepharose, *Clostridium perfringens* neuraminidase (Type X) and staphylococcal Protein A were purchased from Sigma (St. Louis, MO). Beef kidney fucosidase was from Boehringer Mannheim (Indianapolis, IN). Jack bean β-galactosidase free of β-N-acetylhexosaminidase and β-N-acetylhexosaminidase free of β-galactosidase were isolated as described by Li (1973). Specific activity of jack bean β-galactosidase and β-N-acetylhexosaminidase were determined by the method of Li *et. al*. (1975) using P-nitrophenyl-β-D-galactopyranoside and P-nitrophenyl-β-D-N-acetylglucosaminide as substrates respectively.

TABLE 7

Structures of Standard Radiolabeled Oligosaccharide Alditols

Parent Structure	Name or Abbreviation of the Corresponding Reduced Oligosaccharide
Glc	Glucitol
Galβ1-4Glc	Lactitiol
GlcNAcβ1-3Galβ1-4Glc	Lacto-triitol
GalNAcβ1-4Galβ1-4Glc	Ganglio-triitol
Galβ1-3GlcNAcβ1-3Galβ1-4Glc	Lacto-N-tetraitol
Galβ1-4GlcNAcβ1-3Galβ1-4Glc	Lacto-N-neotetraitol
Galβ1-3GalNAcβ1-4Galβ1-4Glc	Ganglio-tetraitol
NeuAcα2-3Galβ1-4Glc	3'Sialyllactitol
NeuAcα2-6Galβ1-4Glc	6'Sialyllactitol
NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	STa* alditol
Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc	STb* alditol
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	STc* alditol
Galβ1-3GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc	GM_1**-ol

** Abbreviations are: STa, STb and STc Sialyltetrasaccharides* a, b *and* c *respectively. **GM_1-ol stands for the alditol derivative of the oligosaccharide released from ganglioside GM_1 after ozonolysis-alkali fragmentation.*

TABLE 8

Human Brain Gangliosides Used as Standards in TLC

Structure	Abbreviation
NeuAcα2-3Galβ1-4Glc-cer	GM_3
GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc-cer	GM_2
Galβ1-3GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc-cer	GM_1
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc-cer	$6'LM_1$
NeuAcα2-8NeuAcα2-3GalβGlc-cer	GD_3
NeuAcα2-3Galβ1-3GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc-cer	GD_{1a}
Galβ1-3GalNAcβ1-4[NeuAcα2-8NeuAcα2-3]Galβ1-4Glc-cer	GD_{1b}
NeuAcα2-8NeuAcα2-3Galβ1-3GalNacβ1-4GalβGlc-cer 3 | NeuAcα2	GT_{1b}

Structures are from Leeden (1982)

3.2 *METHODS*

3.2.1 *Analytical Methods*

Sialic acid was measured by the thiobarbituric acid method (Aminoff, 1961) using N-acetylneuraminic acid as standard, and total hexose content was determined by the phenol-sulfuric acid method using galactose as standard (Dubois *et. al.*, 1956). Tritium was measured by liquid scintillation counting using Ready-Solv EP (Beckman, Fullerton, CA) in a Beckman LS-7500 liquid 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 is then titrated under constant stirring with 4mM sodium thiosulfate until the blue-black color disappears. Ozone concentration in the solution is calculated according to the following equation (Belew, 1969):

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

Gangliosides in thin layer chromatograms were chemically detected by staining by the resorcinol-HCl method (Jourdain 1971).

3.2.2 *Gangliosides from Human Meconium*

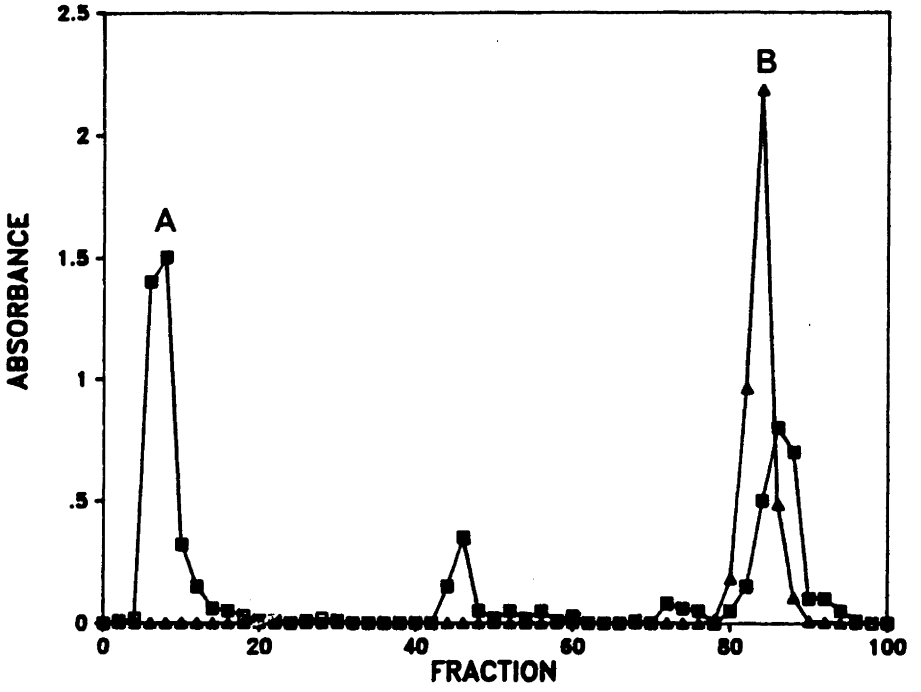
Lipid extracts were obtained according to the method of Svennerholm and Fredman (1980) for the quantitative extraction of human brain gangliosides. Meconium (50 g) was homogenized in 150 ml of water with a single speed waring blender (Waring, New Harthford, CT) at 4°C for 2 min. The homogenate was poured into 504 ml of methanol under constant stirring at room temperature. Chloroform (270 ml) was added and the resulting mixture was stirred for 30 min. This homogenate was centrifuged at 5000 x g for 10 min and the supernatant was transferred to a separatory funnel. The precipitate was rehomogenized in 80 ml of water, poured into 320 ml of chloroform/methanol (2/1) and centrifuged. The supernatants were combined and 270 ml of water were added in order to give a 1/2/1.4 chloroform/methanol/H_2O-tissue ratio. Phases were allowed to separate overnight and the lower phase was reextracted with 125 ml of methanol and 167 ml 10 mM KCl. The upper phases were combined and evaporated to dryness under reduced pressure. The dry material was dissolved in 50 ml of water and dialyzed against four changes of water (1 l each) for 48 h in Spectra/Por dialysis membrane tubing M.W. cutoff 6-8000 (American Scientific Products, McGaw Park, IL). The dialyzed material (7 µmol sialic acid) was suspended in 10 ml of methanol, sonicated in a bath sonicator for 15 min and centrifuged to remove a black precipitate. The supernatant constitutes the upper (aqueous) phase of the lipid fraction of meconium and contains the gangliosides.

3.2.3 *Chromatographic Methods*

The upper phase lipid extract from 50 g of human meconium (7 µmol sialic acid) in 10 ml of methanol was applied to a column of DEAE-Sepharose (1.5 x 20 cm) in the acetate form equilibrated in methanol (Momoi *et. al.*, 1976) (Figure 1) and the column was washed with four column volumes of methanol before eluting the monosialylgangliosides with a solution of 0.01 M potassium acetate in methanol. The monosialylgangliosides detected by sialic acid assay of column fractions (Fig. 1, Peak B) are pooled, dialyzed against distilled water and lyophilized.

Partial separation of monosialylgangliosides of meconium was obtained using column chromatography on iatrobeads (Momoi *et. al.*, 1976). Monosialylgangliosides from meconium (4µmol, Peak B, Fig. 1) were dissolved in 2 ml of chloroform/methanol/water (30/60/4.5) and applied to an iatrobeads column (0.9 x 45 cm) previously equilibrated in the same solvent. The column is eluted first with 4.7 volumes of starting solvent and then with 6.38 volumes of chloroform/methanol (1/1). Fractions (6.5 ml) were collected and aliquots (325 µl) were assayed for sialic acid. Elution of specific gangliosides was monitored by immuno- and resorcinol staining of thin layer chromatograms of individal fractions.

Gangliosides were applied to high performance thin layer chromatographic plates in 1 cm wide bands at 1 cm above the bottom of the plate. Chromatography was carried out in chloroform/methanol/0.25% KCl in water (5/4/1) for 2 h 45 min.



The meconium upper phase of a lipid extract (7 µmol sialic acid) in 10 ml of methanol is applied to a DEAE-sepharose column (1.5 x 20 cm) previously equilibrated with methanol. the column is washed with initial solvent which is then changed to a solution of 0.01 M potassium acetate in methanol as indicated by the arrow. Fractions (4 ml) were collected and aliquots were assayed for total hexose content (-■-■-) and sialic acid (-▲-▲).

Figure 1: DEAE-sepharose Chromatography of Meconium Glycolipids

Sialyl[^{3}H]oligosaccharide alditols derived from meconium monosialylgangliosides after ozonolysis and alkaline fragmentation were separated by DEAE-cellulose (DE-53) chromatography according to the method of Smith *et. al.* (1978) for milk sialyloligosaccharides. Briefly, radiolabeled oligosaccharide alditols were dissolved in 200 µl of 2 mM pyridine-acetate buffer and applied to a DEAE-cellulose column (0.9 x 50 cm). Fractions (2 ml) were collected at a flow rate of 42 ml/h.

Chromatography on Bio-Gel P2 (-400 mesh) was performed by applying [^{3}H]-labeled oligosaccharide alditols plus 800 µg of glucose and 800 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 carbohydrate 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 mono, di, tri, and tetrasaccharide derivatives were identified by their distribution coefficients (K_d).

Descending paper chromatography was performed with Whatman No. 1 paper with the following solvent systems: Solvent A, ethyl acetate/pyridine/acetic acid/water (5/5/1/3); Solvent B, ethyl acetate/pyridine/water (12/5/4); Solvent C, the upper phase of ethyl acetate/pyridine/water (2/1/2), and Solvent D, the upper phase of phenol/isopropanol/formic acid/water (85/5/10/100). Labeled

oligosaccharide alditols were located on 2.5 x 50 cm chromatograms by cutting the strips into 1 cm segments extracting each segment with 1 ml of 0.1 M pyridine-acetate buffer (pH 5.4) and counting aliquots in 7 ml beta vials (Denville Scientific, Denville, NJ) with 4 ml of scintillation cocktail.

3.2.4 *Radioimmune-staining of Glycolipids on Thin Layer Chromatograms*

Goat, anti-rabbit IgG (1 mg/ml in 0.2 M NaH_2PO_4), was radiolabeled by a modified iodogen-catalyzed procedure (Fraker *et. al.*, 1978 and Smith, 1983). A glass tube (10 x 75 mm) was coated with 200 µl of Iodo-Gen (0.5 mg/ml, Pierce Chemical Co., Rockford IL) solution in chloroform/methanol (2/1) by rotating under vacuum in a 37°C water bath until the solvent was evaporated. The antibody (200 µl) and $Na^{125}I$ (0.5 mCi, 5 µl) were added to the coated tube which was rotated every 2 min for 20 min at room temperature. The reaction mixture was then applied to a PD-10 column (Pharmacia, Uppsala, Sweden) previously equilibrated in phosphate buffered saline (PBS, 8.1 mM Na_2HPO_4, 0.14 mM NaCl, 2.7 mM KCl, 0.02% Na azide, pH 7.2) containing 1% polyvinylpyrrolidone (Mr 40,000, Sigma, St Louis, MO). The specific activity of the goat-anti-rabbit IgG was between 6 and 18 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 the cover the plate with a film of the polymer and reduce non-specific protein-binding. The chromatograms were dried, sprayed with a 2% bovine serum albumin (Sigma, St. Louis MO) in cold PBS (buffer A) and immediately soaked in the same buffer for 30 min. The plates are then overlayed with a 1/150 dilution of anti-oligosaccharide sera in buffer A (50 $\mu l/cm^2$). After incubation for 2 h at 4°C the chromatograms were washed by dipping in six succesive changes of cold PBS at 1 min intervals and overlayed with a ^{125}I-labeled goat anti-rabbit IgG (10^6 cpm/ml, 50 $\mu l/cm^2$) solution in buffer A. The chromatograms were incubated for 2 h at 4°C and washed by dipping 11 succesive 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; Du Pont, Wilmington, DW) for 18 h at -73°C.

3.2.5 *Inhibition of Antibody Binding to Gangliosides*

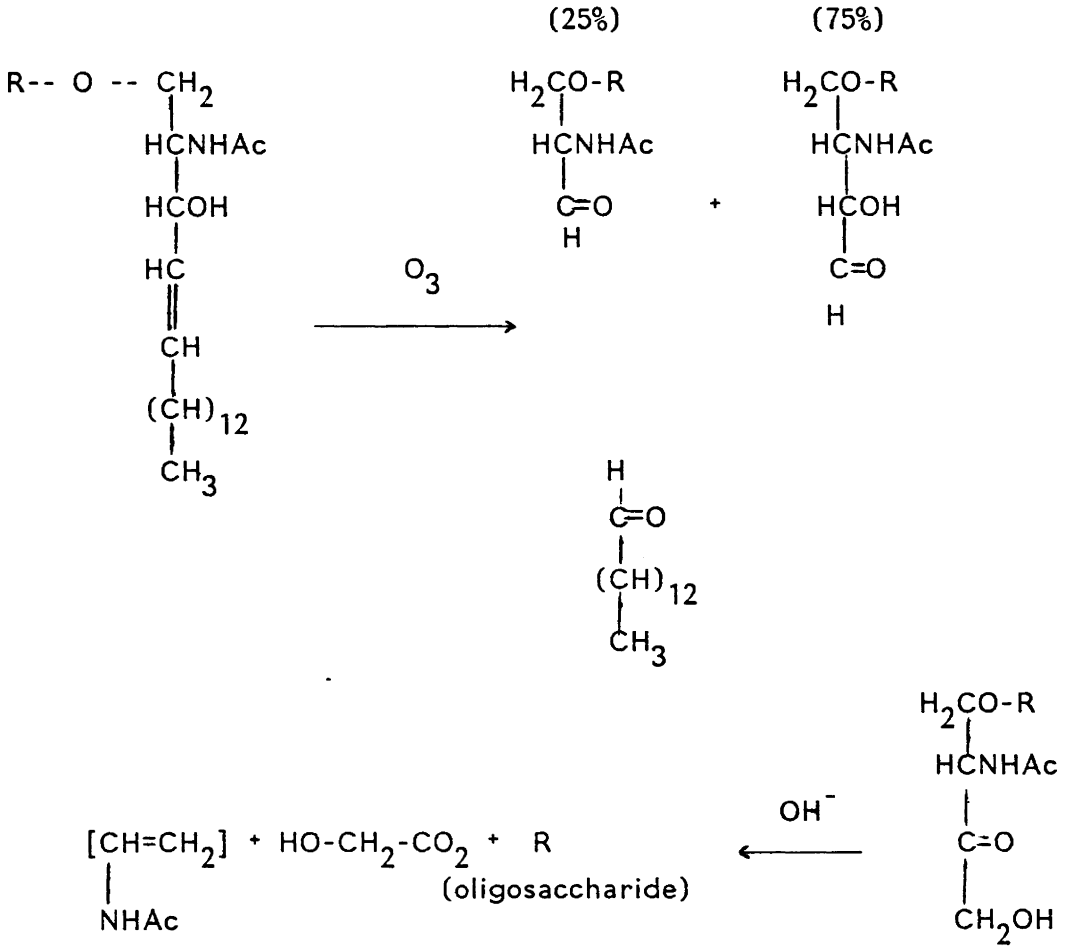
Separate aliquots of anti-sialyltetrasaccharide *b* serum (diluted 1/15 with buffer A) were incubated with either sialyltetrasaccharide *b* (14μM) or lacto-N-tetraose (150 μM), for 18 h at 4°C. A final dilution (1/150) of each preparation was made with buffer A, and the antibody solutions were used to overlay thin layer chromatograms as described above.

