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The Role of Glycoconjugates in Mediating Human Fertilization and Induction of Fetomaternal Tolerance

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THE ROLE OF GLYCOCONJUGATES IN MEDIATING HUMAN FERTILIZATION AND INDUCTION OF FETOMATERNAL TOLERANCE

by

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ABSTRACT

THE ROLE OF GLYCOCONJUGATES IN MEDIATING HUMAN SPERM-EGG BINDING AND IN THE INDUCTION OF FETO-MATERNAL TOLERANCE

Manish Suresh Patankar
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Using the hemizona assay (HZA), a *in vitro* sperm-egg binding assay, we show that specific glycoconjugates known to inhibit immune cell interactions mediated by the selectins, potently block human sperm-egg binding. The selectin ligand sialyl Lewis^x inhibits sperm binding in the HZA by 60% at a concentration of 1 mg/ml. Our data indicates that glycodelin-A, a endometrial glycoprotein known to block sperm-egg binding in the HZA at low concentrations expresses unusual fucosylated lacdiNAc type glycans. The fucosylated lacdiNAc type sugars have been previously shown to be 15-20 fold more potent ligands of E-selectin. Glycodelin-S a seminal plasma glycoform of glycodelin-A does not express such unusual glycans and is not contraceptive. These results support our hypothesis that human sperm-egg binding may involve a selectin-like event.

Periodate oxidation under conditions that affect only the terminal sugar residues results in a 30-40% loss in sperm binding. Treatment of the ZP with neuraminidase and endo-β-galactosidase results in a 2.5 and 4 fold enhancement in sperm binding respectively. However, sequential treatment of the ZP with neuraminidase and periodate results in 80% decrease in sperm binding. These studies strongly indicate that ZP glycans are essential for mediating human gamete binding. Furthermore, efficient initial human

sperm egg binding *in vivo* may require a prior activation event involving desialylation of the gametes.

In this study we provide preliminary evidence that human gametes express the bisecting type glycans. Our studies indicate that these glycans potently inhibit natural killer (NK) cells, the predominant cell type expressed in the uterus during pregnancy. Other glycoconjugates like α-fetoprotein, expressed in the uterus during pregnancy, also carry the bisecting-type glycans. Based on these observations we propose that glycoconjugates expressed during pregnancy protect are responsible for mediating feto-maternal tolerance. We refer to this model for as the Human Feto-Embryonic Defense System hypothesis. Finally our data suggests that parasites like schistosomes, filarial worms, and the human immunodeficiency virus may be evading the host's immune responses by a similar by expressing immunosuppressive glycoconjugates on their coats.

This thesis is dedicated to my parents and my wife Sangeeta.

ACKNOWLEDGEMENTS

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I am extremely grateful to Dr. Frank A. Lattanzio for serving as a member on my dissertation committee and for his help in conducting some of the experiments that were absolutely essential for this thesis.

I greatly appreciate all the help that was given to me Drs. Peter F. Blackmore, Karl A. Schellenberg, and Laura K. Moen, who served on my guidance and dissertation committees.

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Finally I would like to thank Sunil and Anuja Mhatre for their support and friendship over the past several years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AGP- α_1-Acid glycoprotein

EBGase-Endo-β-galactosidase

ES-MS- Electron spray-mass spectroscopy

FAB-MS- Fast atom bombardment-mass spectroscopy

FITC-EPHA- Fluorescein isothiocyanate conjugated erythro agglutinating phytohemaglutinnin

FITC-MAA- Fluorescein isothiocyanate conjugated *Maackia amurensis*

Fuc-Fucose

Gal-Galactose

Glc-Glucose

GlcNAc-*N*-Acetylglucosamine

h-Hour(s)

HZA- Hemizona assay

kDa-KiloDaltons

LC-ES-MS- Liquid chromatography-electron spray-mass spectroscopy

min- Minute(s)

NeuAc- Neuraminic acid or Sialic acid

NK- Natural Killer

PAEP- Progesterone associated endometrial protein

PBS- Phosphate buffered saline

PP14- Placental protein 14

SMP- Sodium *m*-periodate

ZP-Zona Pellucida

CHAPTER I

INTRODUCTION

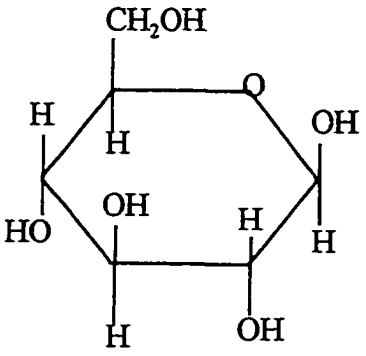
Review of the Literature

Carbohydrates constitute a major component of the diverse biopolymers expressed in both the animal and the plant kingdom. These sugar chains are either found as free oligosaccharides or may be attached to lipid (glycolipids) or protein (glycoproteins) backbones. Such oligosaccharide bearing chimeric molecules are referred to as glycoconjugates. The main purpose of this research is to examine the role of carbohydrates in mediating human fertilization and human pregnancy. A brief review of the chemistry and biochemistry and the possible biological roles of carbohydrates in mammals are provided to acquaint the reader with the various aspects of carbohydrate biology (commonly referred to as glycobiology) and to review the latest developments in this field.

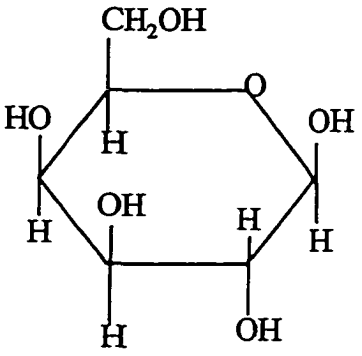
Biosynthesis of Oligosaccharides

The most common type of monosaccharides found in mammals are L-fucose (Fuc), D-glucose (Glc), D-galactose (Gal), D-mannose (Man), D-*N*-acetylglucosamine (GlcNAc), D-*N*-acetylgalactosamine (GalNAc), and *N*-acetylneuraminic acid (NeuAc; also known as sialic acid) (Figure 1). These sugars are linked to each other via *O*-glycosidic bonds to form the oligosaccharides. Synthesis of the oligosaccharide chains of glycolipids or glycoproteins is initiated in the endoplasmic reticulum of the cell and is completed in

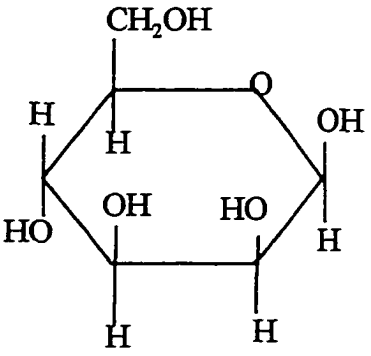
Figure 1: Structures of monosaccharides commonly found in mammalian glycans. The names and abbreviations commonly used for these sugars are given.



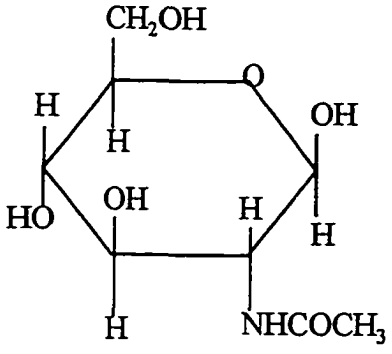
β-D-Glucose (Glc)



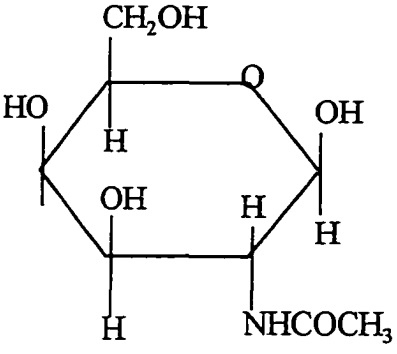
β-D-Galactose (Gal)



β-D-Mannose (Man)



β-D-N-Acetylglucosamine
(GlcNAc)



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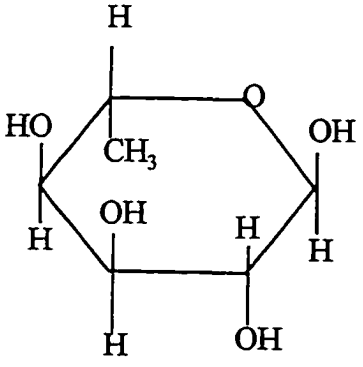
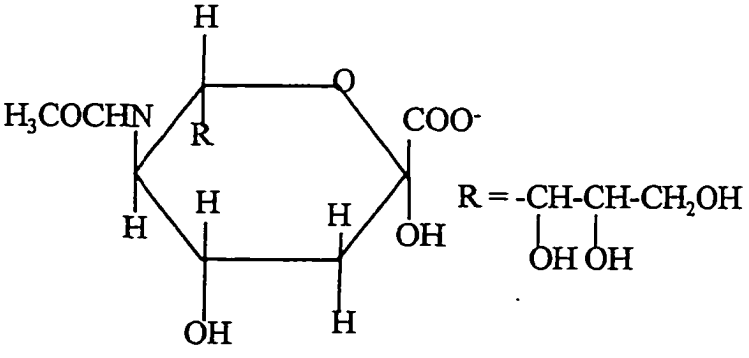
(GalNAc)



α-L-Fucose (Fuc)



N-Acetylneuraminic acid/Sialic acid (NeuAc)

the *trans* compartment of the Golgi apparatus. Two components that are absolutely essential for the synthesis of oligosaccharide chains are the glycosyltransferases and the sugar nucleotides. Glycosyltransferases are enzymes that catalyze the addition of the monosaccharide units to the growing oligosaccharide chain (Paulson and Colley, 1988). For this reaction the glycosyltransferases require sugar nucleotides as the source for the monosaccharide units. Sugar nucleotides carry a monosaccharide attached to the nucleotide via a high energy phosphate bond. The energy required for the addition of the monosaccharide to the oligosaccharide chain is provided by the hydrolysis of this high energy bond. The nucleotide sugars used as substrates by the different types of glycosyltransferases are listed in Table 1.

Many different types of glycosyltransferases have been identified and characterized to date (Paulson and Colley, 1988). The glycosyltransferases are named according to the monosaccharide that they add to the oligosaccharide chain that is being synthesized. Thus enzymes that add Gal to the growing sugar chain are termed as the galactosyltransferases (Gal-T), whereas those adding a GlcNAc residue are *N*-acetylglucosaminyltransferases (GlcNAc-T). Glycosyltransferases are further classified according to the linkage position to which the monosaccharide unit is being added. For example, a fucosyltransferase (Fuc-T) that adds a Fuc residue to the oligosaccharide in the α1-3 position is termed as the α1-3 Fuc-T, whereas the enzyme that adds a Fuc in the α1-2 linkage is referred as the α1-2 Fuc-T. Approximately 100 or more different glycosyltransferases are required for the synthesis of all of the known carbohydrate structures on glycolipids and glycoproteins (Paulson and Colley, 1988). Table 2 shows the commonly found glycosyltransferases that

Table 1: Nucleotide sugars used as donor substrates by different glycosyltransferases.

Glycosyltransferase	Nucleotide Sugar Substrate (Abbreviation)
Glucosyltransferases	Uridine diphosphoglucose (UDP-Glc)
Galactosyltransferases	Uridine diphosphogalactose (UDP-Gal)
Mannosyltransferases	Guanidine diphosphmannose (GDP-Man)
N-Acetyl-glucosaminyltransferases	Uridine diphospho-N-acetylglucosamine (UDP-GlcNAc)
N-Acetyl-galactosaminyltransferases	Uridine diphospho-N-acetylgalactosamine (UDP-GalNAc)
Fucosyltransferases	Guanidine diphosphofucose (GDP-Fuc)
Sialyltransferases	Cytidine monophosphoneuraminic acid (CMP-NeuAC)

Table 2: Some of the glycosyltransferases that are required for the synthesis of terminal sugar residues.

Glycosyltransferase	Sequence Formed
Galactosyltransferase (Gal-T)	
GlcNAcβ1-4-Gal-T	**Galβ1-4**GlcNAc-
Galα1-3-Gal-T	**Galα1-3**Galβ1-4GlcNAc-
Sialyltransferase (ST)	
Galα2-3-ST	**NeuAcα2-3**Galβ1-4GlcNAc
Galα2-6-ST	**NeuAcα2-6**Galβ1-4GlcNAc
Fucosyltransferase (Fuc-T)	
Galα1-2-Fuc-T	**Fucα1-2**Galβ1-4GlcNAc-
GlcNAcα1-3/4-Fuc-T	Galβ1-4(**Fucα1-3**)GlcNAc- Galβ1-3(**Fucα1-4**)GlcNAc-
***N*-Acetylglucosaminyltransferase (GlcNAc-T)**	
GlcNAc-T I	Manα1-6> Manα1-3 >Manα1-6 >Manβ1-4- **GlcNAcβ1-2**Manα1-3
GlcNAc-T II	**GlcNAcβ1-2**Manα1-6 GlcNAcβ1-2Manα1-3 >Manβ1-4-
GlcNAc-T III	GlcNAcβ1-2Manα1-6 **GlcNAcβ1-4** —Manβ1-4- GlcNAcβ1-2Manα1-3
***N*-Acetylgalactosaminyltransferase (GlcNAc-T)**	
Galα1-3GalNAc-T	Fucα1-2(**GalNAcα1-3**)Gal-
GlcNAcβ1-4GalNAc-T	**GalNAcβ1-4**GlcNAc-

are required for the synthesis of the terminal sugars. The sequence specificities of the glycosyltransferases are also shown.

Different Types of Mammalian Glycoconjugates

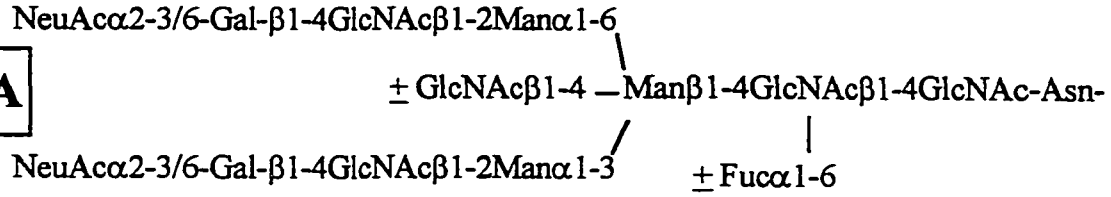
(a) Glycoproteins

Oligosaccharides conjugated to the protein backbone are of two major types, the *N*-linked and the *O*-linked oligosaccharides (Kornfeld and Kornfeld, 1985; Olofsson, 1991) . The *N*-linked oligosaccharides are attached to the protein via an asparagine (Asn) residue whereas the *O*-linked chains are conjugated via serine (Ser) or threonine (Thr) residues of the protein. The Asn residue of a protein flanked by an -X-Ser/Thr (where X is any amino acid) sequence is usually a good consensus site for *N*-glycosylation (Kornfeld and Kornfeld, 1985). However, not all Asn residues in this Asn-X-Ser/Thr motif are *N*-glycosylated. No consensus sequence has been shown to exist for the *O*-glycosylation sites. The typical structures of the *N*- and *O*-linked oligosaccharides are shown in Figure 2.

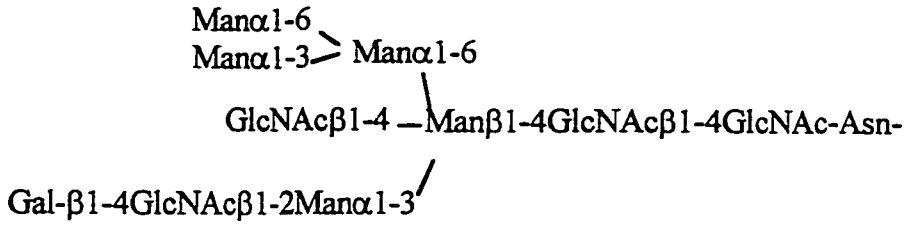
Biosynthesis of the *O*-linked chain is initiated by the addition of a GalNAc to the Ser or Thr residue of the protein (Olofsson, 1991). This reaction is followed by the sequential addition of the other monosaccharide units by the appropriate glycosyltransferases. Recently however, an unusual modification of some proteins has been reported where Ser or Thr residues are modified by a single GlcNAc. The *O*-linked carbohydrate chains may range in size from one to more than 20 sugars with the larger oligosaccharides displaying considerable structural and antigenic diversity (Olofsson, 1991). Typically the *O*-linked oligosaccharides are expressed in clusters of heavily

Figure 2: Structures of some of the commonly found *N*-linked and *O*-linked glycans in mammals. Structures shown in Panel A-C are complex type, hybrid type, and high mannose type *N*-linked sugars. Panel D and E show structures of *O*-linked glycans found on erythrocyte surface glycoproteins.