3.2.6 *Ozonolysis and Alkali-Fragmentation*

Oxidation of unsaturated sphingosines in glycosphingolipids 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 Fig. 2. The release of oligosaccharides from glycosphingolipids by O_3-alkali fragmentation was established by Weighandt and Baschang (1965) who bubbled ozone through glycolipid solutions; however, overoxidation of small quantities of glycolipid by O_3 may result in degradation of oligosaccharides by oxidation of acetals (Deslongchamps, 1967). Oxidation was controlled by adding aliquots of O_3 in CH_2Cl_2 instead of bubbling O_3 through the raction mixture (Smith, 1982).

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 Corporation, Phoenix, AZ) at 1 amp, and O_2 flow rate of 1 l/min, was bubbled through methylene chloride (chilled in an isopropanol/dry ice bath) for 10 min and the O_3 concentration was defined as described under Analytical Methods. Monosialylgangliosides (300 nmol) were lyophilized in a 1.5 ml microcentrifuge polypropylene tube, dissolved in 200 µl of methanol and placed on ice. An aliquot (150 µl) of O_3/CH_2Cl_2 solution (4 mM) was added to the tube containing the gangliosides, and the tube was immediately capped.

R = Oligosaccharide



From Weighandt and Baschang 1965.

Figure 2: Ozonolysis and Alkali-Fragmentation of Glycolipids

Ozonolysis was allowed to proceed at 4°C for 15 min until the reaction was stopped by the addition of 50 µl of dimethylsulfide (Aldrich, Milwakee, WI). The reaction mixture was dried under a stream of N_2 and dissolved in 100 µl of sodium methoxide (0.2 M, 4°C) . After 1 h (4°C) the reaction was stopped by neutralizing with 200 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 addition of 200 µl of distilled water. After vigorous mixing, two phases were separated by centrifugation (Eppendorf microcentrifuge). The upper phase was lyophilized, dissolved in distilled water (100 µl) and applied to a small column (0.5 x 2 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 2 mM pyridine-acetate buffer. The resulting solution was applied to DEAE-cellulose (DE-53, 0.5 x 2 cm) 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 desalted ganglioside-derived oligosaccharide alditols were dissolved in 100 µl of 0.1 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.

3.2.7 *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-3 fold molar excess of $NaB[^3H]_4$ in 0.2 ml of 0.05 M pyridine, pH 8.2 for 4 h. A 100-fold molar excess of $NaBH_4$ was added to ensure complete reduction. Excess acetone (200 µl) was added to destroy unreacted $NaBH_4$. The radiolabeled products were then transferred to "Y shaped" sublimation vessels (Englard, 1969), shell frozen and evaporated. The samples were twice evaporated from 0.1 M pyridine-acetate buffer to ensure removal of volatile tritiated 2-propanol and tritiated water. The labeled oligosaccharide alditols were dissolved in 1 ml of water and combined with two 1 ml washes of the sublimation vessels and applied to an AG-50W (H^+ form) column (0.5 x 4 cm). The column was eluted with 4 ml of deionized distilled water and elution is monitored by assaying aliquots of the eluates for radioactivity. The eluate containing radiolabeled oligosaccharide alditols and boric acid is evaporated under reduced pressure and then evaporated 7 times from methanol in order to eliminate boric acid as its methyl ester. The resulting borate free material contained the $[^3H]$-labeled oligosaccharide alditols.

3.2.8 *Binding of Radiolabeled Oligosaccharide Alditols*

Direct binding of radiolabeled oligosaccharide alditols to specific antisera was performed as described (Gersham, 1972). Aliquots from paper eluates were added to 12 x 75 mm tubes and dried in a speed-vac concentrator. The dried material was dissolved in 0.01 M Tris-HCl buffer pH 7.5 containing 0.14 M NaCl, 5 x 10^{-4} M $MgSO_4$ and 1.5 x 10^{-4} M $CaCl_2$. Specific antiserum (5-20 µl) was added and the mixtures were incubated for 1h at 37°C and overnight at 4°C. The incubation mixtures were filtered through nitrocellulose filters in a vacuum filtration 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 or saved as affinity purified material.

3.2.9 *Affinity Purification on IgG-Affi-Gel Columns*

Anti-sialyltetrasaccharide *a* serum (10 ml) was subjected to ammonium sulfate precipitation (35% saturation), and the precipitate was dissolved in 10 ml of PBS and dialyzed against two changes (4 l) of the same buffer for 24 h at 4°C. An aliquot (3 ml) of the resulting protein solution was applied to a column of protein A-Affi-Gel 10 (1 mg/4 ml of gel)and washed with PBS until absorbance of the eluant was less than 0.01. An affinity purified IgG fraction was recovered by

eluting with two column volumes of 0.2 M sodium acetate adjusted to pH 2.5 with HCl. The IgG fraction was dialyzed against MOPS buffer (0.1 M, pH 7.5) and concentrated using an ultrafiltration cell equipped with a Diaflo YM30 ultrafiltration membrane (Amicon corp. Lexington, MA) to a protein concentration of 1.1 mg/ml. The IgG solution (4 ml) was combined with 4 ml of Affi-Gel 10 and allowed to react overnight with constant rotation at 4°C. The resulting gel (1 mg IgG/ml) was then treated with 400 µl of 0.1 M ethanolamine pH 8.0 for 1 h at room temperature. The gel was then poured into a column (0.9 x 5 cm) and washed with PBS.

Fractions containing sialyl[^{3}H]tetrasaccharide alditol (15,000 cpm in 100 µl of PBS) were applied to the Affi-Gel-IgG column which was then washed with 3 column volumes of starting buffer. Specifically bound [^{3}H]-labeld oligosaccharide alditols were eluted from the column with 0.1 M acetic acid. Appendix A is a summary of data obtained during the preparation and characterization of the affinity column used for the experiments described above.

3.2.10 *Enzyme Digestions*

Radiolabeled sialyloligosaccharide alditols were digested with *Clostridium perfringens* neuraminidase as described (Cassidy *et. al.*, 1965). Briefly, oligosaccharides (25,000-500,000 cpm) were dried in 1.5 ml microcentrifuge tubes and 150 µl of a solution containing 0.25 units of enzyme in 0.1 M potassium acetate buffer (pH 4.8) were added. The

contents of the tube were gently mixed and allowed to incubate for 18 h at 37°C. The incubation mixture was diluted with 1 ml of water and applied to a small column (0.5 x 3 cm) of DEAE-cellulose previously equilibrated in 2 mM pyridine-acetate buffer. The neutral oligosaccharide alditols released during digestion (98%) were eluted in starting buffer and non-digested material remained bound to the column.

Jack bean β-galactosidase and β-N-acetylhexosaminidase were stored at 1 unit /ml in 0.02 M potassium acetate buffer pH 8.0. Digestions with β-galactosidase were carried out at 37°C during 18 h with 5 mUnits or 45 mUnits of enzyme brought to a final volume of 0.05 ml with 0.4 M citrate buffer pH 3.2. Digestions with β-N-acetylhexosaminidase were carried out at 37°C for 18-96 h with 30 mUnits of enzyme brought to a final volume of 0.05 ml with 0.4 M citrate buffer pH 3.2. All incubations included 2 µl of toluene to prevent bacterial growth. Incubations were stopped by application to Bio-Gel P2 or paper chromatography.

Fucosidase digestions were performed as follows: beef kidney fucosidase (Levy, 1961) suspended in 3.2 M ammonium sulfate was centrifuged in a 1.5 ml polypropylene tube and the supernatant was removed. The pellet was dissolved in 0.05 M sodium acetate buffer (pH 5.0) and 0.4 units (0.2 ml) were added to 1 ml polypropylene tubes containing dried substrate (50,000 cpm). The mixtures were incubated for 18 h at 37°C and the reaction products were applied to Bio-Gel P2 chromatography.

Neuraminidase digestions of gangliosides on thin layer chromatograms were performed according to the procedure of Spitalnik *et. al*. (1986). Briefly, developed chromatograms were soaked in polyisobutylmethacrylate in hexane for 1 min, allowed to dry, sprayed with buffer A and then soaked in the same buffer for 30 min. Excess buffer was removed and the chromatograms were overlayed ith a solution of *Clostridium perfringes* neuraminidase (1 unit/ml, 0.05 Unit/cm^2) in 0.05 M sodium acetate, 0.15 M NaCl, pH 5.5). The overlayed chromatograms were incubated for 18 h at room temperature in a humidified chamber. After incubation the plates were washed four times with cold PBS before overlaying with antibody as described above.

IV

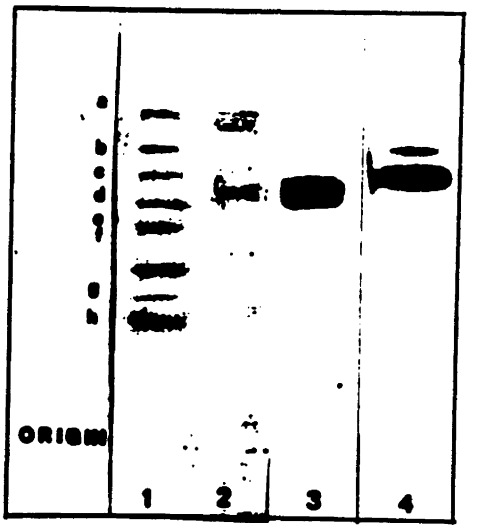
RESULTS AND DISCUSION

4.1 *DETECTION OF GANGLIOSIDES ON THIN LAYER CHROMATOGRAMS*

Anti-sialyltetrasaccharide *b* and anti-sialyltetrasaccharide *c* antibodies bind to monosialylgangliosides of human meconium separated by thin layer chromatography (Figure 3). Human brain ganglioside standards and the two major monosialylgangliosides of human meconium GM_3 and $6'LM_1$, were detected by resorcinol staining (Figure 3, lanes 1 and 2). Anti-sialyltetrasaccharide *b* antibodies detected two gangliosides which did not cochromatograph with any of the meconium gangliosides detected by resorcinol staining (Figure 3, lanes 2 and 4). Based on their known specificity anti-sialyltetrasaccharide *b* and anti-sialyltetrasaccharide *c* sera were presumably reacting with ceramide derivatives of their homologous haptens on thin layer chromatograms. each antiserum has high affinity for the reduced form of its homologous haptens (see Table 1.6). Anti-sialyltetrasaccharide *b* has an apparent binding constant of 4.16×10^6 liters mol^{-1} for sialyltetrasaccharide *b* alditol and anti-sialyltetrasaccharide *c* has an apparent binding constant 1×10^8 liters mol^{-1} for its reduced homologous hapten. However, anti-sialyltetrasaccharide *b* has an apparent binding constant of 6.25×10^5 liters mol^{-1} for unreduced sialyltetrasaccharide *b* and anti-sialyltetrasaccharide *c* has an apparent binding constant of 3.57 x

10^4 for unreduced sialyltetrasaccharide *c*. Anti-sialyltetrasaccharide *c* was able to recognize a ganglioside that co-chromatographs with ganglioside $6'LM_1$ (sialyltetrasaccharide *c*-ceramide), because (based on the apparent binding constants and biinding to glycolipids on thin layer chromatograms) antibodies with apparent binding constants equal or larger than 3.0×10^4 l mol^{-1} for unreduced homologous haptens are able to recognize specific carbohydrate structures as components of glycolipids. The major monosialylganglioside of human meconium is GM_3 (3'-sialyllactose-ceramide). However, anti-3'-sialyllactose was not able to detect GM_3 on thin layer chromatograms. This is consistent with the low affinity of this antiserum for non-reduced 3'-sialyllactose (2.2×10^3). Anti-sialyltetrasaccharide *a* did not react with any glycolipid on thin layer chromatograms of the monosialylganglioside fraction of meconium. This is consistent with the low affinity of this antiserum for native (unreduced) sialyltetrasaccharide *a* (6.66×10^2 lt mol^{-1}). Failure to detect sialyltetrasaccharide *a*-ceramide on thin layer chromatograms does not indicate that the structure is absent from the monosialylganglioside fraction of meconium.

Anti-sialyltetrasaccharide *b* was also used to monitor the purification of sialyltetrasaccharide *b*-ceramide after column chromatography in iatrobeads as shown in Figure 4 . A single fraction (fraction 20) had glycolipid recognized by the antiserum on thin layer chromaograms. However, this fraction was also contaminated with other gangliosides as determined by resorcinol staining (data not shown). This indicated that

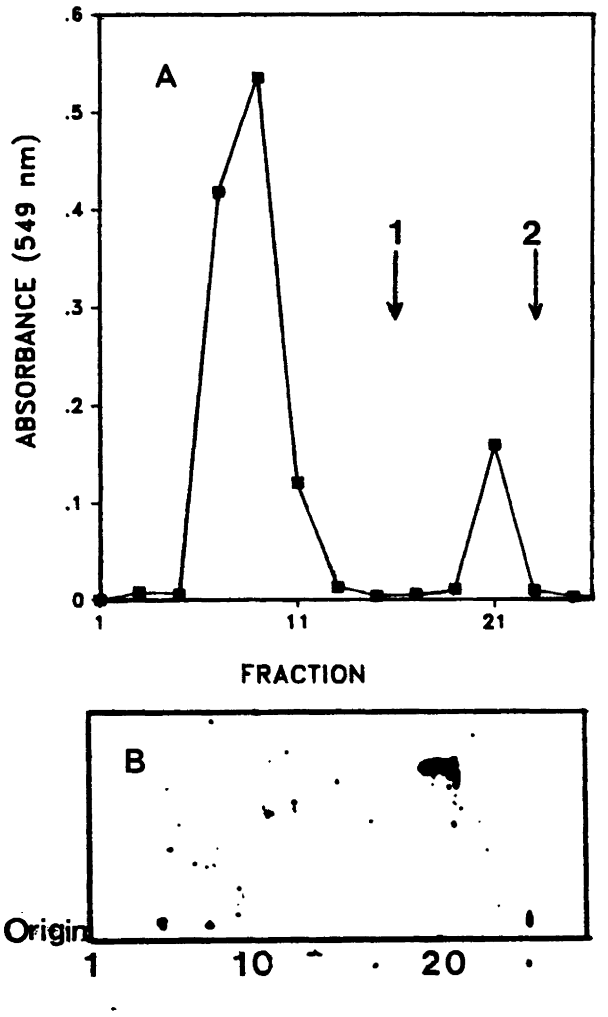


Gangliosides (6 nmol sialic acid per lane) are spotted on high-performance aluminum-backed thin layer plates and chromatographed in chloroform/methanol/0.23% KCl (5/4/1). Human brain ganglioside standards (lane 1) and human meconium gangliosides (lane 2) are visualized by resorcinol staining. Chromatograms of meconium monosialylgangliosides are overlayed with anti-sialyltetrasaccharide *c* (lane 3) and anti-sialyltetrasaccharide *b* (lane 4) sera, and the glycolipid-antibody complexes are visualized by autoradiography after treating with ^{125}I-labeled goat-antirabbit IgG as described under Experimental Procedures. Gangliosides detected by resorcinol on lanes 1 and 2 are: a, GM_3; b, GM_2; c, GM_1; d, $6'LM_1$; e, GD_3; f, GD_{1a}; g, GD_{1b}; and h, GT_{1t}.

Figure 3: Immune-staining of Gangliosdes on Thin Layer Chromatograms

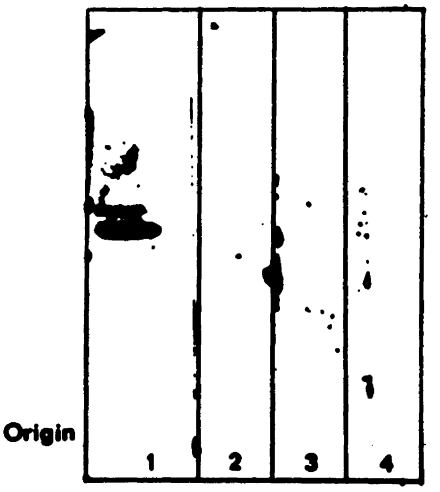
the chromatographic methods employed were not sufficient to purify meconium monosialylgangliosides, but also demonstrated the usefulness of anti-sialyltetrasaccharide *b* as a monitor of sialyltetrasaccharide *b*-ceramide purification.

A ganglioside with the sialyltetrasaccharide *b*-ceramide structure had never been reported from any source; therefore, experiments were carried out to confirm the specificity of anti-sialyltetrasaccharide *b* towards glycolipids on thin layer chromatograms. Anti-sialyltetrasaccharide *b* has a very low apparent binding constant for lacto-N-tetraose (1×10^2 lt mol^{-1}); hence, sialic acid linked α2-6 to N-acetylglucosamine is required for antibody binding. A thin layer chromatogram of the monosialylganglioside fraction of human meconium was directly treated with *Clostridium perfringens* neuraminidase as described under Experimental Procedures. Treatment with neuraminidase eliminated binding of anti-sialyltetrasaccharide *b* antibodies to the chromatograms (Figure 5, lane 4). That the antibodies were reacting with a ceramide derivative(s) of the homologous hapten is supported by the ability of both sialyltetrasaccharide *b* and its reduced derivative to inhibit antibody binding to thin layer chromatogram as shown in Figure 5, lanes 2 and 3. No inhibition of antibody binding was detected with 40 mM sialyltetrasaccharide *c* or 150 mM lacto-N-tetraose (data not shown).



Monosialylgangliosides of human meconium (Fig. 1 , Peak B, 1000 nmol) were dried down and dissolved in 200 µl of chloroform/methanol/water (30/60/4.5) and applied to a column (0.9 x 50 cm) previously equilibrated in the same solvent. (A) Fractions (2 ml) were collected while eluting with initial solvent which was then changed to chloroform/methanol (2/1) and to methanol (arrows 1 and 2 respectively). Aliquots (200 µl) were assayed for sialic acid with the thiobarbituric acid assay. (B), aliquots were also applied to thin layer chromatography and the resulting chromatogram was immunostained with anti-sialyltetrasaccharide *b* serum as described under Experimental Procedures.

Figure 4: Chromatography of Meconium Gangliosides on Iatrobeads

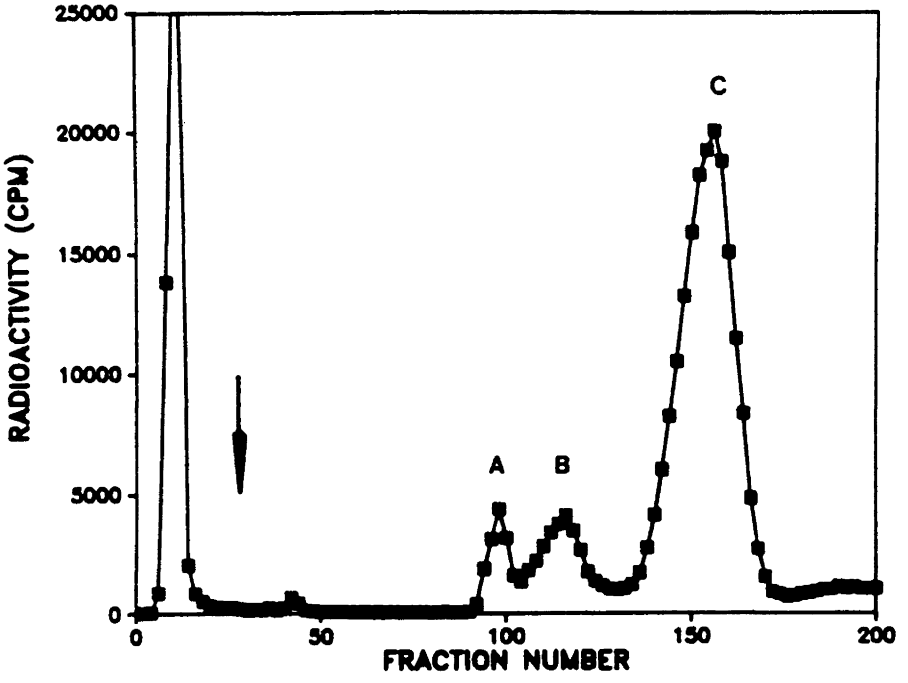


Autoradiography of meconium monosialylgangliosides overlayed as described in Figure 3 with anti-sialyltetrasaccharid b serum (lane 1); with anti-sialyltetrasaccharide b serum plus 17μM reduced sialyltetrasaccharide b (lane 2); with anti-sialyltetrasaccnaride *b* plus 14μM unreduced sialyltetrasaccharide b (lane 3). Lane 4 is the same as lane 1 except that the chromatogram was incubated with a solution of neuraminidase for 8 h prior to overlay with anti-sialyltetrasaccharide b serum as described under Experimental Procedures.

Figure 5: Studies of Inhibition of Antibody Binding to Gangliosides

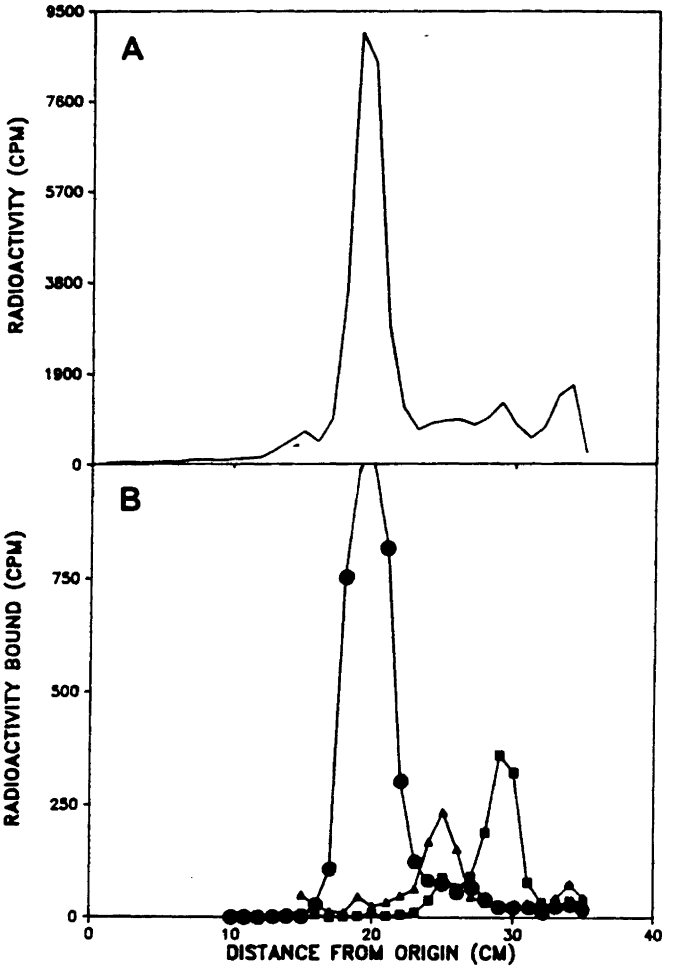
4.2 *GANGLIOSIDE-DERIVED OLIGOSACCHARIDE ALDITOLS FROM MECONIUM*

Although anti-sialyltetrasaccharide *b* and and anti-sialyltetrasaccharide *c* interact with their unreduced homologous haptens, anti-sialyltetrasaccharide *a* and anti-3'-sialyllactose can only recognize their reduced homologous haptens (as alditol derivatives of oligosaccharides). In order to analyze the carbohydrate structures of meconium gangliosides, oligosaccharides were released from their parent meconium glycolipids by O_3-alkali fragmentation and reduced with $NaB[^3H]_4$ as described under Experimental Procedures. The resulting ganglioside-derived [^{3}H]-labeled alditols were applied to DEAE-cellulose (DE-53) chromatography (Fig. 6). Fractions containing [^{3}H]-labeled monosialyloligosaccharide alditols were eluted from the column with 20 mM pyridine-acetate buffer (pH 5.4). Peak A is 3'-sialyl[^{3}H]lactitol as identified by binding of anti-3'-sialyllactose antibody and by cochromatography on paper with authentic 3'-sialyllactitol (data not shown). Peak B is the sialyltetrasaccharide region that has as its major component sialyl[^{3}H]tetrasaccaharide *c* alditol derived from ganglioside 6'LM_1. The sialyltetrasaccharide alditol fraction (Fig. 6 , Peak B) was pooled, lyophilized and subjected to paper chromatography in Solvent A as shown in Figure 7 . The migrations of sialyl[^{3}H]tetrasaccharide *c* (17-22 cm from the origin), sialyl[^{3}H]tetrasaccharide *b* (24-27 cm from the origin), and sialyl[^{3}H]tetrasaccharide *a* (28-31 cm from the origin) were determined by direct-binding of radiolabeled haptens on nitrocellulose using anti-sialyltetrasaccharide *c*, *b* and *a*, respectively.



[^{3}H]-labeled alditols from the monosialylganglioside fraction (300 nmol) were dissolved in 2 ml of 2 mM pyridine-acetate buffer pH 5.4 and applied to a column of DEAE-cellulose (0.9 x 50 cm) equilibrated with the same buffer. The monosialyl[^{3}H]oligosaccharide alditols are partially separated during elution with 20 mM pyridine-acetate buffer pH 5.4 indicated by the arrow. Aliquots (20µl) of 2 ml fractions are assayed for [^{3}H] by liquid scintillation counting.

Figure 6: DEAE-cellulose Chromatography of Oligosaccharide Alditols



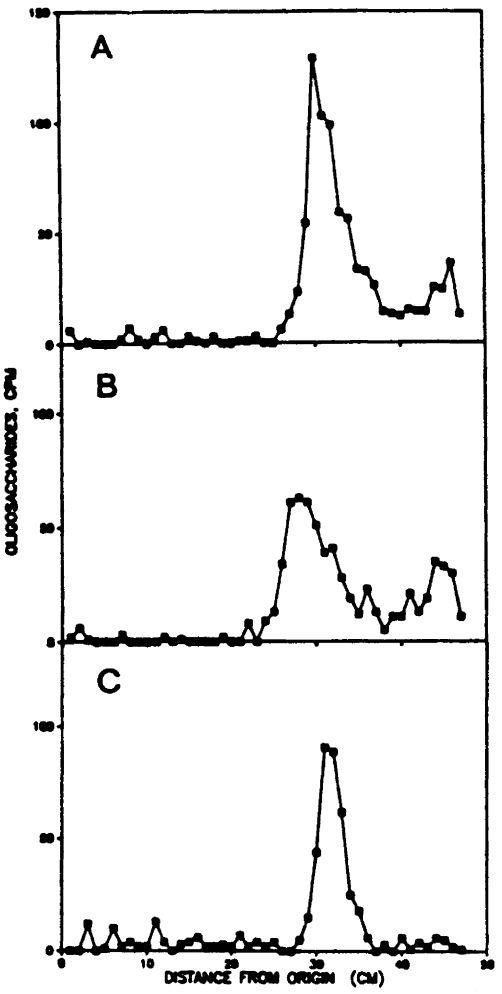
The sialyltetrasaccharides alditols from DEAE-cellulose chromatography (Fig. 6 fractions 104-126) were chromatographed for 6 days on Whatman No. 1 paper using Solvent A. Segments of the papers (1 cm) were eluted with 1 ml of 0.1 M pyridine-acetate buffer pH 5.4 and aliquots (50µl) were assayed for radioactivity (Panel A). Additional aliquots were assayed for binding of anti-sialyltetrasaccharide *c*(-●-●-), anti-sialyltetrasaccharide *b*(-▲-▲-) and anti-sialyltetrasaccharide *a*(-■-■-) sera as described under Experimental Procedures (Panel B).