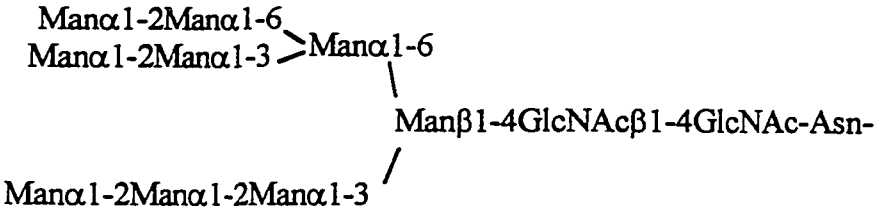
A



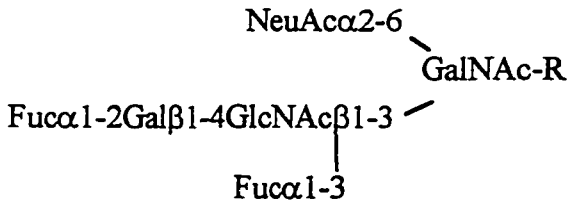
B



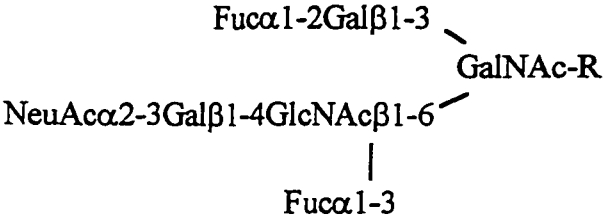
C



D



E



glycosylated domains of the protein. Mucins are a classic example of proteins carrying heavily *O*-glycosylated domains. Two *O*-linked oligosaccharides expressed on human erythrocyte membrane glycoproteins are shown in Figure 2.

N-linked oligosaccharides are broadly classified into the complex type, hybrid, and high mannose type structures (Figure 2). The basic core of *N*-linked oligosaccharide is initially synthesized on a lipid named dolichol (Kornfeld and Kornfeld, 1985). The core structure is then transferred *en block* to the Asn residue of the protein by a specific oligosaccharidyl transferase enzyme. The core structure is further modified by addition and deletion of specific monosaccharide units in the endoplasmic reticulum and Golgi apparatus, to produce the final structures shown in Figure 2.

(b) Glycolipids:

Glycolipids are synthesized by the sequential addition of the sugars to a lipid core. Glycolipids are typically found in animal cells have a sphingosine backbone. These glycolipids are referred to as the glycosphingolipids (GSL) (Basu and Basu, 1982). The GSL are widely distributed in animal tissues, particularly the synaptic membranes and cell surfaces. Most commonly occurring GSL's can be classified into two different families (a) the acidic or NeuAc containing GSL's also known as gangliosides, and (b) the neutral GSL's containing mono-, di-, or oligoglycosyl chains attached to the ceramide backbone. Gangliosides may contain one or more NeuAc residues. The neutral polyglycosyl GSL's are further subdivided into two classes, (a) the blood group active GSL's which contain a core structure of GlcNAcβ1-3Galβ1-4Glcβ1-1Ceramide, and (b) the globosides whose core structure is Galα1-4Galβ1-4Glcβ1-1Ceramide (Table 3). Many of the

Table 3: Structures of different mammalian glycolipid glycans.

STRUCTURE	NAME
Gangliosides	
Glcβ1-1Cer	Glucosylceramide
Galβ1-4Glcβ1-1Cer	Lactosylceramide
NeuAcα2-3Galβ1-4Glcβ1-1Cer	GM3
GalNAcβ1-4Galβ1-4Glcβ1-1Cer NeuAcα2-3	GM2
Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer NeuAcα2-3	GM1
GalNAcβ1-3 NeuAcα2-8Galβ1-4Glcβ1-1Cer NeuAcα2-3	GD2
Blood Group Active	
Fucα1-2(Galβ1-4GlcNAcβ1-3)$_n$Galβ1-4Glc-β1-1Cer	Blood Group H_I (n=1) H_{II} (n=2)
Galα1-3 Fucα1-2(Galβ1-4GlcNAcβ1-3)$_n$Galβ1-4Glc-β1-1Cer	Blood Group B_I (n=1) B_{II} (n=2)
GalNAcα1-3 Fucα1-2(Galβ1-4GlcNAcβ1-3)$_n$Galβ1-4Glc-β1-1Cer	Blood Group A_I (n=1) A_{II} (n=2)
Globosides	
Galα1-4Galβ1-4Glcβ1-1Cer	$GbOse_3Cer$
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	$GbOse_4Cer$
GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	$GbOse_5Cer$/ Forssman

glycosyltransferases that are involved in the synthesis of glycoprotein glycans are also used for GSL oligosaccharide synthesis.

Glycoforms

The final products of glycosylation linked to a single protein are very diverse. Studies indicate that a significant number of the molecules of a glycoprotein may not have the fully formed oligosaccharide chain. For example porcine fibrinogen carries two different forms of oligosaccharides (Figure 3) (Da Silva et al., 1994). A single glycoprotein is homogenous in its protein component but usually heterogeneous in its carbohydrate sequences. A subset of glycoprotein molecules that are homogeneous in both their protein and carbohydrate sequences is referred to as a glycoform. Therefore a single glycoprotein generally consists of a mixture of different glycoforms (Rademacher et al., 1988).

Biological Roles for Oligosaccharides

In recent times, considerable effort has been devoted to understanding the chemistry and biochemistry of glycoconjugates. However, determining the exact physiological roles of these molecules has remained enigmatic. Carbohydrates have been conventionally known to affect the three dimensional structure of proteins (Varki, 1993). Therefore glycans have been postulated to play a major role in determining the folding patterns of proteins. It has been reported recently that simple occupation of key glycosylation sites may be the critical factor regulating protein folding in certain cases (Helenius, 1994; Allen et al., 1995; Feng et al., 1995). For example, occupancy of crucial N-linked glycosylation sites by a single monosaccharide (GlcNAc-Asn) is sufficient to allow proper folding of rat leutenizing hormone receptor to occur (Zhang et al., 1995).

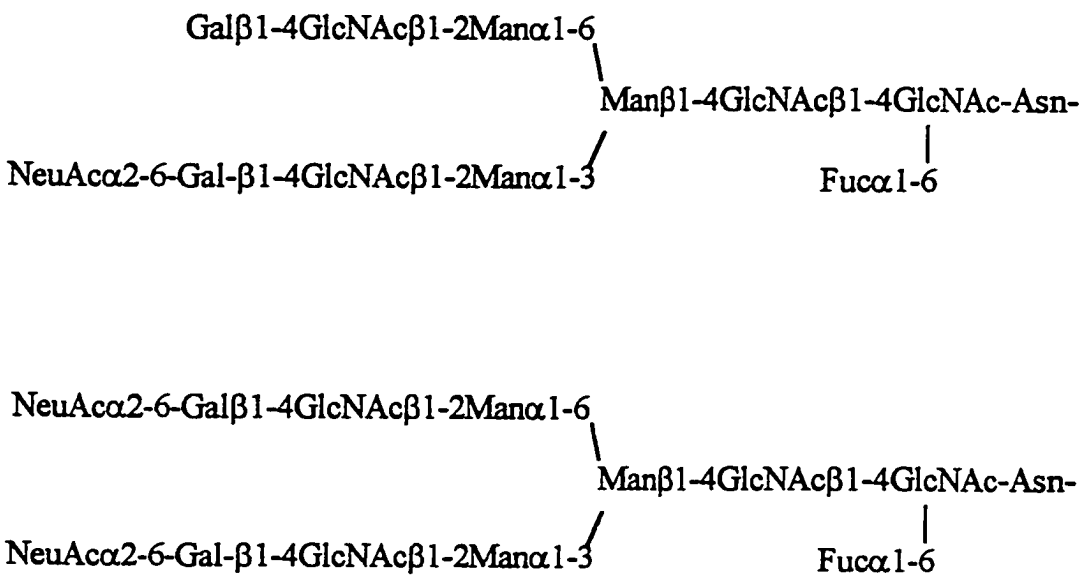


Figure 3: Two different glycans found on porcine fibrinogen.

Another proposed role for carbohydrates is that, being hydrophilic molecules, they help solubilize the proteins better in the intercellular and intracellular milieu (Varki, 1993). While this proposed function may be a valid biological role for the carbohydrates, it is difficult to understand the expression of structural diversity of the oligosaccharides in this context. For example, if the sole purpose of expressing carbohydrates on proteins or lipids was to increase the solubility of these molecules, it would have been more beneficial for the cell to express only a few particular kinds of carbohydrate structures that could help solubilization of the molecules. However, oligosaccharide structures reported so far in the literature are extremely diverse. Furthermore, there have also been reports of the oligosaccharides being modified by acetyl, glycolyl, sulfate, or phosphate groups (Varki, 1992; Kornfeld et al., 1982). Thus each individual cell expends considerable effort in the synthesis of the oligosaccharide structures, sugar nucleotides, and various enzymes like glycosyltransferases and glycosidases involved in the synthesis and the degradation of carbohydrates. It is in fact believed that an individual mammalian cell spends as much as 30 % of its total energy reserve on the synthesis of the oligosaccharides chains.

Few examples are available in the literature that conclusively demonstrate the importance of carbohydrates in mediating any cellular event. Several studies implicating the lack of oligosaccharide production or specific defects in the oligosaccharide processing enzymes in various disease states have been reported. Examples of two such diseases are discussed below to illustrate this point.

Carbohydrate-deficient glycoprotein syndrome (CDGS) is a family of multisystemic congenital diseases which are caused due to the underglycosylation of glycoproteins. The exact biochemical defect responsible for causing this disease has not been pinpointed.

However, recent data suggests an abnormality in one of the steps of glycan assembly on the protein (Charuk et al., 1993). Interestingly, one of the variants of CDGS (designated CDGS II) has been linked to the deficiency of the GlcNAc-T II (Table 2), an enzyme required for the synthesis of *N*-linked oligosaccharides. Individuals suffering from CDGS exhibit severe neurological and physical defects (Charuk, et al., 1993).

Tay-Sachs disease is another prominent example of the link between a deficiency of a proper oligosaccharide processing enzyme and a disease state. Tay-Sachs disease is an autosomal recessive disorder caused by inadequate activity of the enzyme β-N-acetylhexosaminidase, used for degrading gangliosides in the brain. Gangliosides are found in the highest concentration in the nervous system and are continually degraded by the action of glycosidases, like β-N-acetylhexosaminidase (Mahuran, 1995). Deficiency of this enzyme results in the accumulation of the gangliosides in the brain of the infant suffering from Tay-Sachs disease, resulting in severely retarded psychomotor development, impaired vision and spasticity. Tay-Sachs disease is fatal usually before the infant is three years of age.

However, such physiological disorders associated with either the lack of or incorrect expression or degradation of the oligosaccharide sequences do not provide evidence for the functional role for carbohydrates in cellular events. It can still be argued that the reason for these disorders is due to incorrect folding of the proteins that bear the aberrant oligosaccharides thereby inducing them to be inactive or to mediate abnormal functions.

The Selectins

In recent years more evidence has been presented suggesting a direct physiological role for oligosaccharides in mediating many cellular events. Recruitment of the leukocytes to the sites of inflammation has to occur under the influence of extremely high shear forces generated by the high bulk rate of flow of the blood cells in the postcapillary venules (Lasky, 1995). However, such high shear forces do not prevent the tight binding of leukocytes to the inflamed endothelium. The tight binding of these inflammatory cells has been shown to be mediated by the cell adhesion molecules known as integrins. The slow rolling of leukocytes on the surface of the inflamed endothelium occurs due to binding of three specific carbohydrate binding proteins expressed on the surface of the leukocytes and endothelial cells, with their respective ligands. These three receptors, E-, L-, and P-selectins have been identified and very well characterized (Lasky, 1995). E-selectin has been found to be primarily expressed on the inflamed endothelium. P-selectin is expressed in the α- granules of the platelets and the Wiebel-Palade bodies of the endothelial cells. This selectin is rapidly translocated to the cell surface during inflammation. L-Selectin is expressed on various leukocytes.

All three selectins carry a C-type lectin domain that can bind to a specific carbohydrate ligand in the presence of calcium (Lasky, 1995). The expression of the three selectins is also differentially regulated. L-Selectin is constitutively expressed on the surface of the myeloid leukocytes and is rapidly shed by proteolytic cleavage after leukocyte activation where it helps in mediating adhesion to high endothelial venules of peripheral lymph nodes and to cytokine activated endothelial cells (Lasky, 1995). E-selectin is synthesized by endothelial cells after stimulation with cytokines. Expression of E-selectin

peaks 4 hours after stimulation and returns to basal levels within 12-24 hours. P-selectin is rapidly translocated to the cell surface upon activation by agonists such as thrombin or histamine. At the cell surface P-selectin serves as a receptor for the myeloid cells and subsets of lymphocytes.

Considerable effort has been made to identify the exact ligand recognized by the three selectins (Varki, 1994). Sialyl Lewis^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc), a tetrasaccharide expressed on many glycoproteins and glycolipids has been shown to be a ligand for the three selectins. Evidence supporting the role of sialyl Lewis^x in mediating adhesion include:

(1) Antibodies directed against sialyl Lewis^x blocked cell adhesion mediated by both E- and P-selectins (Walz, et al., 1990; Phillips, et al., 1990; Polley, et al., 1991),

(2) Liposomes containing glycolipids carrying the sialyl Lewis^x epitope blocked leukocyte adhesions to the inflamed endothelium (Lasky, 1995)

(3) All three selectins bind with differing affinities to immobilized glycolipids bearing the sialyl Lewisx terminated glycans (Foxall, et al., 1992)

Substantial evidence suggests that sialyl Lewis^x is a low affinity ligand for these selectin molecules (Varki, 1994). Oligosaccharides bearing the sulfo-Lewis^x (SO_3^--3Galβ1-4[Fucα1-3]GlcNAc-) and sulfo-Lewis^a (SO_3^--3Galβ1-3[Fucα1-4]GlcNAc-) sequences on their terminal ends also serve as ligands for the selectins. Other high affinity ligands have since been identified. These high affinity ligands carry clusters of highly sialylated or sulfated oligosaccharides which are responsible for serving as ligands for the selectins. In addition to the presence of C-type lectin domain all of the three selectins also express a

epidermal growth factor-like domain (Lasky, 1995). It is therefore likely that recognition of the oligosaccharide ligand by the selectins could trigger a signal transduction cascade through the epidermal growth factor-like domain. Such signaling events could in turn allow tight binding of the lymphocytes to the inflamed endothelium via other receptors such as the integrins.

The selectins have also proved to be excellent candidates for devising strategies for the prevention of reperfusion injuries most commonly seen in patients with ischemia (McEver, 1995). Efforts are under way to use sialyl Lewis^x as a therapeutic agent for blocking the recruitment of the leukocytes to the ischemic tissue thereby preventing these injuries.

Other Mammalian Lectins

In addition to the selectins, a growing number of studies have indicated the presence of other mammalian lectins on the surfaces of various immune effector cells and also on neural cells (Varki, 1992). Recently, many different C-type and I-type lectins have also been shown to be expressed on different immune cell types. I-Type lectins are specific for sialic acids (Powell and Varki, 1995). CD22 is an example of an I-type lectin that is expressed on the naive B-cells. Absence of CD22 on the naive B-cells results in hyperactivation of the cells, suggesting that this molecule could play an important role in the maturation process of the B-cells (O'Keefe, 1997). Recent reports suggest that the ligand for CD22 is terminal NeuAcα2-6Galβ1-4GlcNAc. Similarly, many C-type lectins like CD94, and NKR-P1A have been shown to be expressed on natural killer cells and various other T-cell subsets (Moretta et al., 1997).

The exact physiological roles of many of these mammalian lectin receptors are currently under intense scrutiny. However, the diversity of the different oligosaccharide chains expressed by the mammalian cells and the presence of specific carbohydrate binding proteins strongly suggests that carbohydrates may be playing significant roles in various physiological events. Research efforts currently undertaken by several groups in various aspects of mammalian glycobiology will definitely help to determine the exact roles of the glycoconjugates.

Role of Carbohydrates in Reproduction

Another critical biological process that has been proposed to be mediated via glycoconjugates is the initial binding of the male and female gametes (Bedford, 1977). Studies performed in many different species throughout the animal kingdom have indicated that specific carbohydrates expressed on the surface of the egg are recognized as ligands for specific egg binding protein(s) expressed on the plasma membrane of the sperm. Considerable evidence obtained by investigators using the murine model system also strongly suggests the involvement of the carbohydrates in mediating mammalian sperm-egg interaction (Wassarman, 1990). Mammalian eggs are surrounded by an extracellular matrix referred to as the zona pellucida (ZP). During fertilization, sperm initially bind the ZP before penetrating into the ooplasm. In the mouse, the ZP is primarily composed of three glycoproteins, ZP1, ZP2 and ZP3 (Wassarman, 1988). Wassarman and co-workers have demonstrated that carbohydrate chains expressed on ZP3 play a major role in mediating murine sperm-ZP binding (Wassarman et al., 1986). Initial studies indicate that human sperm-ZP binding is also mediated by the ZP glycans (Wassarman, 1990). However, conclusive proof of the involvement of glycoconjugates in mediating human gamete binding

has been lacking until recently. Initial studies performed by our group strongly suggested that specific carbohydrate ligands could potently inhibit human sperm-egg binding (Mahony et al., 1991; Oehninger et al., 1991; Patankar et al., 1993). These studies, reviewed in depth in the introduction section of Chapter II, further indicate that the carbohydrate ligands responsible for mediating human sperm-egg binding may also overlap with the same ligands mediating human immune cell interactions (Patankar, et al., 1993). The purpose of the current study was therefore to further investigate the validity of these initial observations made in our laboratory.

Specific Aims of this Study

(1) To conclusively determine if human sperm-egg binding is a carbohydrate mediated event.

(2) To demonstrate the expression of carbohydrate sequences that could possibly play a role in mediating immune cell interactions on the surface of the human gametes.

(3) To demonstrate that carbohydrate sequences expressed on the surface of the gametes could affect immune cell function.

CHAPTER II

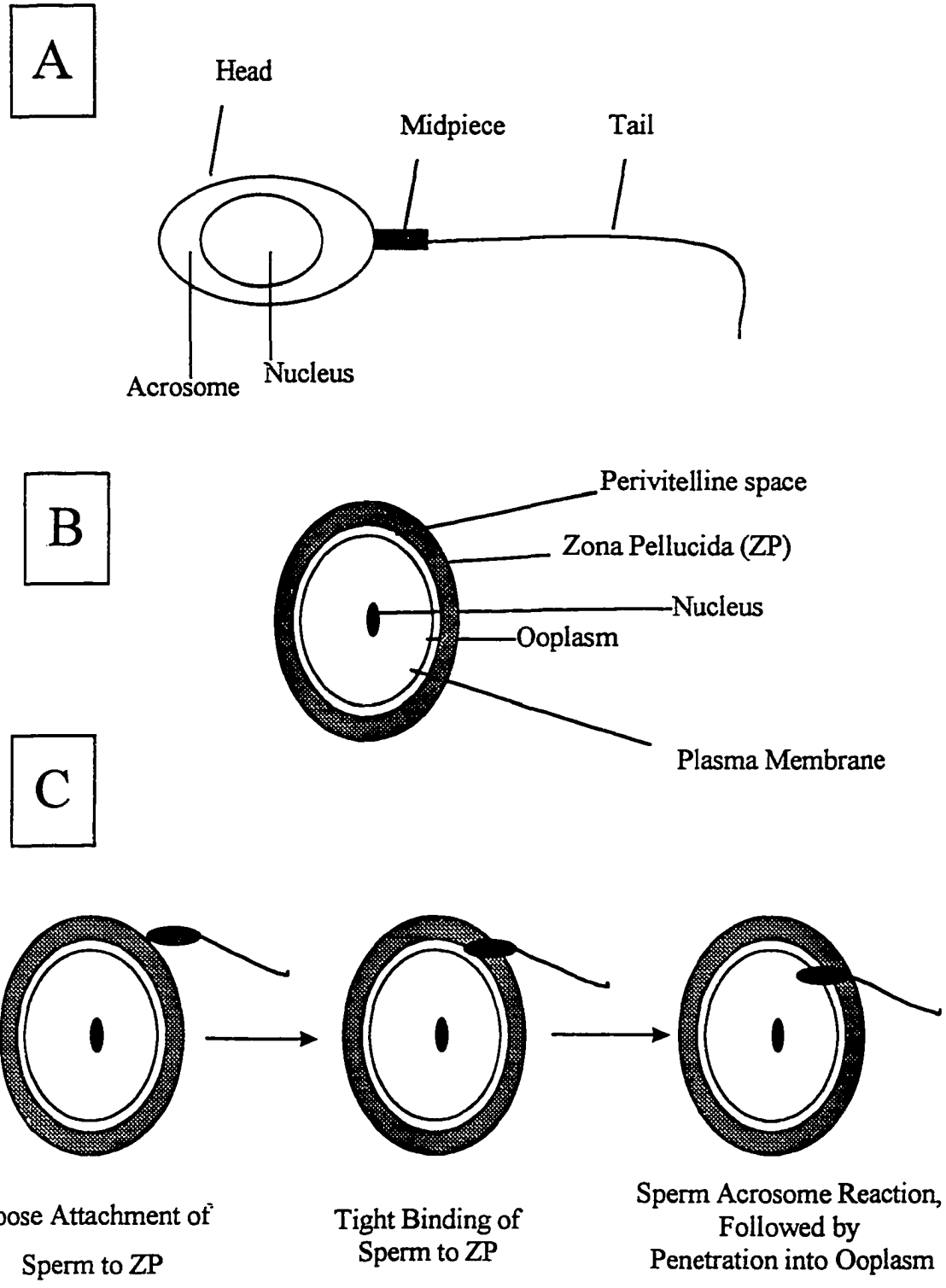
INTRODUCTION

Fertilization of the female gamete is the first committed step in reproduction. Mammalian fertilization is a multi-step process initiated by the binding of sperm to the egg. This initial binding event results in fusion and subsequent transmission of genetic material from parents to the offspring. Research performed using the mouse as the animal model system has been helpful in providing considerable insights in the initial events leading to fertilization. The understanding gained from these experiments and their impact on the human gamete binding system is reviewed here.

Events Leading to Fertilization in Mice

The mammalian egg is surrounded by an extracellular matrix known as the zona pellucida (ZP) (Figure 4). The sperm initially undergo loose attachment to the ZP. This initial loose attachment is followed by a tight binding of the sperm to ZP. Tight adhesion of the gametes is supposed to be mediated by the binding to the receptor expressed on the surface of the sperm head to the ligands expressed on the ZP (Wassarman, 1990) (Figure 4). Lying beneath the surface of the sperm is an organelle referred to as the acrosome (Figure 4). The acrosome is a lysosome-like body which contains proteases and glycosidases. The tight binding of the sperm to the ZP results in fusion of the plasma membrane with the outer acrosomal membrane, blebbing off of the fused membrane and exposure of the inner acrosomal membrane (McLeskey et al., 1998). This process of fusion

Figure 4: Mammalian gametes and major events occurring during fertilization. Panels A and B show the important structural features of the sperm and the egg respectively. The initial loose binding followed by tight adhesion and the penetration of the sperm into the egg is shown in Panel B.



of the acrosomal membrane followed by the release of the acrosomal contents is known as the acrosomal reaction. Proteases released in this step help the sperm to penetrate through the ZP and reach the perivitelline space present between the ZP and the egg (Figure 4). Once the sperm reach the perivitelline space, the plasma membranes of the gametes fuse together to form the zygote.

Initial Sperm-ZP Binding

Considerable advances have been made in understanding the initial binding of mouse sperm to the ZP. As discussed earlier the tight binding of the sperm to the ZP is absolutely essential for the future events of fertilization to occur. A similarity between the binding of leukocytes of inflamed endothelium and sperm-ZP binding is the presence of high shear forces. Murine ZP3 has been isolated and purified from oocytes. Electrophoretic studies indicate that this glycoprotein migrates as a diffuse band with an apparent molecular weight of approximately 83 (kDa) (Wassarman, 1988). ZP3 has also been shown to be sulfated and migrates on isoelectric focusing gels with an average pI of 4.7 (Shimizu et al., 1983). Amino acid analysis of the ZP3 polypeptide chain indicates that it is composed of 402 amino acids with a calculated molecular weight of 44 kDa (Wassarman, 1988). ZP3 expresses 3-4 *N*-linked oligosaccharide chains and an undetermined number of *O*-linked oligosaccharide chains (Salzmann et al., 1983). It has been accepted that the heterogeneous nature of ZP3 observed during electrophoretic analysis of this glycoprotein is primarily due to the posttranslational modification of this protein and not because of any heterogeneous nature of the polypeptide chain.

Several lines of evidence have been presented suggesting that ZP3 mediates binding of sperm to the ZP:

(1) Initially it was observed that ZP3 purified form mouse oocytes bound specifically to the sperm head and not the midpiece or the tail (Bleil and Wassarman, 1986). This finding is consistent with the fact that only the head of the sperm is capable of undergoing tight binding with the ZP

(2) Purified murine ZP3 does not bind to acrosome reacted murine sperm (Bleil and Wassarman, 1986). This observation complements the data that the acrosome reaction of the sperm is preceded by the tight binding of the gametes

(3) Purified ZP3 inhibits murine sperm-ZP binding at relatively low concentrations (Florman and Wassarman, 1985) (Bleil and Wassarman, 1980) (Florman et al., 1984)

(4) Perhaps the most conclusive data demonstrating ZP3 as the primary receptor for the sperm was obtained when it was shown that acrosome intact mouse sperm tightly bound to silica beads coated with murine ZP3 (Vazquez et al., 1989)

(5) ZP3 was also shown to be capable of inducing acrosome reaction in murine sperm, albeit at high concentrations (Bleil and Wassarman, 1983) (Florman, Bechtol et al., 1984)

(6) ZP3 purified from mouse embryos did not bind to the heads of the mouse sperm (Bleil and Wassarman, 1986). This observation is consistent with the fact that mouse sperm do not undergo any attachment with the embryo. Thus considerable evidence has been accumulated so far suggesting that at least in the murine model, sperm bind to the ZP via ZP3.

ZP3 Oligosaccharides Mediate Sperm Binding

As mentioned earlier, murine ZP3 has been shown to be heavily glycosylated. It was initially reported that exposure of ZP3 to high temperatures (100°C), or to denaturants like urea and detergents like sodium dodecyl sulfate (SDS) did not significantly affect its ability to serve as a sperm receptor (Bliel and Wassarman, 1980, Wassarman, 1985; Wassarman, 1989). Exposure to such harsh conditions usually results in denaturation of proteins, in turn resulting in the loss of their biological activities. Carbohydrates have however been shown to be very resistant to such denaturing conditions. Therefore these initial observations indicated that the oligosaccharide chains expressed on ZP3 could be playing a role in mediating sperm-ZP binding. This suggestion perfectly complemented prior observations that various monosaccharides, glycoconjugates, and lectins were capable of abrogating the binding of the sperm to the ZP in the murine system *in vitro* (Huang et al., 1982; Shur and Hall, 1982) (Oikawa et al., 1973) (Ahuja, 1982; Ahuja, 1985) (Shur and Hall, 1982) (Lambert, 1984). It was further observed that some of the ZP3 glycopeptides obtained upon digestion of the glycoprotein with pronase retained the ability to serve as sperm receptor, although they were unable to induce acrosome reaction in the sperm (Florman, Bechtol et al., 1984). More recently it was shown that the *O*-linked and not the *N*-linked ZP3 oligosaccharides were absolutely essential for this molecule to serve as the mouse sperm receptor (Litscher and Wassarman, 1996).

Further studies performed by Wassarman and co-workers indicated that α-linked galactose at the nonreducing terminal of a subpopulation of the *O*-linked ZP3 oligosaccharides was absolutely essential for murine sperm-ZP binding (Wassarman, 1990). Removal of this α-galactose by the enzyme α-galactosidase or by oxidation of the C-6

alcohol of this sugar to an aldehyde by galactose oxidase resulted in complete abrogation of the sperm-receptor function of this oligosaccharide (Bleil and Wassarman, 1988). Recently it has been shown that a synthetic oligosaccharide bearing α1-3 linked galactose terminals could effectively block binding of the murine sperm to the ZP (Litscher et al., 1995). Thus there is considerable evidence to suggest that the oligosaccharides expressed on the murine ZP3 are responsible for mediating sperm-ZP binding.

ZP3 is Found in All Mammalian Species

Studies performed to date have indicated that all mammalian species carry a homolog of murine ZP3. ZP3 from many different species including the human have been cloned and expressed in different cell types. The presence of ZP3 in all the mammalian species indicates that the role of this molecule, i.e. in mediating sperm-ZP binding may have been conserved in all of these species.

Human ZP3

As mentioned above, the human homolog of murine ZP3 has been cloned and isolated. Based on the evidence obtained in the mouse system, it has been suggested that the human ZP3 also may be playing a major role in mediating binding of sperm to the ZP. However, the lack of significant number of human eggs has severely curtailed the ability to isolate human ZP3 and to directly assess its potential as a sperm receptor as performed in the mouse studies. To circumvent this problem, many researchers have attempted to obtain recombinant human ZP3 (Chapman and Barratt, 1996). However, to date, a totally biologically active ZP3 molecule has not been produced. It has been shown that glycoproteins obtained using recombinant DNA technologies do not express the same

biological activities, or at least not with the same potency. For example appropriate expression of the glycan chains is absolutely essential for producing biologically active glycohormones like leutenizing hormones (Thotakura and Blithe, 1995).

Different cell types probably follow a specific program for glycosylation. The differences in the glycosylation patterns within cell types is apparent considering the various types of glycosyltransferases and the cell surface carbohydrates expressed by these cells. For example, the Chinese hamster ovary cell line that is often used to obtain recombinant proteins predominantly expresses high mannose type oligosaccharides on its cell surface. Therefore the proteins being transfected in this cell line often also end up expressing high mannose type oligosaccharide structures. Thus if the expression of specific type(s) of oligosaccharide(s) is required, it is very likely that the recombinant protein would not exhibit the correct biological activity unless it was transfected in the right cell type. A similar situation may be occurring with human recombinant ZP3. Human ZP3 is expressed by the egg cells and therefore may probably have to be expressed in a very specific cell line to obtain biologically active recombinant human ZP3. The inability to obtain a biologically active recombinant ZP3 molecule indirectly suggests that its oligosaccharides may be playing an important role in mediating sperm receptor function.