Figure 7: Identification of Specific Sialyloligosaccharide Alditols

4.3 *GANGLIOSIDE-DERIVED SIALYLTETRASACCHARIDE B ALDITOL FROM MECONIUM*

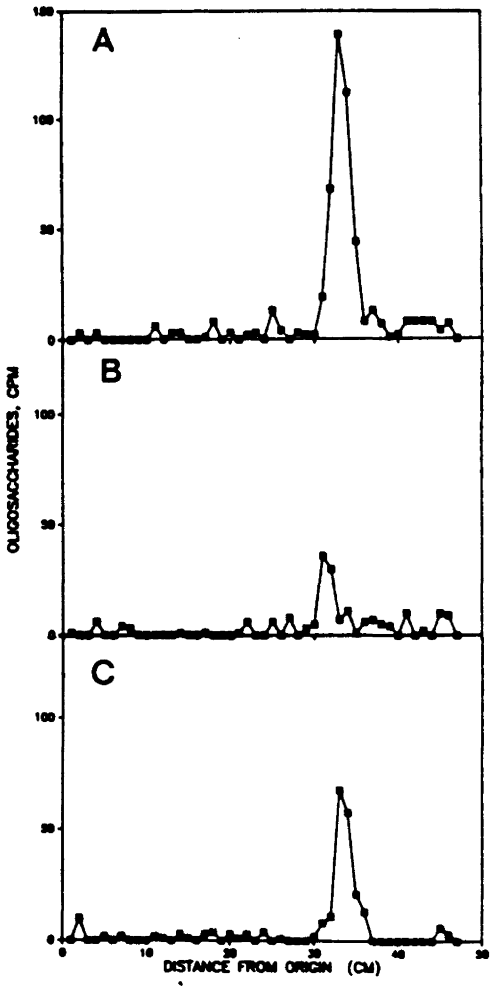
The material specifically recognized by anti-sialyltetrasaccharide *b* (Fig. 7 , 24-27 cm from the origin), was affinity purified by eluting the [^{3}H]-labeled oligosaccharide alditol from antibody-hapten complexes collected in nitrocellulose filters as described in Experimental Procedures. The [^{3}H]-labeled material bound by anti-sialyltetrasaccharide *b* was applied to paper chromatography in Solvent B (Fig. 8 , Panel B). Two components were detected in this chromatogram. The slower migrating cochromatographs with sialy[^{3}H]tetrasaccharide *b* alditol (Fig. 8 , Panels A and C), which suggests that meconium contains a minor monosialylganglioside containing this structure. A faster migrating component may be a degradation product of sialyltetrasaccharide *b* alditol.

The presence of a sialyltetrasaccharide *b* structure was further supported by paper cochromatography of the neuraminidase digestion product of the [^{3}H]-sialyloligosaccharide alditol in Figure 8, Panel B with authentic lacto-N-tetraitol (asialo-sialyl[^{3}H]tetrasaccharide *b* alditol) as shown in Figure 9 These results suggested that the neutral core of the ganglioside-derived sialyl[^{3}H]oligosaccharide alditol was [^{3}H]-labeled lacto-N-tetraitol. Although lacto-N-tetraitol and lacto-N-*neo*tetraitol are not separated by paper chromatography, lacto-N-tetraitol is the most probable neutral tetrasaccharide core based on the specificity of the antibody (Smith and Ginsburg, 1980). The low amount of radioactivity precluded further analysis of the ganglioside-derived sialyl[^{3}H]oligosaccharide alditol.



Paper chromatography of [^{3}H]-labeled oligosaccharides was carried out for 8 days in Solvent A. Radioactivity is determined as described under Experimental Procedures. (A) Mixtures of reduced sialyl[^{3}H]oligosaccharides in (B) and (C), (B) ganglioside-derived sialyl[^{3}H]oligosaccharide alditol (500 cpm, 1.5 pmol) obtained by the affinity purification method using nitrocellulose filters as described under Experimental Procedures. (C) affinity purified sialyl[^{3}H]tetrasaccharide *b* (470 cpm, 1.4 pmol) from human milk

Figure 8: Paper Chromatography of Sialyltetrasaccharide b alditol



The affinity purified sialyl[^{3}H]oligosaccharides (1000 cpm) used for experiments described in Fig. 8 are incubated with *Clostridium perfringens* neuraminidase, filtered through columns (0.3 x 4 cm) of DEAE-cellulose to remove undigested oligosaccharide, and applied to paper chromatography for 4 days in Solvent B. (A) Mixture of [^{3}H]-labeled oligosaccharide alditols in (B) and (C), (B) the asialo-derivative of ganglioside-derived sialyl[^{3}H]oligosaccharide, Fig. 8 , Panel C, and (C) the asialo-derivative of sialyl[^{3}H]tetrasaccharide *b* alditol.

Figure 9: Paper Chromatography of Asialo- Sialyltetrasaccaride b

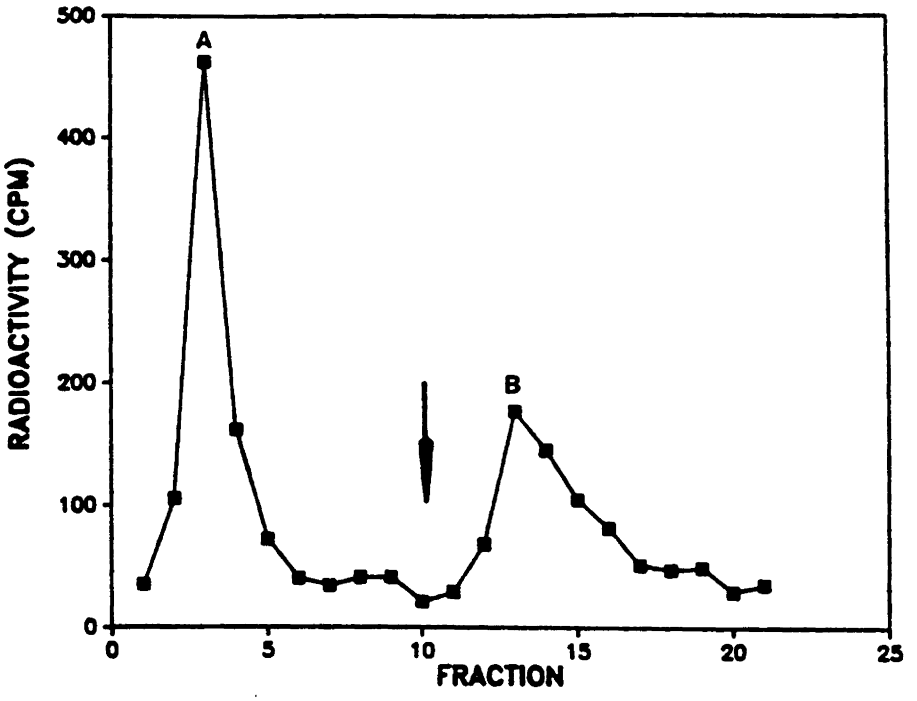
4.4 *GANGLIOSIDE-DERIVED SIALYLTETRASACCHARIDE A FROM MECONIUM*

4.4.1 *Affinity Purification*

Portions (15,000 cpm) of the material specifically recognized by anti-sialyltetrasaccharide *a* (Fig. 7, Panel A, 28-31 cm from the origin) were applied to a column of anti-sialyltetrasaccharide *a* (IgG fraction) coupled to Affi-Gel-10 as described under Experimental Procedures. Unbound [^{3}H]-labeled oligosaccharide alditols eluted in starting buffer (Fig. 10, Peak A) contained no sialyl[^{3}H]tetrasaccharide *a* alditol as determined by direct binding on nitrocellulose filters. The specifically-bound [^{3}H]-oligosaccharide alditol eluted in 0.1 M acetic acid (Fig. 10, Peak B), was pooled, lyophilized and used to confirm the structure as sialyltetrasaccharide *a*.

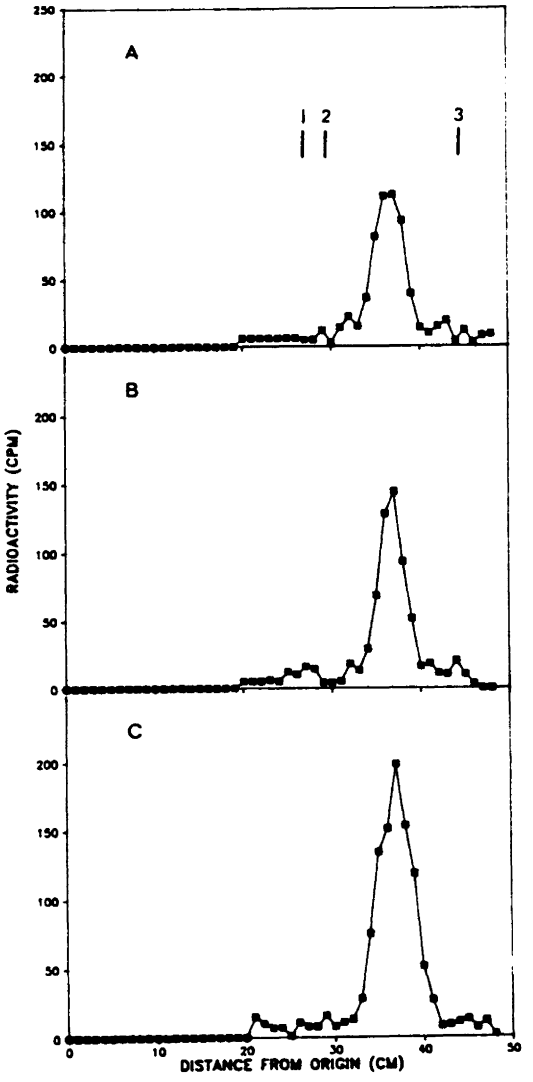
The affinity purified [^{3}H]-oligosaccharide alditol migrated as a single peak (Fig. 11, Panel A) and co-chromatographed with authentic sialyl[^{3}H]tetrasaccharide *a* alditol from human milk (Fig. 11, Panels B and C). The migration positions of sialyl[^{3}H]tetrasaccharide alditols *b* and *c* and the sialyl[^{3}H]oligosaccharide alditol from GM_1 are indicated on the figure.

Based on the strict specificity of anti-sialyltetrasaccharide *a* serum for sialic acid linked α2-3 to the terminal galactose residue of lacto-N-tetraitol neutral core and co-chromatography of the affinity purified oligosaccharide alditol with authentic sialyl[^{3}H]tetrasaccharide *a* alditol from human milk (Fig. 11) the sialic acid was assigned in an α2-3 linkage to the terminal galactose of a tetrasaccharide.



Fractions from paper chromatography containing sialyl[^{3}H]tetrasaccharide *a* (Fig. 7, fractions 28-31) were pooled lyophilized and dissolved in PBS. Portions of this solution (150 µl, 15,000 cpm) were applied to a column (0.9 x 5 cm) of anti-sialyltetrasaccharide *a* coupled to Affi-Gel-10. Fractions (1 ml) were collected while the column was washed with starting buffer. Affinity purified oligosaccharide was eluted with 0.1 M acetic acid as indicated by the arrow.

Figure 10: Affinity Purification of Sialyltetrasaccharide a Alditol



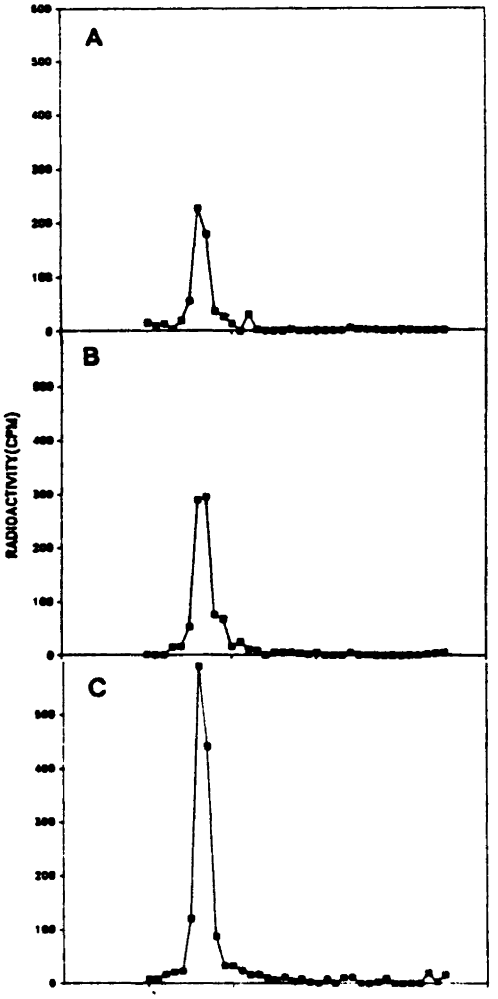
Paper chromatography of [^{3}H]-oligosaccharide alditols is carried out with Solvent B for 10 days. Radioactivity was determined as described under Experimental Procedures. (A) ganglioside-derived sialyl[^{3}H]tetrasaccharide a *from meconium,* (*B*) *authentic* sialyl[^{3}H]tetrasaccharide *a,* (C) mixture of samples A and B. Portions of the following oligosaccharides were used as standards: (1) sialyl[^{3}H]tetrasaccharide *c,* (2) sialyl[^{3}H]tetrasaccharide *b* and (3) [^{3}H]-labeled alditol of GM_1 oligosaccharide.

Figure 11: Paper Chromatography of Sialyltetrasaccharide a Alditol

4.4.2 *Identification of the Neutral Tetrasaccharide Core*

The affinity purified ganglioside-derived sialyl[^{3}H]tetrasaccharide *a* from meconium (25,000 cpm) was incubated with *Clostridium perfringens* neuraminidase as described under Experimental Procedures. The resulting neutral oligosaccharide alditol was applied to a column of Bio-Gel P2 and eluted with a K_D identical to lacto-N-tetraitol (data not shown). During paper chromatography in Solvent system B the [^{3}H]tetraitol migrated as a single peak and comigrated with authentic [^{3}H]-lacto-N-tetraitol as shown in Figure 12

However, since lacto-N-tetraitol and its positional isomer lacto-N-*neo*tetraitol do not separate during paper chromatography, the neutral [^{3}H]-tetraitol was assayed for binding with anti-lacto-N-fucopentaose-I . This antisera was used to differentiate lacto-N-tetraitol and lacto-N-*neo*tetraitol structures (Prieto and Smith, 1980). While the antiserum did not bind authentic [^{3}H]-lacto-N-*neo*tetraitol, the [^{3}H]-labeled asialo-derivative of the oligosaccharide released from ganglioside 6'-LM_1 (Fig. 7, Peak B), binding of the neutral [^{3}H]-tetraitol and authentic [^{3}H]-lacto-N-tetraitol was essentially identical (Fig. 13). In additional experiments anti-lacto-N-fucopentaose I did not bind the [^{3}H]-labeled asialo derivative of the alditol obtained from ganglioside GM_1 after ozonolysis, alkai-fragmentation and reduction with NaB[^{3}H]$_4$ (Galβ1-3GalNAcβ1-4Galβ1-4Glc), nor to [^{3}H]-lacto-triose alditol (data not shown). Binding of anti-Lacto-N-fucopentaose I

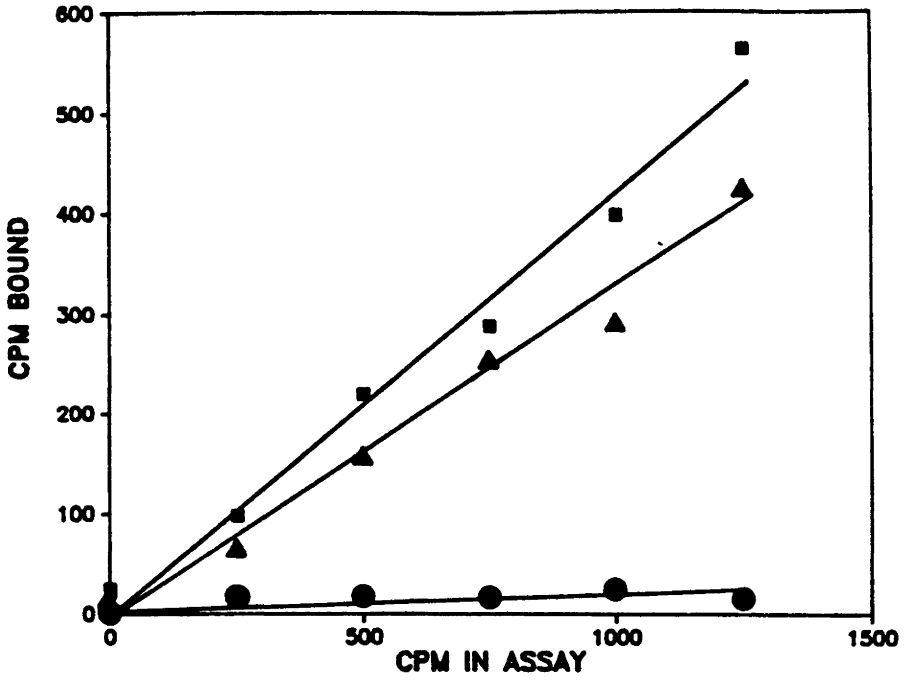


[^{3}H]-Labeled oligosaccharide alditols were chromatographed for 36 h in Solvent B and radioactivity was assayed as described. under Experimental Procedures. (A) asialo derivative of sialyl[^{3}H]tetrasaccharide *a* alditol form meconium (400 cpm), (B) authentic [^{3}H]-labeled lacto-N-tetraitol (400 cpm), and (C) a mixture of oligosaccharides in A and B.

Figure 12: Paper Chromatography of Tetraose Alditols (Solvent B)

antiserum to the neutral tetraitol further supported the assignment of a lacto-N-tetraose core for the monosialyloligosaccharide recognized by anti-sialyltetrasaccharide *a*.

Because of its specificity jack bean β-galactosidase has been used to study structural features of oligosaccharides (Li *et. al.*, 1979). For example, Galβ1-4βGlcNAc... linkages are very susceptible to the hydrolytic activity of the enzyme; however, Galβ1-3GlcNAc... linkages are less susceptible. In order to hydrolyze Galβ1-3GlcNAc... linkages, higher concentrations of enzyme and longer incubation times are required (Li *et.al.*, 1979). That the non-reducing terminal residue of the neutral [^{3}H]-oligosacchride alditol is a β1-3 linked galactose was confirmed by its low succeptibility to jack bean β-galactosidase. Neutral [^{3}H]-tetraitol was incubated with 0.1 unit/ml of β-galactosidase for 18 h at 37°C and the reaction mixture was applied to acolumn of Bio-Gel P2. As shown in Fig. 14 A the radioactivity migrated as a neutral tetraitol indicating resistance to jack bean β-galactosidase. Under identical conditions authentic lacto-N-*neo*tetraitol was completely converted to a trisacccharide alditol (Fig. 14 B). The neutral [^{3}H]-tetraitol was partially converted to a trisaccharide alditol at a higher enzyme concentration (1 unit/ml) at 37°C during a 48 h incubation (Fig 14 C). Although hydrolysis was not complete the identical pattern was obtained with authentic lacto-N-tetraitol that was digested under identical conditions (data not shown). In order to separate the triose alditol from remaining tetraitol, the products of



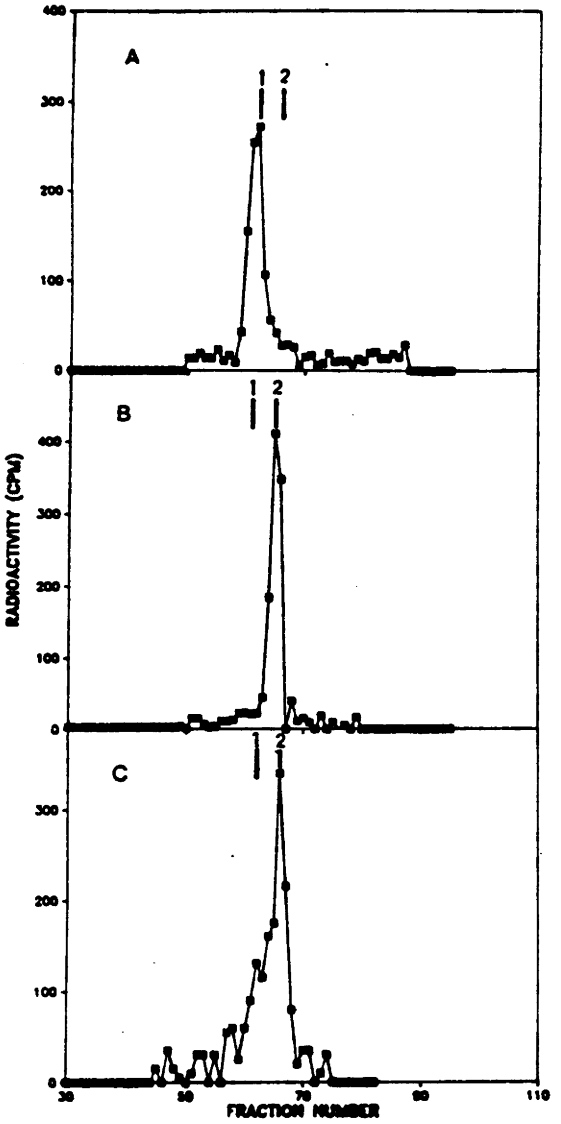
Binding assays were carried out as described under Experimental Procedures using 20 µl of anti-lacto-N-fucopentaose I serum and varying amounts of [^{3}H]-oligosaccharide alditols. [^{3}H]-lacto-N-teraitol (- ■ - ■), neutral tetrasaccharide from ganglioside-derived sialyl[^{3}H]tetrasaccharide *a* from meconium, (- ▲ - ▲ -) and [^{3}H] -lacto-N-*neo*tetraitol (- ● - ●-) or the asialo derivative of the reduced oligosaccharide released from ganglioside GM_1 ($Gal\beta1\text{-}3GalNAc\beta1\text{-}4Gal\beta1\text{-}4Glc_{OH}$.

Figure 13: Binding Curves of Anti-lacto-N-fucopentaose I Antiserum

galactosidase digestion (Fig. 14 , Fractions 59-70) were pooled, lyophilized and applied to paper chromatography (Fig. 15, Panel A). The material obtained after subjecting authentic [^{3}H]-lacto-N-tetraitol to galactosidase treatment and to Bio-Gel P2 chromatography under the same conditions was also applied to paper chromatography (Fig, 15, Panel B). Identical chromatographic patterns indicated that the tetraitol and authentic lacto-N-tetraitol were equally susceptible to jack bean β-galactosidase.

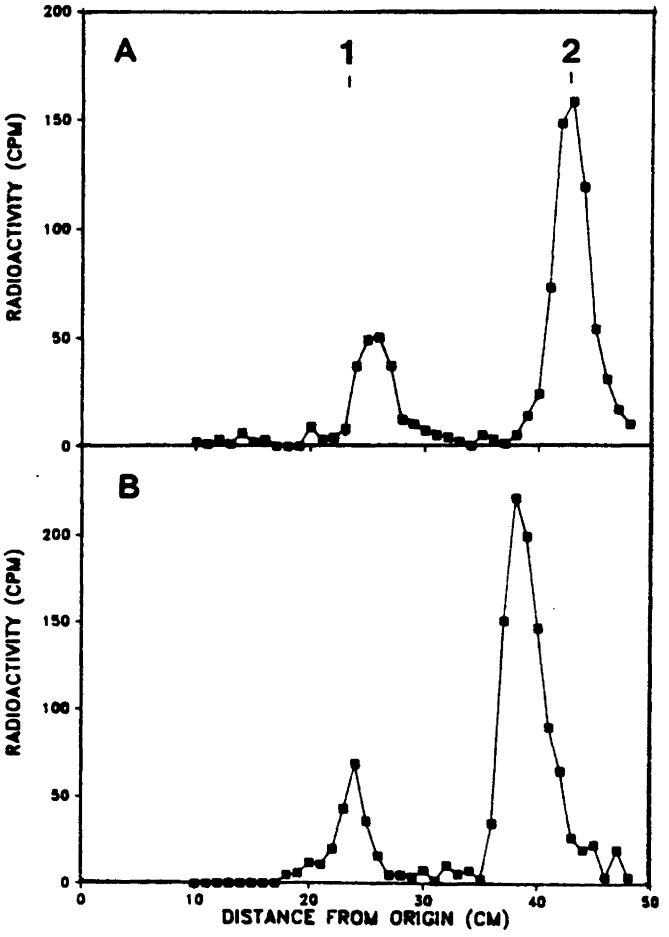
These data were consistent with the preferential activity of jack bean β-galactosidase toward terminal β1-4 linked galactose and confirm the presence of a β1-3 linked galactose as the terminal residue of the neutral [^{3}H]-tetraitol. The [^{3}H]-trisaccharide alditol (Fig. 15 , A 24-32 cm from the origin) migrated on paper as a single peak and co-chromatographed with authentic [^{3}H]-labeled GlcNAcβ1-3Galβ1-4Glucitol in Solvent C as shown in Figure 16 . Chromatography of the [^{3}H]-trisaccharide alditol with authentic [^{3}H]-lacto-alditol was also observed in Solvents B and D. (Figs 17 and 18).

The trisaccharide alditol was digested with jack bean β-hexosaminidase (0.7 units/ml) for 96 h at 37°C and the reaction mixture was applied to a Bio-Gel P2 column. The radioactive product eluted with a K_D identical to [^{3}H]-lactitol and co-chromatographed with authentic lactitol on paper in Solvent B as shown in Figure 19



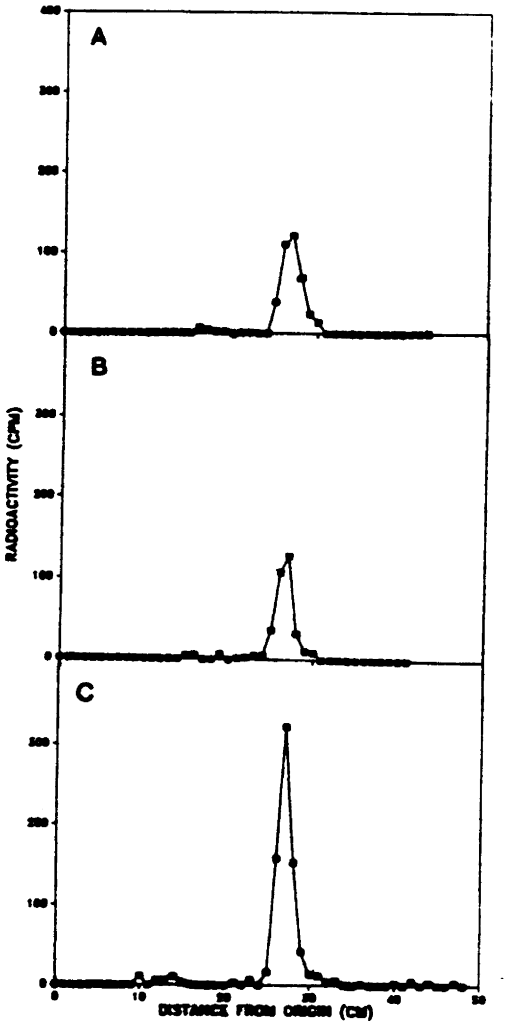
(A)Asialo-derivative of the ganglioside-derived sialyl[^{3}H]tetrasaccharide *a* from meconium (25,000 cpm) was treated with jack bean β-galactosidase (0.1 units/ml, 18 h, 37°C) and the products were applied to Bio-Gel P2 chromatography as described under Experimental Procedures. Aliquots (50 µl) were assayed for radioactivity. (B) lacto-N-*neo*tetraitol treated under the same conditions described for A. (C) asialo-derivative of ganglioside-derived sialyl[^{3}H]tetrasaccharide *a* from meconium treated with jack bean β-galactosidase (1 unit/ml, 48 h, 37°C). Positions for lacto-N-tetraitol (1) and the alditol of lacto-N-triose (2) during chromatography are indicated.