Using the Hemizona Assay to Determine the Role of Oligosaccharides in Human Sperm-ZP Binding

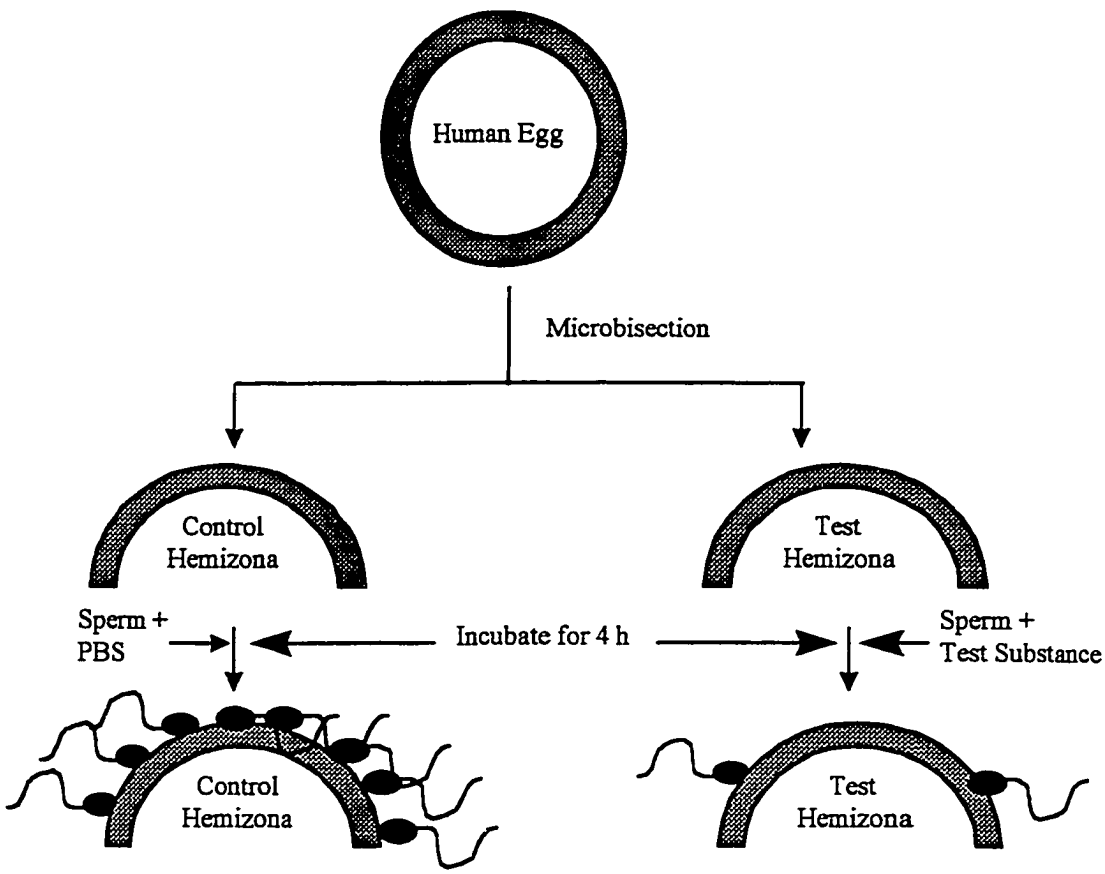
The inability to directly assess the role of the ZP3 oligosaccharides in mediating human sperm-ZP binding due to reasons specified above have therefore forced investigators to search for other alternative strategies to study this interaction at the molecular level. However, even for such indirect methods, the main challenge has been the

availability of human eggs in significant numbers. Furthermore, incubation of live human eggs with sperm could potentially result in the formation of human embryos, thus leading to severe ethical dilemmas. To circumvent these problems, the hemizona assay was developed (Burkman et al., 1988). The initial use of the hemizona assay was restricted to assessing sperm dysfunction in male patients (Burkman, Coddington et al., 1988). The general paradigm for conducting this assay is provided in Figure 5. In the first step, human eggs are microbisected in to two equal halves. The halves are aspirated vigorously to expel the cellular egg contents, leaving behind the two equal halves of the ZP known as hemizona. Of these two hemizonae, one is used as a control and the other as the test (Figure 5). In the clinical application, the control hemizona is incubated with sperm from normal known fertile donor whereas the test hemizona is exposed to sperm obtained from the patient. The incubation is carried out for 4 hours in an 5% CO_2 incubator. The individual hemizona are then washed thoroughly in media to remove any loosely bound sperm. The sperm that are tightly bound to each hemizona are counted. Comparison of the number of sperm bound to the test versus the control hemizona is done to assess the dysfunction of the sperm from the patient. The comparison is done by calculating the hemizona index (HZI) as follows-

HZI= (Number of sperm Bound to the test Hemizona)/(Number of sperm bound to control hemizona) X 100.

An HZI of 100 indicates no difference in the binding capacity of the sperm from the patient. Complete sperm dysfunction is reflected if the HZI is 0.

Figure 5: Schematic diagram of the modified hemizona assay (HZA) to test the effect of different substances on human sperm-ZP binding. The human eggs are initially bisected and the zona pellucida is isolated from the ooplasm. The test hemizona is incubated with the sperm in the presence of the test substance and the control is incubated with sperm in the presence of PBS (or any other suitable placebo substance). The number of sperm bound to the hemizona after 4 h incubation are counted and the hemizona index (HZI) is calculated as shown.



$$HZI = \frac{\text{Number of Sperm Bound to Test Hemizona}}{\text{Number of Sperm Bound to Control Hemizona}} \times 100$$

The main advantage of the HZA is that it provides an internally controlled test as the matched hemizonae are derived by microbisection of the same egg (Burkman, Coddington et al., 1988). The use of the same egg as the control and test also is beneficial in reducing the number of eggs required to do such studies. Considering these advantages, the HZA was modified to perform various studies to assess the role of different chemical and biochemical agents to affect human sperm-ZP binding (Oehninger et al., 1990). The test hemizona was incubated with sperm from healthy proven fertile donor (defined as a donor who has fathered a child in the past two years), in the presence of the test substance and the control hemizona was incubated with the sperm from the same donor in the absence of the test substance (Figure 5). The HZI is calculated by counting the number of sperm bound to the test versus the control. The HZI indicates if the test substance had any effect on human sperm-ZP binding as discussed above. The inhibitory effect of the test substance in the HZI is calculated by the formula-% inhibition in the HZA = (100 - HZI).

A number of different substances were initially screened in the HZA for their effect on sperm-ZP binding (Oehninger, et al., 1990). It was hypothesized that the test substance that could potently inhibit sperm binding in the HZA would at least be mimicking the natural ligands expressed on the surface of the zona. The identification of such a substance could therefore provide valuable insights about the molecular mechanisms involved in mediating this adhesive event.

Fucoidan, a Potent Inhibitor of Sperm Binding in the HZA

Studies on mouse sperm-ZP binding had already indicated that the oligosaccharide chains attached to the ZP could be playing an important role in this event. Therefore,

initially Oehninger and co-workers, tested different glycoconjugates for their ability to inhibit in the HZA (Mahony, et al., 1991; Oehninger, et al., 1991). Of all the glycoconjugates tested at that time, fucoidan, an algal polysaccharide primarily composed of Fuc and sulfate, was found to be an extremely potent inhibitor of human sperm-ZP binding in the HZA (Mahony, et al., 1991). Incubation of the test hemizona with the sperm in the presence of 50 μg/ml of fucoidan resulted in 80% inhibition of binding as compared to the matched control. Other sulfated polysaccharides like dextran sulfate, chondroitin sulfate did not have any effect on this interaction in the HZA (Oehninger, et al., 1991). Furthermore it was found that neither Fuc nor sodium sulfate had any major effect on the binding (Oehninger, et al., 1991). These control experiments therefore indicated that fucoidan was specifically able to block sperm-ZP binding in the HZA.

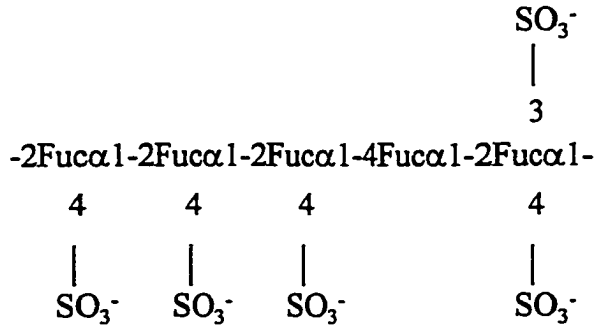
Previously, Huang et al., (1982) had reported that fucoidan could block sperm-ZP binding in humans. They also reported that fucoidan was also a potent inhibitor of sperm-ZP binding in other mammalian species (Huang and Yanagimachi, 1984). For example fucoidan could block the sperm binding in the mouse and the guinea pig system. However, the action of fucoidan in these lower mammalian species was significantly different than that observed in humans. In the lower mammals when sperm binding assays were conducted in the presence of fucoidan, the sperm could undergo initial tight binding to the ZP but would then eventually fall off (Huang and Yanagimachi, 1984). In humans however, short term incubation (0.25-0.5 h) of fucoidan potently inhibited the initial binding interaction (Huang et al., 1982; Clark and Oehninger, unpublished observations). Thus in the lower mammalian species, fucoidan inhibited the secondary binding interaction whereas, in humans the initial or primary binding was inhibited by this algal polysaccharide.

Reevaluation of the Structure of Fucoidan

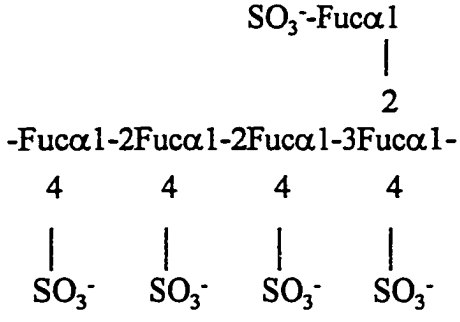
Other than demonstrating that an algal polysaccharide like fucoidan could inhibit human sperm-ZP binding, these studies also indicated that similar to the mouse system, specific glycoconjugates could be playing a major role in mediating human sperm-ZP binding. To understand the molecular basis of the inhibition of fucoidan we undertook a study to characterize the structure of this polysaccharide. The structure of fucoidan was first studied in the 1950's using the technology available at that time (Percival, 1967) (Figure 6). With the availability of modern structural analysis tools like gas chromatography-mass spectroscopy (GC-MS), a more sensitive and critical analysis of the structure was performed (Patankar, Oehninger et al., 1993). Our analysis indicated that contrary to the findings of the early studies, the structure of fucoidan showed substantial differences when analyzed by GC-MS. Previous studies performed by Percival et al (1967) indicated that fucoidan was primarily a polymer of α1-2 linked Fuc with relatively small number of branches of Fuc extending from the core in α1-4 linkages (Figure 6). A majority of the sulfates were shown to be present on the 4- position hydroxyl group. Our studies indicated that the core structure of fucoidan is composed of α1-3-linked Fuc chain that is highly branched with α1-2 and α1-4 fucose units attached to the α1-3 linked core structure (Patankar, et al., 1993). This work constituted the major analysis completed for the fulfillment of the Masters thesis of this candidate. Comparison between the previous and the new model obtained for fucoidan are shown in Figure 6.

Figure 6: The new average structure of fucoidan. Panel A and B show the previously reported structure of this polysaccharide (Percival et al., 1967). The new average structure reported for fucoidan is shown in Panel C.

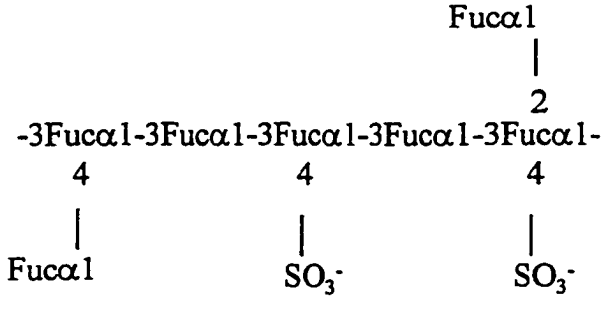
A



B



C



Fucoidan also Inhibits Selectin Mediated Events

In addition to its ability to block mammalian sperm-ZP binding fucoidan was also shown to be a very potent inhibitor of L-selectin mediated attachment of the leukocytes to the inflamed endothelium (Foxall et al., 1992). As mentioned earlier, sulfo-Lewis^x and sulfo-Lewis^a terminated glycans are potent ligands for the selectins (Varki, 1994; (Yuen, et al., 1992; Green, et al., 1992). Sulfo-Lewis^x carries a α1-3 linked Fuc and the sulfo-Lewis^a structure has a α1-4 linked Fuc attached to a GlcNAc residue. Our data indicated the presence of α1-3 and α1-4 branches of Fuc in fucoidan (Patankar, et al., 1993). The concentration dependence of fucoidan's inhibition of selectin mediated processes and of sperm-ZP binding in the HZA was very similar (Foxall, et al., 1992). We therefore hypothesized that fucoidan may be inhibiting both these processes by similar molecular mechanisms (Patankar, et al., 1993). The natural extension of this hypothesis was that human sperm-ZP binding could also involve a selectin-like binding process. This observation indicated that a considerable overlap probably existed between the carbohydrate ligands responsible for mediating human sperm-ZP binding and the recruitment of leukocytes to the inflamed endothelium. We therefore proposed that initial human sperm-ZP binding could be a selectin-like event (Patankar, et al., 1993). However more proof was needed to support this hypothesis. In the first portion of this study we provide more evidence supporting an overlap of the carbohydrate ligands in the gamete binding and the immune system. Our analysis suggests that the known selectin ligands inhibit human sperm-ZP binding in the HZA.

Direct Evidence Demonstrating the Role of Carbohydrates in Human Sperm-ZP Binding

Saling and co-workers recently suggested that a 95 kDa tyrosine kinase activity, ZRK, expressed on human sperm was the primary receptor responsible for mediating gamete binding (Burks, et al., 1995; McLeskey, et al., 1998). Two peptide sequences derived from the extracellular domain of ZRK were shown to inhibit human sperm-ZP binding. An analysis of the primary protein sequence of ZRK did not indicate the presence of a carbohydrate binding domain. Therefore the results obtained with ZRK did not support earlier evidence suggesting a role for carbohydrates in mediating initial human gamete binding. A review of the literature did not reveal any conclusive studies that could counter the suggestions made by Saling and co-workers (Burks, et al., 1995; McLeskey, et al., 1998). We therefore focused our attention on obtaining direct evidence for the involvement of carbohydrates in initial human sperm-ZP binding. Specific chemical and enzymatic manipulations of the ZP oligosaccharide chains conducted in this study clearly indicate the importance of the glycan chains in mediating human gamete binding.

MATERIALS AND METHODS

All of the chemicals utilized in this study were analytical grade and were obtained from Sigma Chemical Company, U.S.A unless otherwise indicated. Plates used for tissue culture were obtained from Falcon, U.S.A. Prior consent from the Internal Review Board was obtained for collection of the human gametes. Eggs were obtained from donors undergoing *in vitro* fertilization at the Jones Institute for Reproductive Medicine. Eggs were stored in high salt solutions prior to use (Franken et al., 1989). Sperm samples were obtained from healthy donors who had fathered a child in the past two years.

Isolation of Hemizona

Flame drawn micropipets were utilized for micromanipulation of the eggs and the hemizonae. Salt stored eggs were rinsed in HAMS-F10 media containing 0.5% human serum albumin (HAMS-HSA). The eggs were then bisected by a microsurgical cutting blade which was manipulated using a Narishige micromanipulator on an inverted phase contrast microscope (Nikon, Japan). The bisected eggs were aspirated vigorously in HAMS-HSA to remove the ooplasmic contents. Both the hemizona were transferred to a 50 µl droplet of salt storage solution and stored at 4°C until used. To prevent evaporation the droplets were stored under mineral oil.

Isolation of Human Sperm

Human sperm were isolated from semen using the swim-up procedure. After liquefaction, semen was pipeted into a 15 ml conical centrifuge tube. To this was added 5 ml of HAMS-HSA that had been aseptically prepared and stored overnight at 37°C in a 5%

CO_2 incubator. The diluted semen sample was centrifuged in a table top centrifuge at 400 X g for 7 min. The supernant was discarded and the precipitate was resuspended in 2 ml of HAMS-HSA. The sample was centrifuged again at 400 X g for 5 min as before. The supernatant was discarded. To the pellet, which was highly enriched in the sperm, was carefully added 1 ml of HAMS-HSA. The tube was capped loosely and kept at an angle of 45° in the 5% CO_2 incubator set at 37°C for 1 h. The swim-up sperm were then carefully pipeted and placed in a 5 ml round bottom capped plastic sample tube. The concentration and the motility parameters of the swim-up sperm were analyzed by using a Hamilton-Thorne Motion Anıalyzer, specially programmed to analyze human sperm motion parameters according to the World Health Organization (WHO) guidelines. Only sperm samples that exhibited progressive motility above 70% were used.

Hemizona Assay

Hemizona assay (HZA) was performed as described previously (Burkman, et al., 1988). Matched hemizona were always used in the assay. Salt stored hemizonae were washed thoroughly by aspiration in HAMS-HSA. The control hemizona was then transferred to 25-50 µl droplets of swim-up sperm (0.7 million sperm/ml media) and kept under mineral oil to prevent evaporation. The test hemizona were transferred into identical droplets containing sperm (0.7 million sperm/ml media) from the same donor premixed with the known amount of the test substance. The control and the test droplets were stored under mineral oil to prevent evaporation. The incubation of sperm and hemizona was carried for 4 h in a 37°C 5% CO_2 incubator as previously described (Burkman, et al., 1988). Prior to the assay the sperm were incubated for 4 h with the test substances to check for any toxic effects and were assayed for motility as mentioned before. None of the

substances tested in the HZA in this study significantly affected the motion parameters of the sperm. When analyzed by the Hamilton-Thorne sperm motion analyzer, the progressive motility of the sperm in the presence of the test substances was > 75%.

After incubation for 4 hr, the control and test hemizonae were individually pipeted out and placed in 100 µl HAMS-HSA droplets. Each hemizona was then washed 15 times in separate HAMS-HSA droplets. The washed control and test hemizonae were placed in a 50 µl HAMS-HSA droplet and the sperm bound to each were visually counted. To control for the quality of the hemizona, only the points where at least 20 sperm bound to the control hemizona were considered. For statistical accuracy, each assay was performed at least nine times. The effect of the test substance on sperm-ZP binding in the HZA was measured by calculating the HZI.

Periodate Oxidation of Hemizona

Oxidation of the oligosaccharides expressed on the ZP was performed by treating the hemizona with sodium *m*-periodate (SMP). For selective oxidation of the terminal NeuAc, the test hemizona were placed in a 50 µl ice cold droplet of phosphate buffered saline (PBS; 20 mM sodium phosphate pH 7.4 containing 150 mM sodium chloride) containing 1 mM SMP. The control hemizona were placed in 50 µl droplets of PBS containing 1 mM SMP and 100 mM ethylene glycol. Ethylene glycol was added to stop the SMP reaction. The droplets were covered with mineral oil to prevent evaporation and were kept on ice for 10 min. After incubation, the test hemizonae were promptly drawn out of the droplets and rinsed thoroughly by aspiration in PBS. The control hemizonae were also similarly rinsed with PBS. The test and the control hemizona were then separately placed

under mineral oil in 50 µl droplets of PBS containing 100 mM sodium borohydride. This step was performed to reduce any aldehydes that may have formed during the oxidation with SMP on the hemizona. The reduction with sodium borohydride was performed for 1 h at room temperature. Following reduction, the control and the test hemizonae were washed thoroughly by aspiration in PBS and stored in salt storage solution until used in the hemizona assay.

A similar procedure was used for the oxidation of the terminal sugars except that 10 mM concentration of SMP was used. The test hemizona were placed in a 50 µl droplet of 10 mM SMP in PBS. The oxidation was performed for 1 hr at room temperature. The control hemizona were treated under identical conditions except that the incubation was done in the presence of ethylene glycol (100 mM). After treatment with SMP, the test and the control hemizonae were subjected to sodium borohydride reduction as described previously. The test and control hemizonae were stored in salt storage solution until used in the HZA.

Chemical Desialylation of Human α_1-Acid Glycoprotein

The NeuAc residues present on oligosaccharides expressed on human α_1-acid glycoportein (AGP) were removed by acid hydrolysis as described previously (Varki and Diaz, 1984). AGP (10 mg) was dissolved in 2 ml 2 M acetic acid and heated at 80°C for 2 hr. The acid was neutralized with pyridine. The reaction mixture was then concentrated to 250 µl in an Amicon 10 kDa cutoff Centricon™ cartridge. The concentrated protein was resuspended in 2 ml of PBS and was again concentrated to 100 µl. This step was repeated at least 5 times to completely exchange the pyridine acetate present in the reaction mixture.

Total protein retained was determined by assaying for absorbance at 280 nm which indicated a 70% recovery. A stock solution of 7 mg protein in 140 µl PBS was prepared for use in the hemizona assay.

Neuraminidase and Endo-β-Galactosidase EBGase Treatment of ZP

Hemizona were obtained by microbisection of human eggs as described before. The hemizonae were washed thoroughly with PBS. Test hemizonae were placed in a 50 µl droplet of PBS containing 1 unit/ml *Clostridium perfringens* type X neuraminidase (Sigma). The control hemizona were placed in a 50 µl PBS droplet containing heat inactivated *Clostridium perfringens* type X neuraminidase under otherwise identical conditions. The droplets were covered with mineral oil to avoid any evaporation of the buffer and were incubated overnight at 37°C in a 5% CO_2 incubator. After incubation the control and test hemizona were individually washed in PBS and stored in salt storage solution until further use in the HZA.

The conditions employed for EBGase treatment of the hemizonae was identical to the one used for neuraminidase digestion, except that 4 unit/ml of EBGase enzyme (V Labs) was used.

Staining of Hemizonae with FITC-MAA

FITC-MAA lectin was used to determine the effect of chemical and enzymatic treatments on the test and the control hemizonae. Test and the control hemizonae were separately incubated for 15 min at room temperature in the dark in a 25 µl droplet of PBS containing either 5 or 10 µg/ml of FITC-MAA. The hemizonae were immediately removed and washed thoroughly in PBS. The washed hemizonae were placed on a slide under a

drop of Antiquench solution. Lectin binding to the hemizona was detected under a fluorescent scope (Nikon Microphot FX equipped with a 627 nm bandpass filter). The stained hemizona were photographed using the on-stage camera (FX-35WA). To make a quantitative estimate of the amount of FITC-MAA bound to the hemizona, the fluorescent microscope was set on an auto exposure mode and the amount of time of exposure estimated by the camera in order to get a clear picture was noted. An arbitrary scale for the amount of fluorescent staining of the hemizona was set as follows- (++++) 1-5 sec; (+++) 6-10 sec; (++) 11-20 sec; (+) 21-30 sec; (+/-) 31-40 sec; and (-) >40 sec.

RESULTS

Sialyl Lewis^x Inhibits Human Sperm-ZP Binding in the HZA

The structure of fucoidan resembled that of sialyl Lewis^x, the known selectin ligand. We therefore decided that sialyl Lewis^x was a good candidate to be tested in the HZA for its effect on sperm-ZP binding. Incubation of the test hemizona with 1 mg/ml (10 mM) of sialyl Lewis^x resulted in approximately 60% inhibition (HZI = 36 $\pm$ 14 [standard error])of sperm binding as compared to the matched control (Figure 7) (Clark et al., 1995). Human α_1-acid glycoprotein (AGP), an acute phase glycoprotein derived from plasma glycoprotein and known to express minor amounts of sialyl Lewis^x on its *N*-linked oligosaccharides also inhibited sperm-ZP binding in the HZA. Approximately 50% inhibition (HZI = 46 $\pm$ 22) of sperm binding was observed when the test hemizona was incubated with 1 mg/ml of AGP whereas 5 mg/ml of this glycoprotein resulted in 99.3% inhibition (HZI = 1) in the HZA (Clark, et al., 1995). Desialylated AGP obtained by chemical hydrolysis of the terminal NeuAc did not exhibit significant inhibition of sperm-ZP binding when assayed at a concentration of 1 mg/ml (HZI = 102 $\pm$ 24) as shown in Figure 7. Desialylated AGP when tested at 5 mg/ml resulted in about 80% inhibition (HZI = 22 $\pm$ 10) (Clark, et al., 1995). However, at 5 mg/ml concentration of both the sialylated or desialylated AGP it is very likely that non-specific effects may ensue resulting in strong inhibition of sperm binding in the HZA.

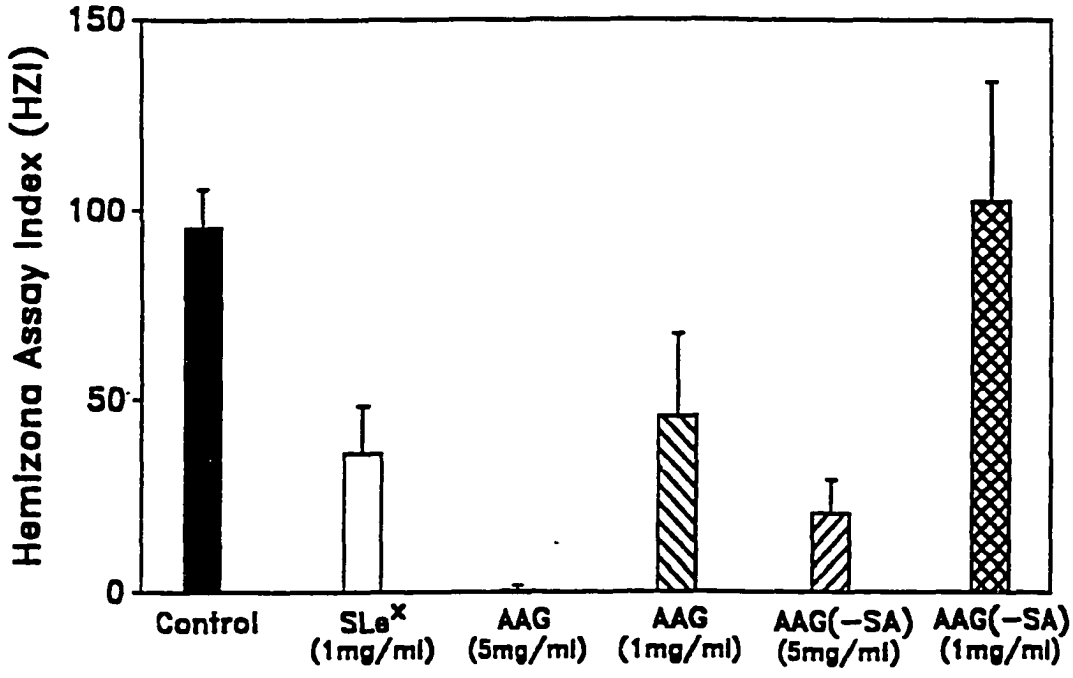


Figure 7: Inhibition of human sperm-ZP binding in the HZA by sialyl Lewis^x and AGP.

Glycodelin-A

Dr. Oehninger in collaboration with Dr. Markku Seppala of the University of Helsinki, Finland, observed that placental protein 14 (PP14, previously also known as progesterone associated endometrial protein, PAEP) was the most potent inhibitor of human sperm-ZP binding in the HZA compared to other substances tested thus far (Oehninger et al., 1995). PP14 is a glycoprotein that is temporally expressed in the uterus during pregnancy (Julkunen et al., 1985; Wahlstrom et al., 1985; Julkunen et al., 1986). This glycoprotein is abundantly found in the amniotic fluid from where it is isolated by using immunoaffinity chromatography (Julkunen, et al., 1985).

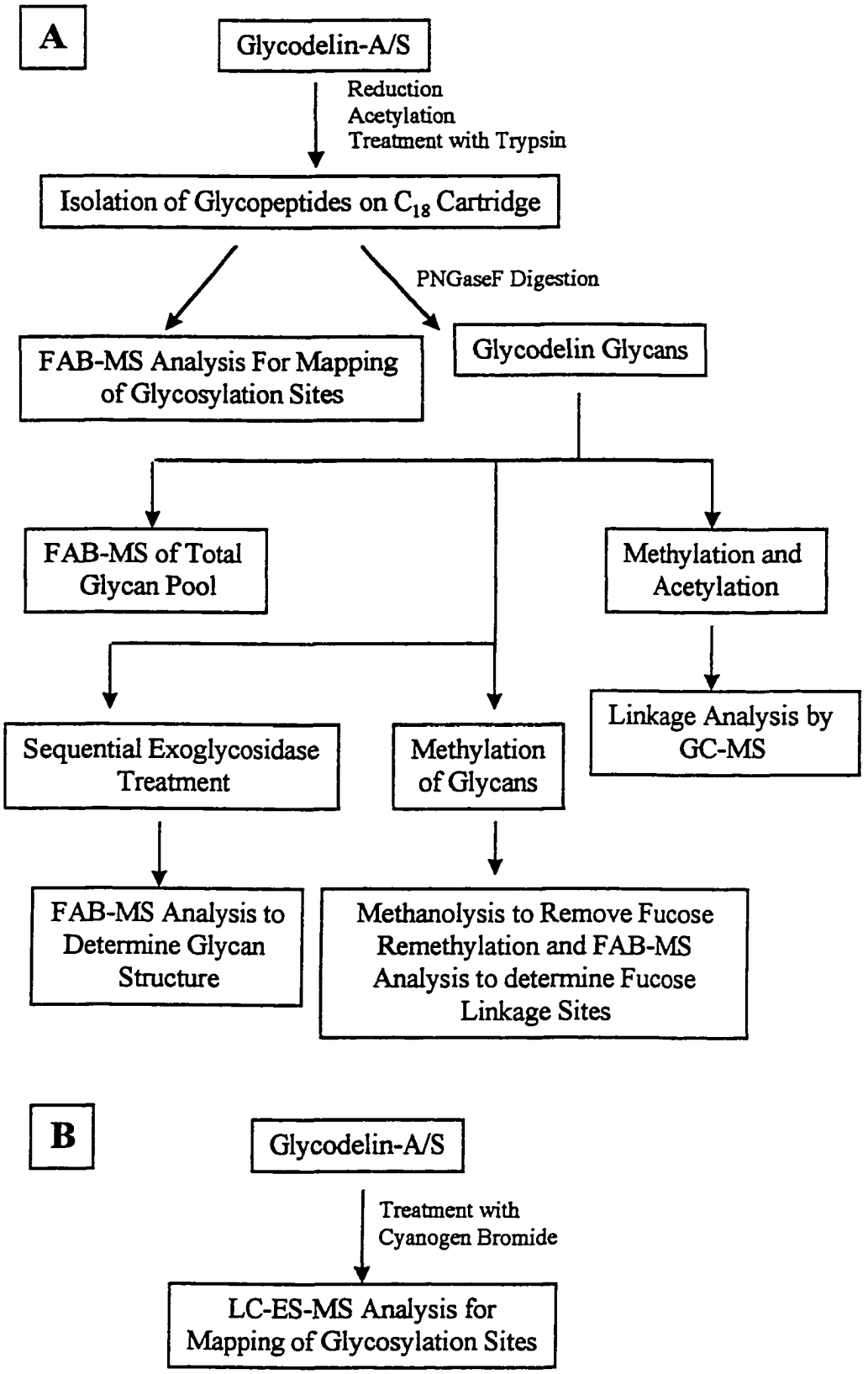
Contrary to previous reports, PP14 is synthesized by the secretory and decidualized endometrium and not by the placenta (Julkunen et al., 1986; Julkunen et al., 1988). Expression of this glycoprotein is also not regulated by progesterone. Therefore, the names PP14 and PAEP given to this glycoprotein were misnomers. In addition, PP14 was also found to be expressed in the seminal plasma and by the hematopoietic cells of the bone marrow (Julkunen et al., 1984; Kamarainen et al., 1994). The results that will be discussed below, clearly suggest a significant biological role for the glycans expressed on PP14 obtained from the amniotic fluid. Therefore to avoid confusion about the nomenclature of the different forms of PP14 we have renamed PP14 or PAEP as Glycodelin (Gd) (Dell et al., 1995). PP14 obtained from amniotic fluid is now referred to as glycodelin-A (Gd-A) whereas the seminal plasma isoform is now named as glycodelin-S (Gd-S). Similarly the new name given for the hematopoietic isoform of PP14 is glycodelin-H (Gd-H) (Dell, et al., 1995). This nomenclature will be followed throughout the remaining portions of this thesis.

Structural Characterization of Gd-A Oligosaccharides

In addition to its ability to block sperm-ZP binding in the HZA, Gd-A was also shown to be an extremely potent suppressor of specific immune responses (Bolton et al., 1987; Pockley et al., 1988; Pockley et al., 1989; Pockley and Bolton, 1989; Pockley and Bolton, 1990; Okamoto et al., 1991). Our studies with fucoidan, sialyl Lewis^x and AAG strongly suggested that the oligosaccharide ligands involved in mediating human sperm-ZP binding closely mimicked the sugars recognized during specific immune responses. Therefore, we thought it likely that the contraceptive and immunosuppressive properties of Gd-A could be manifested by the glycans expressed on this glycoprotein. We undertook a study to characterize the structure of the Gd-A oligosaccharides.

The characterization of the Gd-A oligosaccharides was performed in collaboration with Drs. Anne Dell and Howard Morris from Imperial College, (London) and Dr. Markku Seppala. Fast atom bombardment-mass spectroscopy (FAB-MS) and electron spray-mass spectroscopy (ES-MS), two extremely sensitive tools for carbohydrate and peptide analysis were used for this analysis. The structural characterization studies were mainly done by the group at Imperial College and therefore only the main results of this collaborative study are discussed here as they provide valuable information absolutely essential for the conclusions of this present study. Therefore, the exact methodologies used for the structural characterization of the glycosylation sites and the oligosaccharides of Gd-A are also not described in detail here. A succinct flow chart of the various methodologies used is however included (Figure 8). For more information the reader is directed to the original manuscript (Dell, et al., 1995).