Figure 14: Bio-Gel P2 Chromatography of Oligosaccharide Alditols



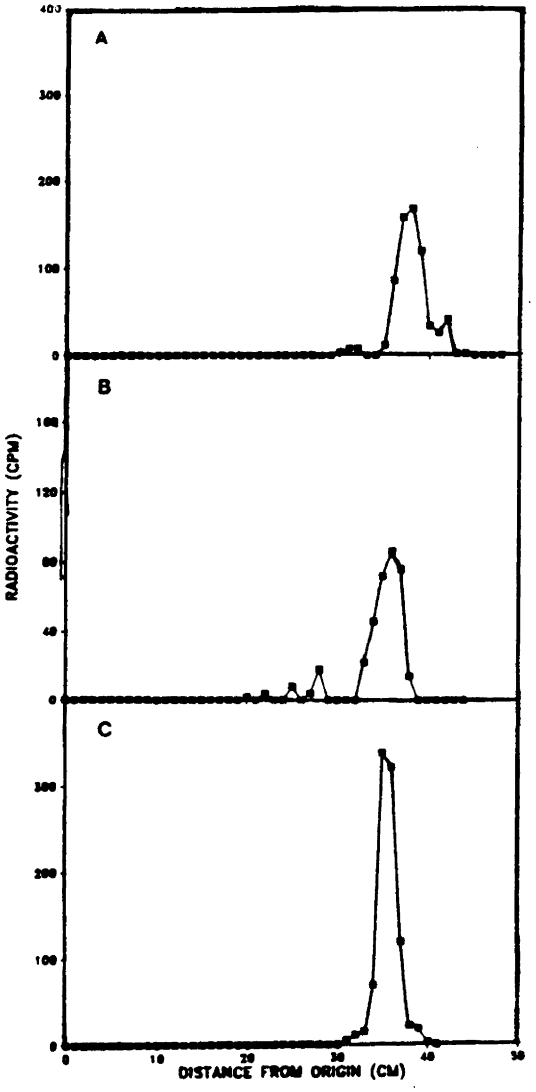
(A) The asialo derivative of the ganglioside-derived sialyl[^{3}H]tetrasaccharide *a* alditol from human meconium after jack bean β-galactosidase treatment (Fig 14 , C, fractions 59-70) was applied to paper chromatography for 3 days in Solvent B and aliquots from paper eluates (20 µl) were assayed for radioactivity as described under Experimental Procedures. (B) The same as (A) but with authentic [^{3}H]-lacto-N-tetraitol. Migration positions of lacto-N-tetraitol (1) and triose alditol (2) are indicated.

Figure 15: Paper Chromatography of the Tetraitol After Galactiosidase



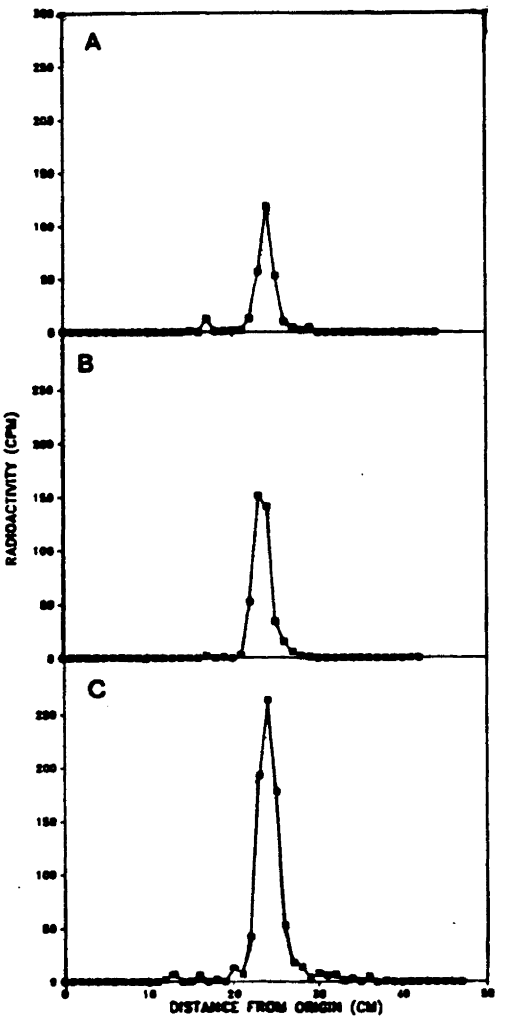
[^{3}H]-labeled oligosaccharide alditols were chromatographed for 36 h in Solvent C and aliquots of the eluates were assayed for radioactivity as described under Experimental Procedures. (A) [^{3}H]-labeled triose alditol from meconium ganglioside derived sialyl[^{3}H]tetrasaccharide α(400 cpm), (B) Authentic lacto-triose alditol (400 cpm) and (C) A mixture of the alditols in A and B.

Figure 16: Paper Chromatography of Triose Alditols (Solvent C)



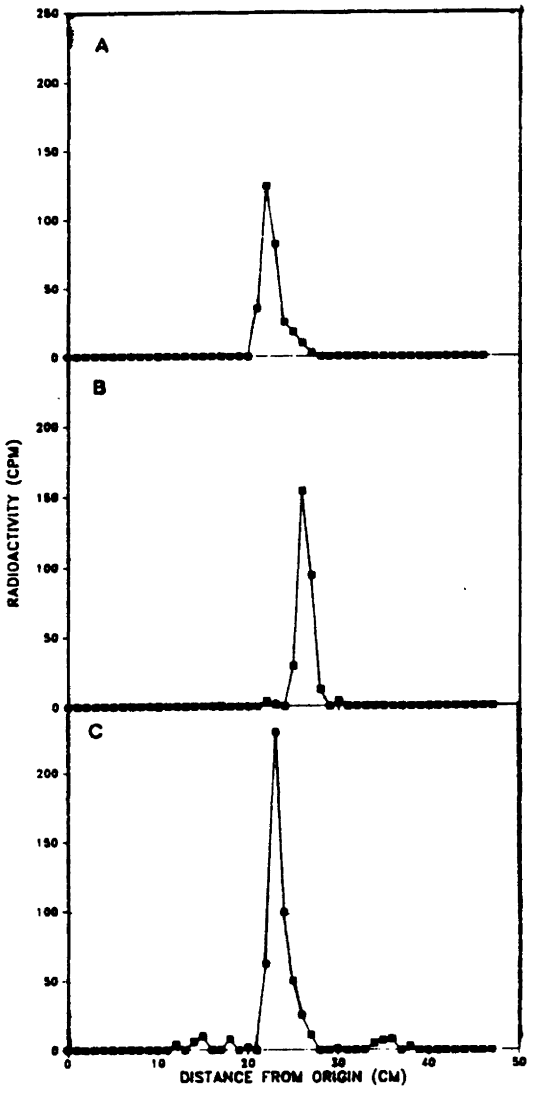
[^{3}H]-labeled oligosaccharide alditols are chromatographed for 36 h in Solvent B and aliquots from the eluates were assayed for radioactivity as described under Experimental Procedures. (A) [^{3}H]-trisaccharide alditol (1000 cpm), (B) authentic lacto-trisaccharide alditol (1000 cpm) and (C) a mixture of oligosaccharide alditols in A and B.

Figure 17: Paper Chromatography of Triose Alditols (Solvent B)



[^{3}H]-labeled oligosaccharide alditols are chromatographed for 36 h in Solvent D and aliquots from the eluates were assayed for radioactivity as described under Experimental Procedures. (A) [^{3}H]-trisaccharide alditol (400 cpm), (B) authentic lacto-trisaccharide alditol (400 cpm) and (C) a mixture of oligosaccharide alditols in A and B.

Figure 18: Paper Chromatography of Triose Alditols (Solvent D)



[^{3}H]-labeled oligosaccharide alditols were chromatographed for 36 h in Solvent A and aliquots from the eluates were assayed for radioactivity as described under Experimental Procedures. (A) [^{3}H]-disaccharide alditol (1000 cpm), (B) authentic lactitol (1000 cpm) and (C) a mixture of oligosaccharide alditols in A and B.

Figure 19: Paper Chromatography of Disaccharide Alditols

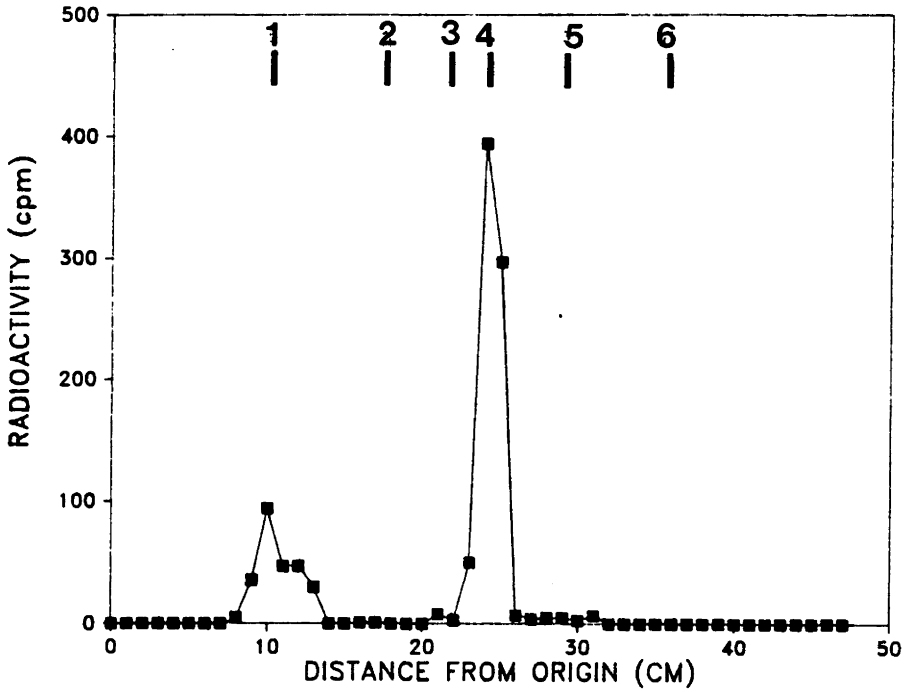
The [^{3}H]-disaccharide alditol was then incubated with jack bean β-galactosidase (0.7 units/ml) for 72 h at 37°C, and the resulting [^{3}H]-alditol cochromatographs with authentic [^{3}H]-glucitol on paper in Solvent C as shown in Figure 20

Antibody binding and cochromatography of the products of sequential enzymatic degradation of the ganglioside-derived sialyl[^{3}H]tetrasaccharide a with known standards were consistent with the presence of a ganglioside with the folowing structure in human meconium:

NeuAcα2-3Galβ1-3GlcNAcβ1-3GalβGlc-ceramide

4.4.3 *Detection of the Sialyl-Le^a Antigen in Human Meconium*

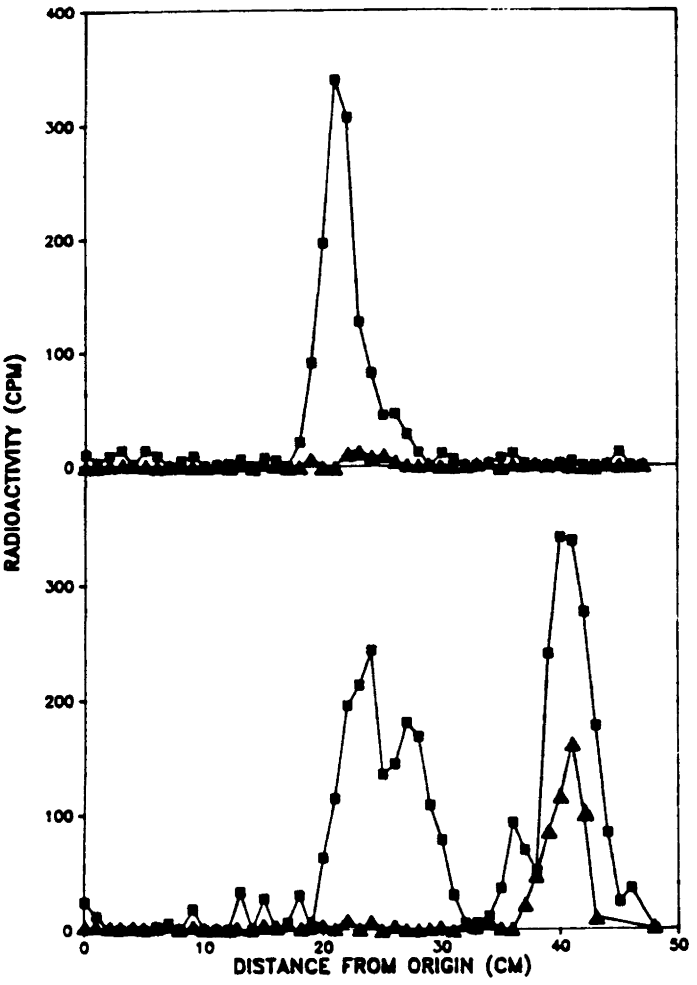
As mentioned above, Magnani *et. al.* (1982) detected the sialyl-Le^a antigen (fucsyl-sialyltetrasaccharide *a*-ceramide) in the monosialylganglioside fraction of human meconium. In order to test the ability of anti-sialyltetrasaccharide *a* to detect minor components with structures related to its homologous hapten, the antiserum was used to detect the release of sialyltetrasaccharide *a* alditol after fucosidase treatment of the sialyl[^{3}H]pentasaccharide alditol fraction derived from human meconium gangliosides. The ganglioside-derived sialyl[^{3}H]pentasaccharide alditols (Fig 7 A, 12-16 cm from the origin) migrated on paper slightly slower than sialyl[^{3}H]tetrasaccharide *c* and presumably contained the sialyl-Le^a hapten. When the



The [^{3}H]-labeled oligosaccharide alditol obtained after galactosidase digestion of the disaccharide alditol, was applied to paper chromatography in solvent C and the resulting chromatogram was assayed for radioactivity as described under Experimental Procedures. Migration positions of the following standards is indicated: (1) lactose, (2) N-acetylglucosamine, (3) galactose, (4) glucose, (5) mannose and (6) fucose

Figure 20: Paper Chromatography of the Monosaccharide Alditol

sialyl[^{3}H]pentasaccharide fraction was applied to paper chromatography the profile shown in Figure 21 ,A was obtained. Analysis of the chromatogram by direct binding on nitrocellulose filters using anti-sialyltetrasaccharide *a* serum indicated no binding of fucosylated derivatives (Fig. 21 ,A). However, if the sialyl[^{3}H]pentasaccharide alditol fraction was digested with beef kidney fucosidase prior to paper chromatography, the profile shown in Figure 21 B was obtained. Analysis of this chromatogram with anti-sialyltetrasaccharide *a* serum indicated that the homologous haptens of the antibody was generated from the sialyl-Le^{a} hapten after α fucosidase digestion (Fig. 21 ,B ,38-43 cm from the origin). These data indicated that although anti-sialyltetrasaccharide *a* binds only the reduced form of its homologous hapten it has strict specificity and does not bind the sialyl-Le^{a} hapten. However the antibody was useful to identify the presence of the sialyl-Le^{a} antigen in meconium and could also be useful to detect larger structures containing the sialyltetrasaccharide *a* structure if these structures are first treated with glycosidases.