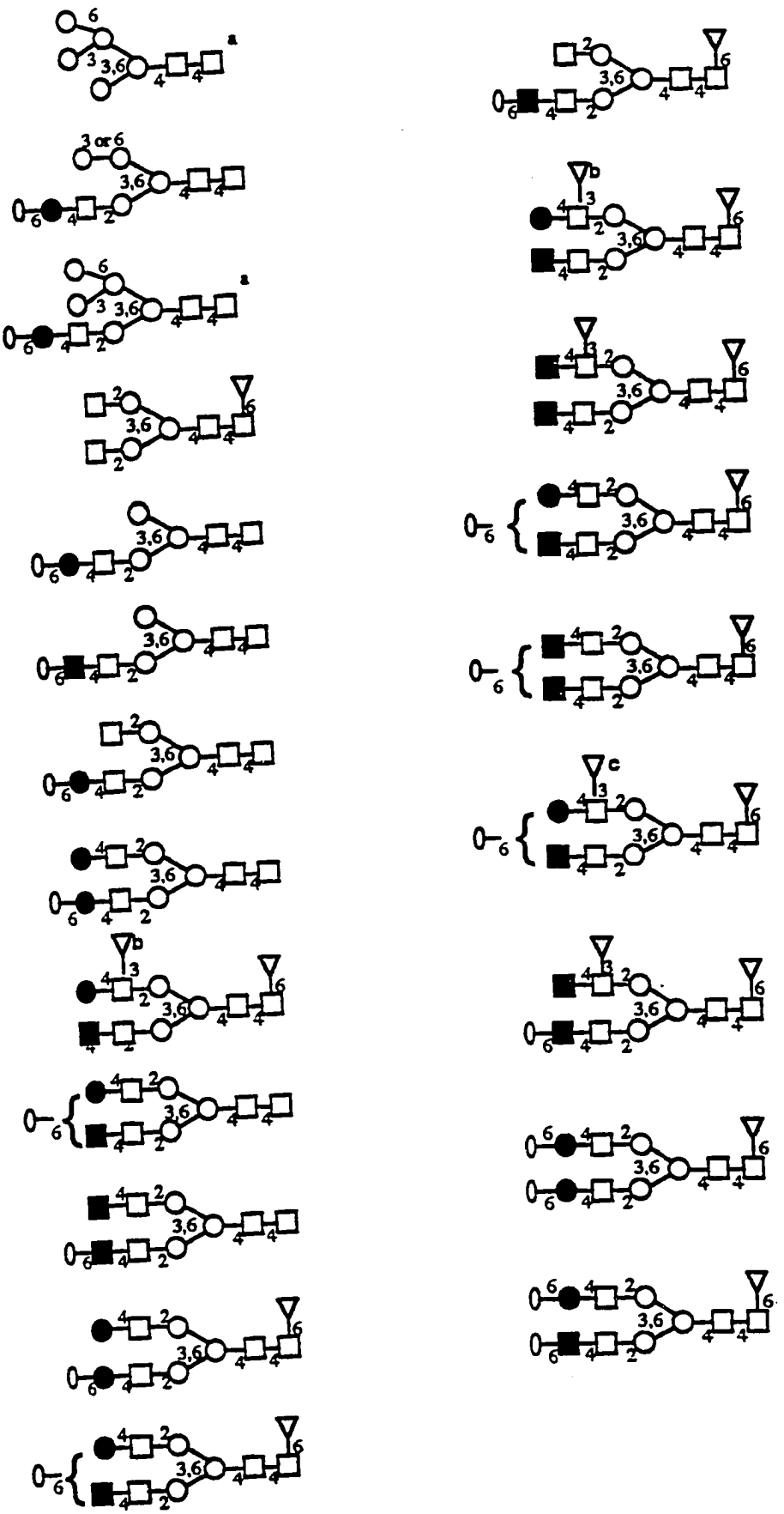
Figure 8: Schematic diagram of the strategies utilized for the structural characterization of the glycodelin glycans and for mapping of their glycosylation sites. For mapping of the glycosylation sites, the glycodelins were either treated with trypsin and analyzed by FAB-MS or treated with cyanogen bromide. Cyanogen bromide results in cleaving the peptide sequence at the methionine residues. The cleaved peptides obtained were analyzed by LC-ES-MS. For structural characterization of the glycans, the proteins were treated with trypsin and the glycopeptides were isolated. FAB-MS profiles of the total glycan mixture were determined. In other experiments, the glycodelin glycans were released from the glycopeptides by the enzyme PNGase F. The free oligosaccharides were further analyzed using three different strategies. (1) The free glycans were subjected to sequential exoglycosidase digestions and the FAB-MS profiles recorded after each round of enzyme treatment. (2) For linkage analysis, the glycans were methylated and then hydrolyzed. The free hydroxyl groups of the released methylated monosaccharides were acetylated and analyzed by GC-MS. (3) To determine the fucosyl linkages, the free glycans were permethylated and then subjected to mild methanolysis to remove the Fuc residues. The free hydroxyl groups were remethylated and the glycans analyzed by FAB-MS. The data obtained from all of these strategies was collectively used to determine the structures of the glycodelin glycans.



The primary protein sequence of Gd-A carries three consensus sites for *N*-linked glycosylation. The glycosylation sites in Gd-A were determined by mapping the tryptic Gd-A glycopeptides by FAB-MS or by microbore liquid chromatography-electron spray-mass spectroscopy (LC-ES-MS) of the glycopeptides obtained after treatment with cyanogen bromide. FAB-MS and LC-ES-MS data indicated that only two of the sites, Asn 28 and Asn 63 were glycosylated. No *O*-linked sugars were detected on this glycoprotein.

Analysis of the *N*-linked oligosaccharides was performed using FAB-MS techniques coupled with specific glycosidase digestions as described previously by Dell and co-workers (Dell, 1987). The glycans expressed on Gd-A were either biantennary or triantennary. FAB-MS analysis of these glycans indicated the presence of highly unusual oligosaccharide chains on this glycoprotein (Figure 9). A vast majority of the *N*-linked glycans found to be expressed on various mammalian glycoproteins carry the Galβ1-4GlcNAc sequence on their non-reducing terminals (Figure 2). This sequence is referred to as the lacNAc sequence. However, approximately 50% of the Gd-A glycans expressed GalNAcβ1-4GlcNAc (lacdiNAc sequence) at their terminal ends in addition to the commonly observed lacNAc glycans (Figure 9). The expression of the lacdiNAc sequence in humans is highly restricted. It has been shown to be expressed on various pituitary glycohormones and also on recombinant human protein C expressed in the human kidney 293 cell line (Grinnell et al., 1991; Grinnell et al., 1994). The terminal lacdiNAc glycans present in Gd-A were further modified by NeuAc and Fuc. Approximately 60% of the glycans were sialylated and 20% were fucosylated (Dell, et al., 1995). All of the NeuAc was attached to the terminal Gal or GalNAc via a α2-6 linkage. The Fuc was attached to

Figure 9: Structures of the oligosaccharides present in Gd-A. The panel on the left shows the different glycans present on Asn 28 whereas the panel on right shows all of the glycans present on Asn 63. Open circles-Man, closed circles-Gal, open ellipse-NeuAc, open inverted triangle-Fuc, open squares-GlcNAc, and closed squares-GalNAc.



the penultimate GlcNAc in a α1-3 linkage. The α1-3 fucosylation and sialylation of the same antenna were found to be mutually exclusive events. A significant number (30%) of the binantennary glycans carried both the lacNAc and lacdiNAc antennae within a single structure.

Structural Characterization of Gd-S Oligosaccharides

As mentioned above, a seminal plasma isoform of Gd-A is also present. Since Gd-A exhibited potent contraceptive activities, it was initially puzzling why a potentially contraceptive glycoprotein could also be expressed in the seminal plasma as it could severely affect the sperm-ZP binding. It was considered very likely that Gd-S may not be expressing the unusual glycans expressed on Gd-A and could therefore not be contraceptive. Initial studies conducted in Dr. Seppala's laboratory indicated major differences in the lectin binding profiles of both Gd-A and Gd-S. *Wisteria floribunda* agglutinin (WFA) is a lectin that selectively binds the GalNAc (Koistinen et al., 1996). In an ELISA-type assay, WFA was found to bind strongly to Gd-A. However, Gd-S did not bind WFA (Koistinen, et al., 1996). Similarly, Gd-S did not bind *Sambucus nigra* agglutinin (SNA) a lectin that binds to α2-6 linked sialic acids (Koistinen, et al., 1996). These two observations indicated the absence of lacdiNAc and the α2-6 linked sialic acid sequences in Gd-S. Gd-S was however found to avidly bind to *Lotus tetragonolobus* lectin, a lectin that binds to α1-3 and α1-2 linked Fuc (Koistinen, et al., 1996). The lectin binding studies therefore indicated major differences in the structures of Gd-A and Gd-S glycans.

To conclusively determine the differences in the glycan structures of Gd-A and Gd-S, the oligosaccharides attached to Gd-S were characterized by using ES-MS and FAB-MS

(Morris et al., 1996). The strategies adopted for the characterization were similar to the methodologies utilized for the characterization of Gd-A glycans (Morris, et al., 1996) (Figure 8).

FAB-MS and LC-ES-MS analysis confirmed the differences in the Gd-S and Gd-A glycans observed by the lectin binding studies. As in the case of Gd-A, Gd-S also carried two *N*-linked glycosylation sites but no *O*-linked oligosaccharides. Asparagine (Asn) 28 and asparagine (Asn) 63 were both glycosylated as in the case of Gd-A. The first glycosylation site (Asn 28) carried only high mannose type glycans (Figure 10). All of the glycans present on Asn 63 were biantennary complex type oligosaccharides (Figure 10). However, none of these biantennary glycans were of the unusual lacdiNAc type but were instead composed of the commonly occurring lacNAc sequences. Contrary to the Gd-A glycans which were highly sialylated, the Gd-S oligosaccharides were not sialylated. More than 80% of the glycans on asparagine 63 carried between 3 and 5 fucose residues per glycan. A substantial percentage of the glycans were terminated with the Lewis^x (Galβ1-4[Fucα1-3]GlcNAc-) and the Lewis^y ([Fucα1-2]Galβ1-4[Fucα1-3]GlcNAc-) sequence. The Lewis^y sequence was totally absent in Gd-A.

Gd-S Does Not Inhibit in the HZA

FAB-MS analysis clearly indicated major differences in the structures of the Gd-S and Gd-A glycans. It was therefore reasoned that if the contraceptive activities exhibited by Gd-A in the HZA were mediated by its glycans, then Gd-S may exhibit differential activities in this gamete binding assay. To test this hypothesis, a comparative study of the effect of Gd-A and Gd-S in the HZA was performed at 1, 10 and 25 μg/ml concentration of the two

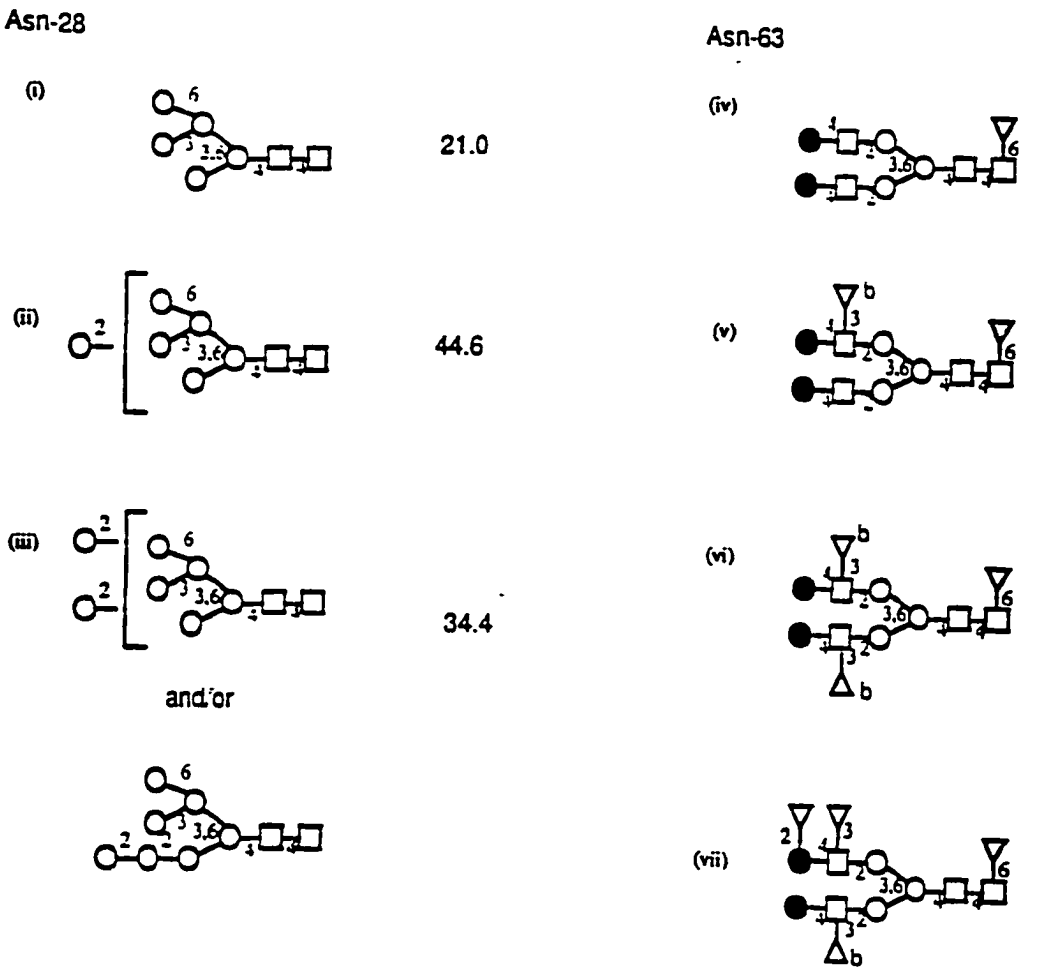


Figure 10: Glycans expressed on Gd-S. The structures shown in the left panel are the glycans found on Asn-28 and the ones on the right are the glycans expressed on Asn-63. Open circles-Man, closed circles-Gal, open ellipse-NeuAc, open inverted triangle-Fuc, and open squares-GlcNAc.

glycodelins (Morris, et al., 1996) (Figure 11). Gd-A at 25 μg/ml inhibited sperm-ZP binding in the HZA by 90% (HZI = 6.6). At the same concentration Gd-S did not produce any effect on the number of sperm binding to the ZP (HZI = 93.3 ± 20). Similarly at 1 and 10 μg/ml concentration of Gd-A approximately 30% (HZI = 70 ± 33) and 70% inhibition (HZI = 33.3 ± 10) of sperm-ZP binding was observed respectively. At these concentrations, Gd-S did not inhibit but instead was found to slightly increase the number of sperm binding to the ZP (Figure 11).

Oxidation of Terminal Sugars with SMP

SMP selectively oxidizes molecules carrying vicinal (adjacent) hydroxyl groups. Sugars carry hydroxyl groups on vicinal carbon atoms which can be oxidized by SMP (Figure 12). Under mild conditions (10 mM SMP at room temperature for 1 h) selective oxidation of cell surface associated oligosaccharides can be achieved (Van Lenten and Ashwell, 1971; Kalyan et al., 1982;Vacquier and Moy, 1977). Oxidation of these vicinal hydroxyl groups results in the cleavage of the furanose or pyranose ring structure of the sugars and the formation of aldehydes on both sides of the oxidation site. The sites for SMP oxidation of galactose and the products obtained in this reaction are shown in Figure 13.