Panel A; The sialylpentasaccharide fraction of ganglioside-derived alditols from human meconium was pooled and lyophilized. A portion of this fraction was dissolved in water and applied to paper chromatography on Whatman No. 1 paper for 11 days on Solvent A. Radioactivity (-■-■-) and binding to anti-sialyltetrasaccharide *a* were assayed as described under experimental procedures. Panel B; A portion of the same fraction used in A is treated with beef kidney fucosidase and then applied to paper chromatography. Radioactivity (-■-■-) and binding to anti-sialyltetrasaccharide *a* (-▲-▲-)are assayed under the same conditions as in A.

Figure 21: Chromatography of Sialylpentasaccharides After Fucosidase

V

CONCLUSIONS

Rabbit antibodies raised against milk oligosaccharides were used to detect and characterize two previously unidentified minor components (<1%) of the monosialylganglioside fraction of human meconium. These two gangliosides were not detected in previous studies of meconium gangliosides in which traditional chemical methods were employed (Nilsson *et. al.*, 1981) (see Table 9).

Although specific anti-oligosaccahride sera can be used for structural analysis of oligosaccharides it is important to recognize the limitations of the methods employed in this dissertation. For example, chemical compositional analyses were not performed on the novel gangliosides or their ganglioside-derived oligosaccahride alditols because the low amounts in which the gangliosides are present in meconium precluded these analyses. Data obtained from Mass spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectrometry are considered unequivocal evidence for assignment of carbohydrate structures. However, the use of anti-oligosaccharide antibodies for isolation and characterization of novel structures has proven to be a valid approach to structural analysis. For example, a minor sialyloligosaccharide from human milk (fucosyl-sialyltetrasaccharide *b*) was detected and characterized using anti-sialyltetrasaccharide *b* (Prieto and Smith, 1984). The same structure was later described by Wieruszeski and

coworkers (1985) using chemical methods on a large pooled sample of human milk.

The anti-oligosaccharide antibodies used in this dissertation react better with the reduced forms of their homologous haptens that with non-reduced oligosaccharides. In order to reduce carbohydrate moieties of glycolipids it is necessary first to release the carbohydrate from the lipid by chemical methods. This was accomplished by subjecting gangliosides to an ozonolysis-alkali fragmentation procedure prior to the reduction of the oligosaccharides with high specific activity $NaB[^3H]_4$.

The high specific activity of the radiolabeled products increased the sensitivity of the assay facilitating the detection and isolation of minor components. The ability of the ozonolysis procedure to release intact oligosaccharides from their parent glycolipids was demonstrated by the similarity between concentration values for the major monosialylgangliosides (GM_3 and $6'LM_1$) obtained by O_3-alkali fragmentation and those values obtained by chemical methods (see Table 9). For example, Nilsson *et. al.* (1981) determined that the concentrations of gangliosides GM_3 and $6'LM_1$ in human meconium were 170 µml/Kg and 20 µmol/Kg of meconium respectively and the values obtained during this investigation were of 156 µmol/Kg for $GM_3$and 18 µmol/Kg for $6'LM_1$. Antisera were used in this investigation as sensitive probes for specific carbohydrate sequences. For example, sialyltetrasaccharide *b*-ceramide was present in a

TABLE 9

Concentration of Monosialylgangliosides of Meconium

Abbreviation	Concentration[1] µmol/Kg (% of fraction)	Concentration[2]
GM_3	170(87)	156(80)
$6'LM_1$	20(10)	18(9)
STa-cer	not detected	0.8(0.4)
STb-cer	not detected	0.2(0.1)
Sialyl-Le^a	not detected	0.4(0.2)

1.- According to Nilsson (1981)
2.- Determined by the experiments described in this dissertation.

concentration of approximately 0.2 μmol/Kg of meconium. Less than 5 pmol of the ganglioside were detected on thin layer chromatograms and only 2 pmol of its ganglioside-derived alditol were needed to detect the sialyltetrasaccharide *b* strucure in a complex mixture of ganglioside-derived [^{3}H]-oligosaccharide alditols from human meconium. Specific rabbit antisera directed against milk oligosaccharides are being used in our laboratory as probes for specific carbohydrate structures on glycolipid extracts obtained from established cancer cell lines. The same antibodies will be used to analyze lipid extracts from normal tissues and the results will be compared with those obtained from the studies on cancer cells in order to identify cancer-associated structures. If cancer-associated antigens are also present in human meconium, the results from these studies could enrich the existing inventory of oncofetal antigens.

The structures defined in this dissertation are two sialyltetrasaccharide ceramide derivatives that had never been reported as gangliosides in any tissue;

NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ceramide

and

Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc-ceramide

The detection of these gangliosides was possible because antibodies directed against milk oligosaccharides were able to detect their carbohydrate structures in quantities that are beyond the limits of detection of available chemical methods. The procedures employed in the experiments described in this dissertation can be applied to the study of cell surface carbohydrates structures from samples available only in limited quantities.

LITERATURE CITED

Aminoff, D. (1961). Methods for the Quantitative Estimation of N-acetylneuraminic Acid and their Applicatio to Hydrolisates of Sialomucoids. *Biochem. J. 81:304-388*

Antonowicz, I. and Shwachman, H. (1979). Meconium in Health and Disease. *J. Adv. Ped. 26:275-310*

Belew, J.S (1969). In: *Oxidation*. Chapter VI-"Ozonization". (Augustine, R.L. ed.) Marcel Decker, Inc. New York, NY. Vol I:259-331

Buchanan, D.J. and Rapoport, S.A. (1951). A Chemical Study of Blood Specific Substances Found in Individual Meconium. *J. Biol. Chem. 19:251-254.*

Buehler, J., Quan, E., DeGregorio, M.W. and Macher, B.A. (1985). Biosynthesis of Glycolipids by Human Myeloid Leukemia Cells. *Biochemistry 24:6978-6984*

Cassidy, T., Jourdain, G.W. and Roseman, S. (1965). Purification and properties of sialidase from *Clostridium perfringens*. *J. Biol. Chem. 240*

Clamp, J.R. and Gough, M. (1979). Study of the Oligosaccharide Units from Mucus Glycoproteins of Meconium from Normal Infants and from Cases of Cystic Fibrosis with Meconium Ileus. *Clinical science 57:445-451*

Deslongchamps, P., Atlani, P. Frehel, D. Malaval, a. and Moreau, C. (1974). Oxidation of Acetals. *Can. J. Chem. 52:3651-3664*

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric Method for Detection of Sugars and Related Substances. *Anal. Chem. 28:350*

Englard, S. and Hanson, K.R. (1969). Stereo Specifically Labeled Citric Acid Intermediates. In: *Methods in Enzymology*. Academic Press, New York, NY *13:567-598.*

Feizi, T. and Childs, R.A. (1985). Carbohydrate Structures of Glycolipids and Glycoproteins as Differentiation Antigens, Tumour Associated Antigens and Components of Receptors. *T.I.B.S. 10:24-29*

Feizi T. (1985). Demonstration by Monoclonal Antibodies that Carbohydrate Structures of Glycoproteins and Glycolipids are Oncofetal Antigens. *Nature 314:53-57.*

Fracker, P.J. and Speck, J.C. (1978). Protein and Cell Membrane Iodinations with a Sparingly Soluble Chloramide 1,3,4,6-tetrachloro 3a,6a Diphenylceuril. *Biochem. Biophys Res. Comm. 80:849-857*

Gersham, H., Power, E. Lawrence, L. and VanVunakis, H. (1972). Radioimmunoassay of Prostaglandins, Angiotesin Digoxine, Morphine and Adenine 3'-, 5'- cyclic monophosphate Using Nitrocellulose Membranes. *Prostaglandins. 1:407-412*

Ginsburg, V. (1972). Biochemical Basis for Some Blood Groups in Man. In: *Biochemistry of the Glycosidic Linkage.* PAABS symposium. *2:387-391.*

Ginsburg, V., Zopf, D.A., Yamashita, K. and Kobata, A. (1976). Oligosaccharides of Human Milk: Isolation of a New Pentasaccharide, Lacto-N-Fucopentaose V. *Arch. Biochem. Biophys. 175:565-568*

Grumuald, B.G., Fredman, P., Magnani, J.L., Trisler, D., Ginsburg, V. and Nierenberg, M. (1985). Monoclonal Antibody 18B8 Detects Gangliosides Associated with Neuronal Differentiation and Synapse Formation. *Proc. Natl. Acad. Sci. US. 82:4008-4012.*

Hakomori, S.-I. (1981). Blod Group ABH and Ii Antigens of Human Erythrocytes: Chemistry, Polymorfism and their Developmental Change. *Seminars in Hematology (January 1981), 18:39*

Hakomori, S.-I. (1984). Glycosphingolipids as Differentiation-Dependent Tumor-Associated Antigens and as Regulators of Cell Proliferation. *T.I.B.S. 9:453-456*

Hakomori, S.-I. (1984). Tumor Associated Carbohydrate Antigens. *Ann. Rev. Immunol. 2:103-126.*

Hannisch, K.-G., Egge, H., Petr-Katalinic, Uhlenbruck, G., Dienst, C. and Fangman, R. (1985). Primary Structures and Lewis Blood-group-Dependent Expression of Mayor Sialylated Saccharides from Mucus Glycoproteins of Human Semminal Plasma. *Eur. J. Biochem. 152:343-351.*

Hansson, G.C. and Zopf, D.A. (1985). Biosynthesis of the Sialyl Le^a Antigen. *J. Biol. Chem. 260:9388-9392*

Harper,P.A. and Juliano, R.L. (1981). Two Distinct Mechanisms of Fibroblast Adhexion. *Nature 290:136-138.* Harris, J.T. (1978). Meconium in Health and Disease. *Medical Bulletin 34:75-78.*

Hellerquist, C.G., Sinatra, R. and Siegel, J. (1985). Role of VWF Oligosaccharides in Platelet Adhesion. *Procedings of the IX International Symposium on Glycoconjugates. 1:389 (Abstract)*

Herlant-Peers, M.C., Montreuil, J. and Strecker, G. (1981). Structures of Fifteen Oligosaccharides Isolated from New-Born Meconium. *Eur. J. Biochem 117:291-300*

Herlyn, M., Steplewski, Z., Herlyn, D. and Koprowski, H (1979). Colorectal Carcinoma-Specific Antigens: Detection by Means of Monoclonal Antibodies. *Proc. Natl. Acad. Sci. US. 76-1438-1442.*

Holmgren, J. Lonnroth, I., Mansson, J.-E. and Svennerholm, P. (1975). *Proc. Natl. Acad. Sci. US 72:2520:2524.*

Holsclaw, D.S., Keith, H.H. and Palmer, T.(1978). Meconim Screening for Cystic Fibrosis. *Pediatric Annals. 7: page 29.*

Jeffrey, A.M., Zopf, D.A. and Ginsburg, V. (1975). Affinity Chromatography of Carbohydrate-Specific Immunoglobulins: Coupling of Oligosaccharides to Sepharose. *Biochem. Biophys. Res. Comm. 62:608-613.*

Jourdain, G.W. and Roseman, S. (1971). The Sialic Acids, Periodate-Resorcinol Method for the Quantitative Estimation of Free Sialic Acid and its Glycosides. *J. Biol. Chem. 240:3501-3506.*

Karlsson, K.A. and Larson, G. (1981). Molecular Characterization of Cell Surface Antigens of Fetal Tissue. *J. Biol. Chem. 258:3512-3524.*

Kobata, A., Tsuda,M. and Ginsburg, V. (1969). Oligosaccharides of Milk: Isolation and Characterization. *Arch. Biochem. Biophys. 130:*

Kobata, A. (1972) Isolation of Oligosaccharides of Human milk. In: *Methods in Enzymology. (Ginsburg, V. ed.) 28:262-271.*

Kohn, D. (1978). Relationships in the Functions of Receptors for Glycoprotein Hormones, Bacterial Toxins and Interferon. In: *Receptors and Recognition.* (Cuatrecasas, P. ed.) Chapram and Hall ed. London Gr. Br. *Series A: 133-212.*

Kolberg, H. and Hellsing K. (1975). Screening Cystic Fibrosis by Analysis of Albumin in Meconium. *Acta Pediatrica Scand. 64:477-482*

Kopito, L. and Shwachman H. (1966). Mineral Composition of Meconium. *J. Pediatr. 313:68-71.*

Korhonen, T.K., Vaisanen.-R., V. Rehn, M., Pere, A., Parkinen, J. and Finne, J. (1984). *Escherichia coli* Fimbriae Recognizes Sialyl-Galctosides. *J. of Bacteriology 159:762-766*

Ledeen, R.W. and Yu, R.K. (1982). Gangliosides, Structure, Isolation and Analysis. In: *Methods in Enzymology* (Ginsburg, V. ed.) Academic Press, New York NY, *83:139-190*

Levy, G.A. and McAllan, A. (1961). Mammalian Fucosidases. *Biochem. J. 80:433-439*

Li, S,-C., Mazzola, Y. Chen, F. and Li, Y.-T. (1976). Isolation and Characterization of jack bean β-galactosidase. *J. Biol. Che. 250:6786-6791*

Li, Y.-T. and Li, S.-C.(1973). In: *Advances in Enzymology*. α-mannosidase, β-glactosidase andβ-N-acetylhexosaminidase from jack bean. Academic Press, New York, NY. *28:702-713*

Magnani, J.L., Brockhaus, M., Smith,D.F. and Ginsburg, V. (1981). A Monosialylganglioside is a Monoclonal Defined Antigen of Colon Carcinoma. *Science. 212:55*

Magnani, J.L, Nilsson, B., Brockhaus, M., Zopf, D., Steplewski, Z., Kropowski, H. and Ginsburg, V. (1982). A Monoclonal Antibody Defined Anigen Associated with Gastrointestinal Cancer is a Ganglioside containing sialated LNF II. *J. Biol. Chem. 257:14365-14369.*

Momoi, T., Ando, S. and Magai, Y. (1976). High Resolution Preparative Chromatography System for Gangliosides Using DEAE-Sephadex and a New Porous Silica,Iatrobeads. *Biochim. Biophys, Acta. 441:488-497.*

Nilsson, O., Mansson, J.-E., Tibblin, E. and Svennerholm, L. (1981). Gangliosides of Human Meconium-Detection of a Possible Fetal Antigen. *FEBS letters 133:197-200*

Openheimer, S.B., (1982). In: *Cancer, A Biological and Clinical Introduction*. Allyn and Bacon ed. Boston, MA. Page 48.