Many cell surface associated oligosaccharide chains are terminated with a NeuAc residue. NeuAc does not carry any vicinal hydroxyl groups within its ring structure. However, vicinal hydroxyl groups are present on the glycerol side chain attached to the C_6 carbon of NeuAc. Very mild oxidation conditions (1-2 mM SMP, at 0-5°C for 10 min) are sufficient to attack this glycerol chain of the NeuAc residues (Figure 14). Previous studies

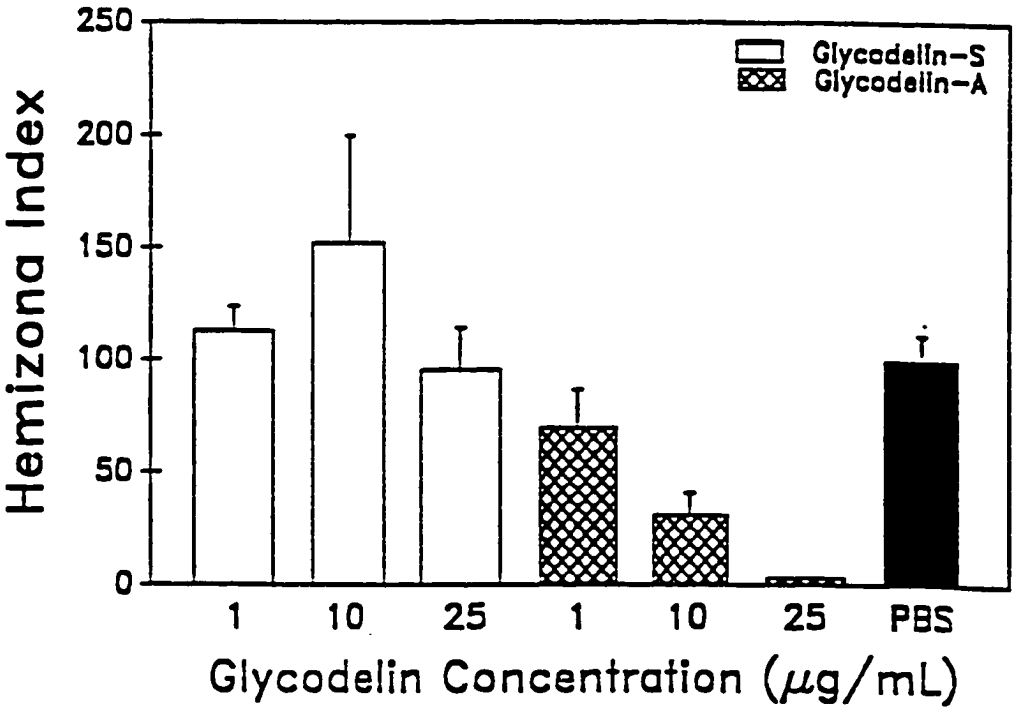


Figure 11: Effect of Gd-S and Gd-A on human sperm-ZP binding in the HZA.

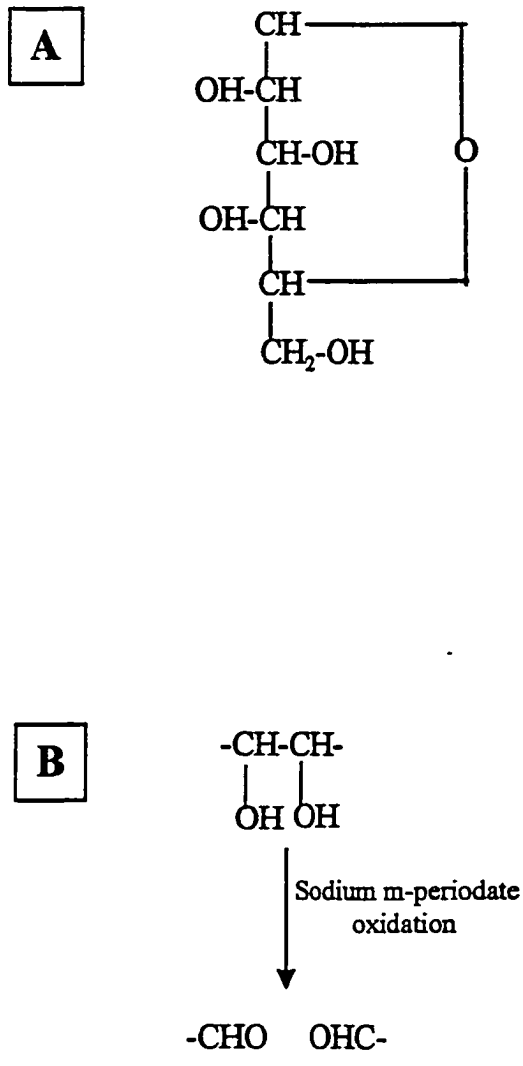


Figure 12: Fisher projection model of galactose is shown in panel A. Note the adjacent or vicinal hydroxyl groups present on the C_2, C_3 and C_4 carbon atoms. Oxidation of vicinal hydroxyl groups by sodium *m*-periodate results in the formation of aldehydes as shown in panel B.

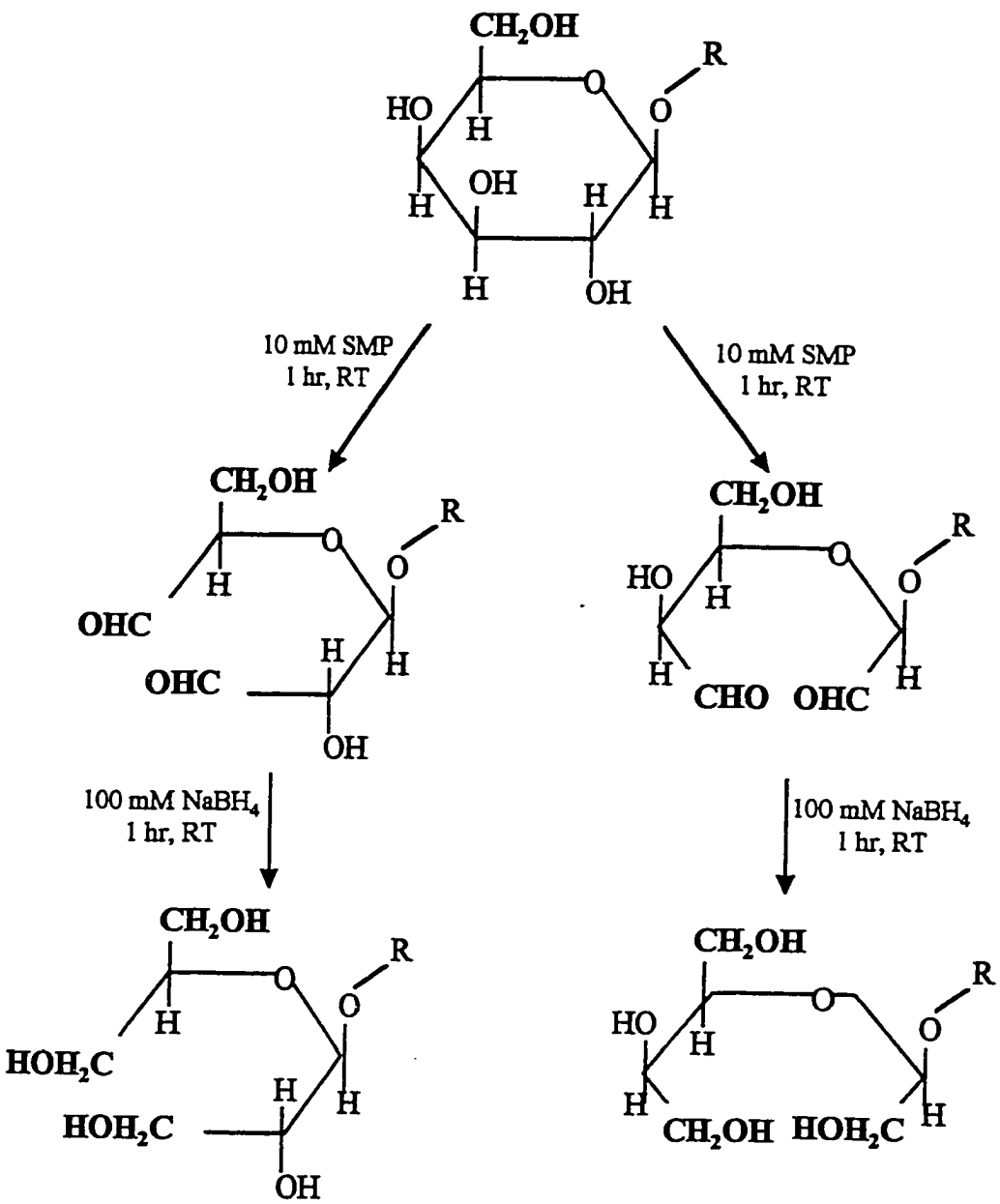


Figure 13: Mild SMP oxidation of a Gal residue.

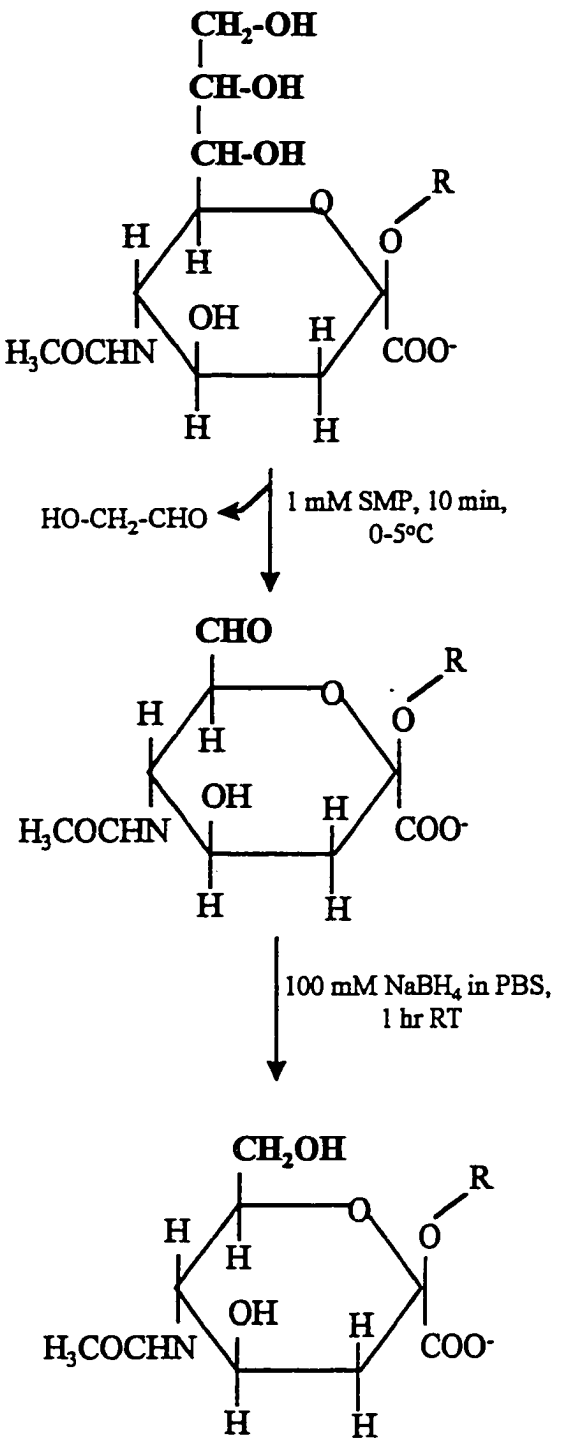


Figure 14: Very mild SMP oxidation of the Terminal NeuAc. R= the rest of the glycan chain.

indicate that under very mild conditions, the vicinal hydroxyl groups present on the C_7-C_8 positions of NeuAc are predominantly attacked resulting in the formation of a six carbon sugar referred to as heptulosonic acid (Figure 14). The internal sugars present in the oligosaccharide chain are not affected by this chemical treatment. Reactive aldehydes are generated after SMP oxidation reaction. Such reactive aldehydes can form imine linkages (>C=NH), also known as Schiff's base, with primary amine groups present in proteins. Such secondary reactions may pose problems when bioassays are being performed. The reactive aldehydes can however be converted to the less reactive alcohols by reduction with sodium borohydride (100 mM at room temperature for 1 h) permitting the use of SMP oxidation to assess the role of the oligosaccharides in a bioassay. Therefore to assess the requirement for the human ZP oligosaccharide chains in mediating sperm-ZP binding, the hemizonae were treated with SMP, reduced with sodium borohydride and assessed for their ability to bind sperm using a standard HZA protocol.

Very Mild SMP Oxidation

Very mild SMP oxidation was performed to oxidize the terminal NeuAc residues. To control for any side effects that SMP or any possible impurities may have on the hemizonae, the control hemizonae were also treated under identical conditions with the same concentration of SMP. However, a 100-fold molar excess of ethylene glycol (100 mM) was added to inactivate the periodate, thereby blocking oxidation of the ZP oligosaccharides. The test and the control hemizonae were then subjected to treatment with sodium borohydride.

The effect of very mild oxidation on hemizona was apparent when the test and the

control hemizonae were assayed for their ability to bind to MAA, a lectin that specifically binds α2-3 linked sialic acids (Figure 15). Lectin binding studies performed in our laboratory and those reported by others had indicated significant binding of FITC-MAA to the human ZP. The test hemizonae obtained after very mild SMP oxidation showed significantly less binding to FITC-MAA as compared to the controls (Figure 15). When tested for sperm-ZP binding in the HZA, approximately 30% inhibition was observed in the case of the test hemizona as compared to their matched controls (Table 4). This result clearly indicated that specific oxidation of the sialic acids expressed on the human ZP affected their ability to bind sperm.

Mild SMP Oxidation of ZP Oligosaccharides

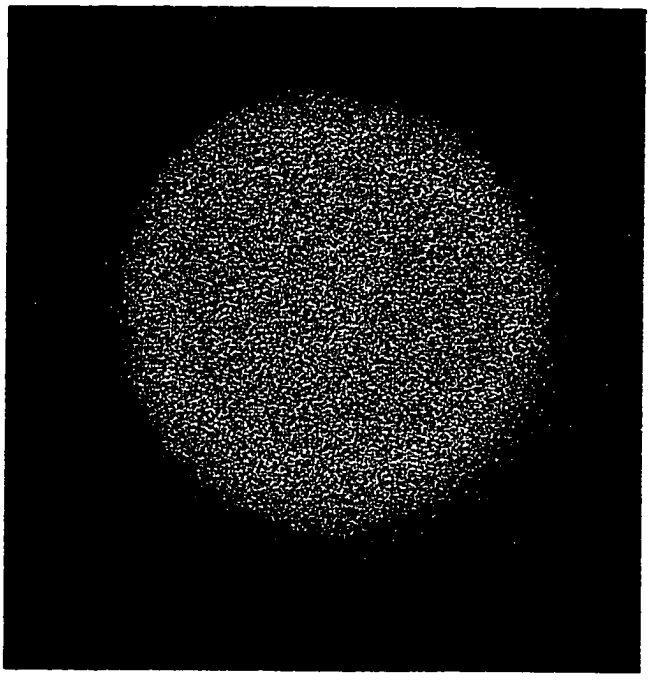
Test hemizonae were treated under mild oxidation conditions with SMP (10 mM, 1 hr at room temperature), to oxidize vicinal hydroxyl groups on both the ring and straight chains. The controls were treated under identical conditions except that 1 M ethylene glycol was added to inhibit the oxidation. Test and control hemizonae were subjected to sodium borohydride treatment. The HZA conducted with the test and the control under standard assay conditions indicated approximately 40% inhibition of sperm binding to the test hemizona (Table 4).

Enzymatic Treatment of ZP With Neuraminidase and EBGase Increases Sperm Binding

SMP oxidation of the ZP under mild and very mild conditions resulted in a significant decrease in sperm binding in the HZA indicating a role for the terminal sugar residues in mediating this interaction. We therefore decided to test the effect of enzymatic removal of some of terminal sugars of the ZP on sperm binding in the HZA. Two enzymes

Figure 15: Reduced binding of FITC-MAA to the SMP treated hemizona. Panel A is a control hemizona. Panel B is a matched test hemizona that was treated under mild SMP oxidation conditions. The control and the test zona were incubated with FITC-MAA, washed and visualized under a fluorescent microscope.

A



B

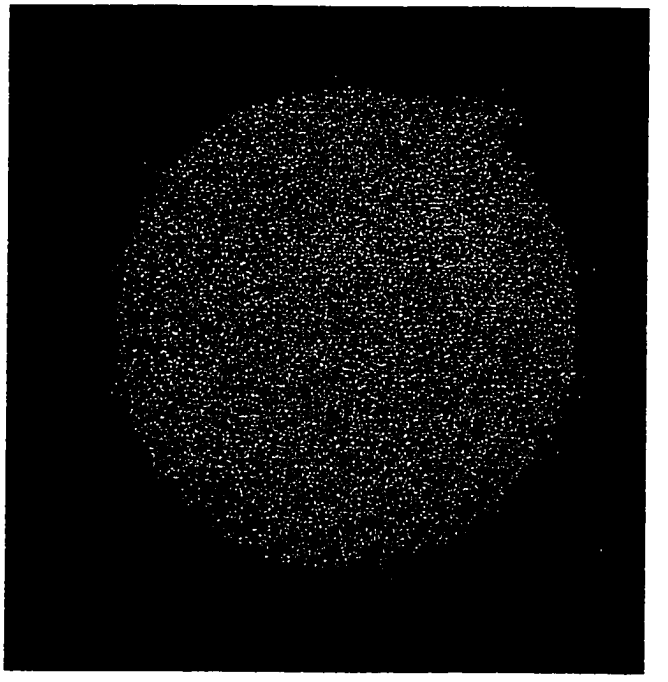


Table 4: Effect of chemical and enzymatic treatments on human sperm-ZP binding in the HZA.