Pappas, J.J., Keaveney, W.P., Ganeher, E. and Berger, M. (1966). A New and Convenient Method for Converting Olefins to Aldehides. *Tetrahedron Letters 36:4273-4278.*

Prieto, P.A. and Smith, D.F. (1984).Anew Oligosaccharide from Human Milk: Isolation and Characterization Using Anti-oligosaccharide Antibodies. *Arch. Biochem. Biophys.*

Roberts, D., Monslin, D.L., Frates, R.C., Chernick, M.S. and Ginsburg, V. (1986). A Serum Test for Cystic Fibrosis Using Monoclonal Antibody 19-9. *Arch. Biochem. Biophys. 254:292-293.*

Ryley, H.C., Lynne, M.N., Brogan, P.D. and Bray, P.T. (1979). Screening for Cystic Fibrosis in the Newborn by Meconium Analysis. *Arch. Disease in Chilhood. 54:92-97.*

Shen, L., Grollman, E.F. and Ginsburg, V. (1968). An Enzymatic Basis for Secretor Status and Blood Group Specificity in Humans. *Proc. Natl. Acad. Sci US. 59:224-230.*

Smith, D.F., Zopf, D.A. and Ginsburg, V. (1978). Fractionation of Sialyloligosaccharides of Human Milk by Ion Exchange Chromatography. *Anal. Biochem. 85:602-608.*

Smith, D.F. and Ginsburg, V. (1980). Antibodies Against Sialyloligosaccharides Coupled to Protein. *J. Biol. Chem. 255:55-59.*

Smith, D.F., Magnani, J.L. and Ginsburg, V. (1981). Glycolipids of Cell Surfaces: Isolation of Specific Sugar Sequences Using Carbohydrate Binding Proteins. *Arch. Biochem. Biophys. 209:52-56*

Smith, D.F. (1983). Glycolipid-Lectin Interactions: Detection by Direct Binding of Lectins to Thin Layer Chromatograms. *Biochem. Biophys. Res. Comm. 115:360-367*

Smith, D.F., Prieto, P.A. and Torres, B.V. (1985). Rabbit Antibodies Against the Human Milk Sialyloligosaccharide Alditol of LS-Tetrasaccharide a. *Arch. Biochem. Biophys. 241:298-303.*

Spitalnik, S.L., Schwartz, J.F., Magnani, J.L., Roberts, D.D., Spitalnik, P.F. Ciuin, C.I., and Ginsburg, V. (1986). *Blood. (in press)*

Svennerholm, L. and Fredman, P. (1980). A Procedure for the Quantitative Isolation of Brain Gangliosides. *Biochem. Biophys. Acta 6117:97-109*

Vitala, J. and Jarnfelt, J. (1985). The Red Cell Surface Revisited. *T.I.B.S. 10:392-395*

Watanabe, K. and Hakomori, S.-I. (1976). Status of Blood Group Carbohydrate Chains in Ontogensis and in Oncogenesis. *J. of Exp. Medicine. 144:644-651.*

Watkins, W.M. (1980). Biochemistry and Genetics of the ABO, Lewis and P Blood Group Sistems. In: *Advances in Human Genetics.* (Harris, H. and Hirschan, K. ed.). *10:1-131*

Weighandt, H. and Baschang, G. (1965). Die Geuing des Zuckeranteiles der Gykosphingolipide durch Ozonolyse and Fragmentierung. *Z. Naturfoschg. 20b:164-166*

Wieruszeski, J.M., Chekkor, A., Bouquelet, S., Mountreuil, G.S, Katalinc, J.P. and Egge, H. (1985). *Carbohydrate Research. 137:127-138.*

Yamshita, K., Tachibana, Y. and Kobata, A. (1976). Oligosaccharids of Human Milk: Isolation and Characterization of Three New Disialylfucosyl hexasaccharides. *Arch. Biochem. Biophys. 174:582-591.*

Zopf, D.A. and Ginsburg, V. (1974). Carbohydrate Antigens of Cell Surfaces. In: *Biology and Chenistry of Eukaryotic Cell Surfaces.* Miami Winter Symposia *Vol 7.* Academic Press, New york, NY.

Zopf, D.A., Ginsburg, A. and Ginsburg, V. (1975). Goat Antibody Directed Against the Human Le-b Blood Group Hapten, Lacto-N-Difucohexose I. *J. of Immunol. 115:*

Zopf, D.A., Tsai, C. and Ginsburg, V. (1979). Antabodies Against Oligosaccharides. In: *Carbohydrate and Protein Interaction. ACS sympsium. 88:90-101.*

Appendix A

PREPARATION AND CHARACTERIZATION OF AFFI-GEL 10-IGG COLUMNS

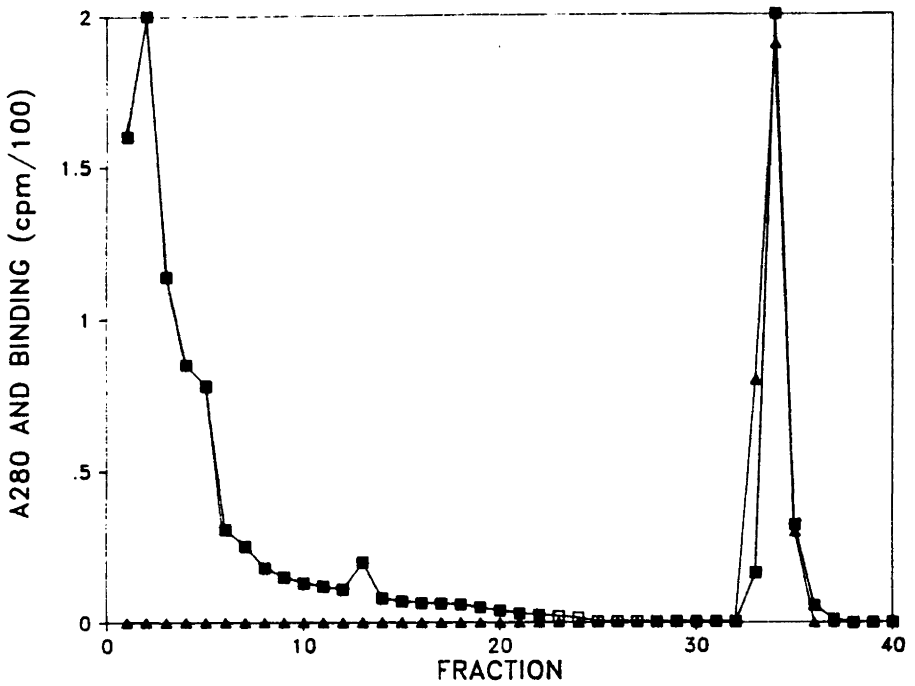
Affi-Gel 10 is an agarose based resin which contains N-hydroxysuccinamide esters. These esters are attacked by free amino groups to produce covalent amide linkages[1]. Affi-Gel 10 (3ml) was washed with 4 volumes of cold distilled water and a 1 mg/ml solution of protein A (1 ml, in 0.1 M MOPS buffer pH. 7.5) was added. The mixture was allowed to incubate overnight under constant rotation at 4°C. After incubation the resulting gel was poured into a column (0.9 x 5 cm) and washed with three column volumes of PBS. The eluate contained no protein as measured by absorbance at 280 nm after neutralization with 0.01 M HCl. The resulting gel (0.25 mg protein A/mg gel) was then treated with 400 µl of 0.1 M ethanolamine pH 8.0 for 1 h at room temperature in order to prevent further reaction of active succinamide groups in the resin. An anti-sialyltetrasaccharide *a* IgG rich fraction was prepared by ammonium sulfate precipitation as described under Experimental Procedures. This IgG rich fraction was applied to an Affi-Gel 10-protein A column as shown in Figure 22 . Non-bound material was immediately eluted from the column in starting buffer (Fig. 22, Peak A), and affinity purified IgG (Fig. 22, Peak B) was eluted with two column volumes of 0.2 M sodium acetate adjusted to pH 2.5 with HCl. Fractions (2.2 ml) were collected and assayed for

[1] *Bio Rad bulletin 1085 (1982)*

absorbance at 280 nm. 180 µl of 1 M Tris-buffer were added to neutralize the fractions and avoid long exposure of the IgG to low pH. Only specifically bound IgG (Fig. 22, Peak B) bound sialyltetrasaccharide *a* in the direct binding nitrocellulose assay.

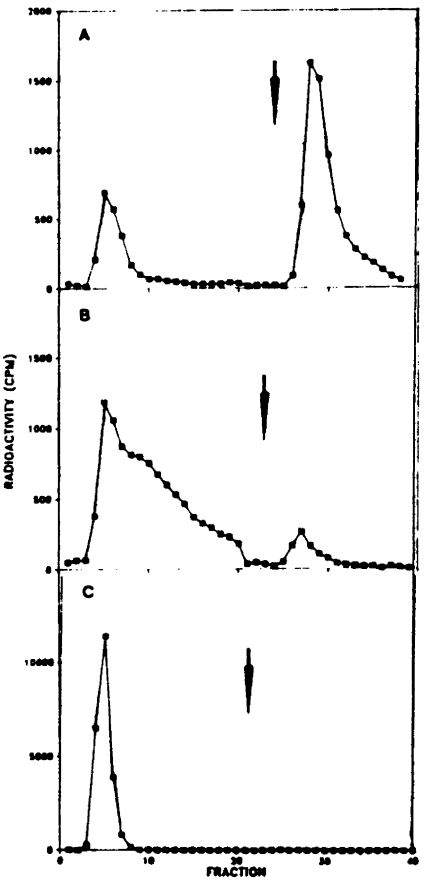
IgG containing fractions were pooled, dialyzed against MOPS buffer (pH 7.5) and concentrated in an ultrafiltration apparatus to 1.1 mg of protein/ml (4 ml). The resulting protein solution was incubated with 4 ml of Affi-Gel 10 , washed and treated with ethanolamine as described above. The Affi-Gel 10-IgG resin was poured into a column and washed with PBS. Reduced sialyl[^{3}H]tetrasaccharides were used to determine the specificity and capacity of the column as shown in Figure 23 As seen in Panel A 72% of 9,600 cpm of sialyl[^{3}H]tetrasaccharide *a* alditol in PBS loaded onto the column were specifically bound and were recovered when the column was eluted with 0.1 M acetic acid (Fig. 23, Panel A, Peak B). Sialyl[^{3}H]tetrasaccharide *b* alditol was slightly retarded (Fig. 23, Panel B) but only 2% was specifically bound. A small amount of sialyltetrasaccharide *a* may be present as a contaminant in the sample used for this experiment since sialyl[^{3}H]tetrasaccharide *b* moves very close to sialyl[^{3}H]tetrasaccharide *a* in paper chromatography and the oligosaccharides used were purified by paper chromatography. Sialyl[^{3}H]tetrasacchride *c* alditol (Fig. IgG, Panel C) was not retarded by the column.

The IgG columns are specific for sialyltetrasaccharide *a* alditol. The main advantage of this method is that it allows repeated use of antibody



Anti-sialyltetrasaccharide *a* serum was subjected to ammonium sulfate precipitaion and dialyzed against PBS as described under Experimental Procedures. The resulting protein fraction (5 ml, 11 mg of protein/ml) was applied to Affi-Gel-protein A chromatography as described under Expeimental Procedures. The column was washed with 15 column volumes of PBS which was then changed to a solution of 0.2 M sodium acetate (pH 2.5 adjusted with HCl) as indicated by the arrow. Fractions (2.2 ml) were assayed for absorbance at 280 nm (Panel A) and aliquots were assayed for binding of sialyl[^{3}H]tetrasaccharide *a* alditol as described under Experimental Procedures (Panel B).

Figure 22: Affinity Purification of IgG Fraction



[^{3}H]-Labeled sialyltetrasaccharide alditols in PBS (200 µl) were applied to the Affi-Gel 10-IgG column described under Experimental Procedures. The column was first eluted with PBS which was then changed to 0.1 M acetic acid as indicated by the arrow. Fractions 1 ml were collected and 200 µl aliquots were assayed for radioactivity. (A) sialyl[^{3}H]tetrasaccharide *a* alditol (9,600 cpm), (B) sialyl[^{3}H]tetrasaccharide *b alditol (9,000 cpm) and* (C) sialyl[^{3}H]tetrasaccharide *c* alditol (12,000 cpm). All oligosaccharides were reduced in the same experiment; therefore, have the same specific activity.

Figure 23: Standarization of Affi-Gel 10-Anti sialyltetrasaccharide a column

which is not possible when direct binding on nitrocellulose filters is used to affinity purify oligossaccharide alditols.

The two page vita has been removed from the scanned document. Page 1 of 2

The two page vita has been removed from the scanned document. Page 2 of 2