Treatment	HZI	# of Sperm Bound to Control	# of Sperm Bound to Test	Number of Eggs used in Study	*p*-value
Mild SMP Oxidation	61 ± 10	73 ± 23	26 ±4	22	<0.01
Very Mild SMP Oxidation	70 ± 11	42 ±16	36 ± 11	24	<0.05
Neuraminidase	297 ± 45	60 ± 10	150 ± 22	22	<0.001
EBGase	710 ± 232	28 ± 10	104 ± 25	14	<0.001
Neuraminidase + Mild SMP Oxidation	20.7 ± 4.4	76 ± 10	12 ± 3	16	<0.001

used in this study were neuraminidase and EBGase. Neuraminidase is a exoglycosidase that specifically cleaves NeuAc from the terminal ends of the oligosaccharides. In this study, neuraminidase (Type X, Sigma) obtained from *Clostridium perfringens* was used. This neuraminidase is known to be a highly purified preparation and is not found to contain any significant activities of other glycosidases or proteases. The *Clostridium perfringens* enzyme cleaves sialic acids in α2-3 linkage with high affinity, although it also hydrolyses the α2-6 linked NeuAc at a slower rate.

We had previously observed that treatment of the ZP with this neuraminidase (1 unit/ml) for 1 h did not significantly affect sperm-ZP binding in the HZA (Clark, et al., 1995). However, NeuAc residues in mammals have been shown to carry glycolyl or acetyl groups. In some cases, α2-8 linked NeuAc has also been reported. It was therefore likely that if α2-8 linked NeuAc or a chemically modified NeuAc residues were expressed on the human ZP, the *Clostridium perfringens* neuraminidase would either be unable to desialylate the oligosaccharides or could cleave the NeuAc at a very slow rate. Human ZP were therefore treated with the *Clostridium perfringens* neuraminidase for 8 h before analyzing the effects of desialylation in the HZA. The extent of desialylation was determined by the binding of FITC-MAA to the test hemizonae. Results similar to the one shown in Figure 15 were obtained when the neuraminidase treated hemizona were labeled with FITC-MAA. Test hemizona were treated with the neuraminidase and the control hemizona were treated with heat inactivated neuraminidase.

Desialylated hemizona when assayed for sperm binding under standard HZA conditions gave an unexpected result (Table 4) . Although oxidation of terminal NeuAc of

the ZP oligosaccharides resulted in a decrease in sperm binding, enzymatic removal of this sugar caused a 2.5-fold increase in sperm binding to the test hemizona as compared to the matched controls which were treated with heat inactivated enzyme.

Previous studies have clearly indicated the presence of lactosaminoglycan sequences on murine and porcine ZP (Hokke, et al., 1994; Nagdas, et al., 1994). Therefore human hemizona were digested with a specific enzyme that degrades lactosaminoglycans (EBGase) by cleaving internal β1-4-linked Gal residues within these repeating co-polymers (Fukuda, et al., 1984). Digestion of the test hemizona with EBGase also resulted in approximately 4-7 fold increase in the number of sperm binding (Table 4).

SMP Oxidation of Neuraminidase Treated ZP Inhibits Sperm Binding

The increase in sperm binding observed after treatment of the ZP with neuraminidase and EBGase could be explained by the fact that the egg binding protein on the sperm recognize an internal oligosaccharide sequence. Therefore enzymatic removal of the outer NeuAc residues or polylactosamine type chains resulted in better binding of this egg binding protein to its ligands. In previous experiments with fucoidan it was observed that SMP was unable to oxidize the Fuc branches. It was thought that the high degree of sulfation present in fucoidan inhibited the chemical action of SMP, possibly due to the electrostatic effects (Clark and Patankar, unpublished results). A similar problem could possibly exist in the case of digestion of the ZP with SMP, where the high degree of sialylation of the ZP could result in inhibition of the SMP and so the internal sequences that were recognized by the egg binding receptor were not completely oxidized. In addition, SMP oxidation does not completely remove terminal sugar residues. For example, SMP oxidation results in the modification of the terminal NeuAc to heptulosonic acid, which

remains attached to the oligosaccharide chain of the ZP (Figure 14). Such modified glycans could perturb the presentation of the ZP glycans and may therefore not be recognized by the egg binding protein of the sperm. Therefore incomplete oxidation or the presence of chemically modified terminal sugars could result in only a 40% inhibition of sperm binding to the mildly oxidized hemizona rather than a complete abrogation (Table 4).

To address this possibility, the test and the control hemizona were treated with neuraminidase as explained in the previous experiment. The neuraminidase treated hemizona were then subjected to mild SMP oxidation as mentioned before (10 mM SMP at room temperature for 1 h). These manipulations resulted in a 80% inhibition of sperm binding to the test hemizona as compared to the control (Table 4). Therefore removal of sialic acid followed by periodate oxidation resulted in substantial inhibition of binding.

DISCUSSION

Specific Inhibition of Sperm-ZP Binding by Sialyl Lewis^x

Based on the structural and biological properties of the algal polysaccharide fucoidan, we had suggested that initial human sperm-ZP binding may involve a selelctin-like interaction (Patankar, et al., 1993). The results obtained in this study provide further evidence supporting this hypothesis. In previous studies it was observed that the Lewis^x (Galβ1-4[Fucα1-3]GlcNAc), Lewis^b or Lewis^a (Galβ1-3[Fucα1-4]GlcNAc) oligosaccharides were unable to block sperm binding in the HZA (Clark and Oehninger unpublished results). It was also observed that fetuin, a bovine glycoprotein expressing *N*-linked glycans similar to AGP but lacking the sialyl Lewis^x epitope exhibited no inhibitory effect when assayed in the HZA (Clark and Oehninger unpublished observations). In this context the significant inhibition of sperm-ZP binding by sialyl Lewis^x and AGP in the HZA is likely a specific effect (Figure 7). We have also demonstrated that desialylation of AGP renders it ineffective in inhibiting in the HZA (Figure 7). It is therefore very likely that the sialyl Lewis^x oligosaccharide mimics the actual oligosaccharides expressed on the human ZP that are required to mediate the gamete interaction.

Relatively high concentration of sialyl Lewis^x or AGP was required to obtain any significant inhibition of sperm binding in the HZA. However, it has been reported that sialyl Lewis^x also inhibits selectin mediated adhesions in the same concentration range required in this study to inhibit human gamete binding (Varki, 1994). None of the carbohydrate ligands identified so far are capable of binding to the selectins with sufficient adhesive strength

required to counter the extremely high shear forces that are experienced by the leukocytes (Varki, 1994). One possible reason that has been presented for this discrepancy is that the key to producing a very high affinity interaction lies in the manner in which the oligosaccharide ligands are being presented on the proteins or lipids. It has been suggested that these ligands when presented in patches or clusters serve as more effective ligands (Varki, 1994). Recognition of these clusters by the selectins results in a very high avidity interaction, leading to the binding of leukocytes to the endothelium even in the presence of high shear forces. Therefore, although sialyl Lewis^x is the correct ligand for the selectins, its presentation in a monovalent fashion rather than a polyvalent manner results in low affinity interaction requiring considerably higher concentration of this oligosaccharide to block E-selectin mediated events in *in vitro* assays.

It is likely that a similar situation exists with the human sperm-ZP binding in the HZA. In the present study sialyl Lewis^x was tested as a monovalent ligand. We have made several attempts to couple multiple molecules of different oligosaccharides to carrier proteins or to other polymeric supports. However, the presence of multiple hydroxyl groups on the sugar molecules poses a major problem in achieving specific labeling of the oligosaccharide chain. The reducing end (a hemiacetal) of the oligosaccharide is the only position that can possibly be used for tagging the molecule with chemical linkers, enabling polyvalent attachment of the sugars to carrier proteins or polymeric supports. The most convenient method of tagging the reducing end of the sugars is to react it with linker arms bearing primary or secondary amines leading to the formation of imines which could eventually be reduced to amines using sodium cyanoborohydride, a reaction sequence commonly known as reductive amination. We have synthesized various biotinylated

reagents that carry 8-12 carbon atom linker arms with primary amine groups at their terminal ends. Our intention was to biotinylate the oligosaccharides of interest with these reagents which could allow us to present the sugars in a tetravalent manner when coupled with avidin, a protein known to bind up to four moles of biotin. A similar strategy was used by Rothenberg and co-workers (Rothenberg et al., 1993) for biotinylation of oligosaccharides. However, our studies indicate that although efficient coupling of mono- and disaccharides can be achieved, coupling of higher oligosaccharides with these reagents results in extremely low yields (data not shown). We have therefore been unable to test sialyl Lewis^x presented in a polyvalent fashion in the HZA.

In addition to polyvalent presentation, sialyl Lewis^x may have to be presented in the context of an N-linked glycan on a protein platform. This possibility can be supported by the inhibition shown by AGP. Only 20-24% of the total glycans expressed on AGP carry the sialyl Lewis^x epitope (Walz et al., 1990). As mentioned above, fetuin has no effect on sperm-ZP binding in the HZA. The only major difference in the glycans expressed on fetuin and AGP is the presence of sialyl Lewis^x epitopes on the latter. Assuming that the protein structure of AGP does not play a role in the inhibition of sperm binding in the HZA (an assumption clearly supported by studies performed with desialylated AGP), it is clear that only 6% of the total glycoforms of AGP must be producing the inhibitory effect. Thus a 1 mg/ml concentration of AGP which produces 50% inhibition in the HZA actually corresponds to only 200-240 μg/ml concentration of the AGP glycoform that bears the sialyl Lewis^x epitope on its glycan chain. It is therefore possible that the sialyl Lewis^x bearing AGP glycoform if isolated selectively could lead to a much more potent inhibition of sperm-ZP binding in the HZA. In this context it can therefore be argued that precise

presentation of the appropriate oligosaccharide ligand(s) may be the key to obtaining very effective inhibition of the gamete binding process at low concentrations.

Specific inhibition of human sperm-ZP binding in the HZA by fucoidan, sialyl Lewis^x oligosaccharide and AGP suggested that these glycoconjugates may be mimicking the structure of the actual ligand expressed on the ZP and thereby preventing its interaction with the egg binding protein expressed on the surface of the sperm. Our data indicate that sialyl Lewis^x oligosaccharide inhibited both human sperm-ZP binding and E-selectin mediated adhesion over the same concentration range. These observations gave further support to our hypothesis that human sperm-ZP binding may involve a selectin-like event. More evidence in favor of this hypothesis was gained from the oligosaccharide structural analysis and the HZA inhibition profiles of the glycodelins.

Gd-A Glycans Inhibit Sperm-ZP Binding

Antibody binding studies performed by Seppala and co-workers indicated that all of the monoclonal antibodies produced against Gd-A also recognized Gd-S in Western blot analysis and also in dot blot assays (Seppala and Koistinen, personal communications). Sequencing data of 22 amino acids from the *N*-terminal of Gd-A and Gd-S was exactly identical (Seppala and Koistinen, personal communications). LC-ES-MS analysis further indicated that fragmentation pattern of the peptides obtained from both the glycodelins was identical. All of this evidence therefore strongly indicates that the primary amino acid sequence of both Gd-A and Gd-S is identical. It is therefore very likely that the tertiary structure of both the glycodelins is also similar. Indeed both Gd-A and Gd-S exhibit similar electrophoretic mobilities when analyzed by non-denaturing or denaturing polyacrylamide gel electrophoresis (PAGE). Both the glycodelins have also been shown to form dimers in

solution. Thus for all practical purposes it must be assumed that Gd-A and Gd-S are different glycoforms of the same protein synthesized by different tissues.

Inhibition studies performed in the HZA clearly indicate that while Gd-A is a potent inhibitor of sperm-ZP binding, Gd-S has no effect on this interaction (Figure 11). Therefore, considering the similarities between the protein structures of these two glycodelins it must be concluded that the differences in the biological properties exhibited by these glycoproteins are due to the expression of their significantly different *N*-linked glycans (Figure 9, 10). The glycodelins therefore provide additional proof that only specific glycoconjugates are capable of blocking initial attachment of the human gametes. The current goal in our laboratory is to synthesize the unusual glycans expressed on Gd-A to test their effect in the HZA. It is likely that these glycans could serve as efficient inhibitors of the gamete binding process and could therefore be used effectively for further research purposes (e.g. identification of the egg binding receptor present on human sperm) or could also be used to devise novel contraceptive agents. As will be discussed in Chapter IV of this thesis we have developed a new technique for the synthesis of glycopeptides. Using this method we have been able to efficiently synthesize the fucosylated lacdiNAc type biantennary glycopeptides and confirm their structures by FAB-MS analysis. We are currently collaborating with Dr. Thomas Plummer (Division of Molecular Medicine, New York State Department, NY) in order to synthesize all of the different Gd-A glycans and test their efficacy to block sperm-ZP binding in the HZA. It should however be noted that as in the case of sialyl Lewis^x, the proper presentation may be an absolute requirement for the Gd-A glycans to exhibit inhibitory activities in the HZA.

Human Sperm-ZP Binding as a Selectin-Like Event

Previous studies indicated that decidual extracts containing Gd-A were capable of suppressing thymidine uptake in both normal and mitogen-stimulated human mixed lymphocyte cultures (Bolton, et al., 1987). However selective removal of Gd-A from these extracts using anti-Gd-A antibodies resulted in a loss of these inhibitory responses. Gd-A was also shown to decrease the synthesis of interleukin-1 (IL-1) and interleukin-2 (IL-2), two cytokines that play a prominent role in initiation and maintenance of cytotoxic immune responses (Pockley, et al., 1989; Pockley and Bolton, 1989; Pockley and Bolton, 1990). Furthermore, this glycoprotein also inhibited the synthesis of IL-2 receptors by mitogen-stimulated cells (Pockley, et al., 1988). Additionally, Okamoto and co-workers had shown that Gd-A suppressed killing of the erythroleukemia cell line K562 by human natural killer (NK) cells in an *in vitro* assay (Okamoto, et al., 1991). The K562 cells have been conventionally known to be extremely good targets for the human NK cells. Based on the significant contraceptive and immunosuppressive effects exhibited by Gd-A coupled with our observations with fucoidan and sialyl Lewis^x, we hypothesized that the oligosaccharide chains expressed on Gd-A could also be responsible for mediating its potent immunosuppressive activities.

The highly unusual fucosylated lacdiNAc type glycans have been previously shown to be expressed on human recombinant protein C by Yan and co-workers (Grinnell, et al., 1994). This group of researchers have also shown that the fucosylated lacdiNAc sequence is a 15-20 fold more potent inhibitor of E-selectin binding to human umbilical vein endothelial cells (an experimental system used extensively to study the E-selectin) than sialyl Lewis^x (Grinnell, et al., 1994). Our structural analysis of the Gd-A glycans therefore

indicated that very potent selectin ligands are expressed on this glycoprotein (Figure 9). The presence of selectin ligands on Gd-A and its potent contraceptive and immunosuppressive activities therefore added support to our hypothesis that human sperm-ZP binding may involve a selectin-like interaction.

Initial Human Sperm-ZP Binding- A Carbohydrate Mediated Event

Extensive studies performed by many investigators have clearly indicated the involvement of the ZP oligosaccharides in mediating gamete binding in the mouse (Wassarman, 1990). However, unambiguous evidence required for proving a similar role for the ZP glycans in human sperm-ZP binding had not been obtained. Studies performed by Saling and co-workers have suggested that a 95 kDa zona receptor kinase (ZRK) expressed on the human spermatozoa is the actual egg binding protein (Leyton and Saling, 1989; Burks et al., 1995). Since all of the known protein tyrosine kinases can only bind to different peptides, the presence of ZRK has clearly instilled doubt about the role of the ZP oligosaccharides in mediating human gamete interaction (Aitken, 1995). Experiments performed with fucoidan have clearly indicated that specific glycoconjugates were capable of inhibiting sperm binding to the ZP (Mahony, et al., 1991). The present study was therefore undertaken to help resolve this controversy. The results obtained in our studies clearly indicate a role for the oligosaccharides in mediating human sperm-ZP binding.

The effect of chemical and enzymatic treatments on the ZP performed in this study provide strong evidence for the role of carbohydrates in mediating human sperm-ZP binding. They also illuminate the molecular events that may be occurring during this initial gamete interaction. Desialylation or EBGase treatment of the ZP results in a 2.5-3 fold increase in the number of sperm being bound to the ZP (Table 4). At least two reasons

were postulated to explain this substantial increase in sperm binding to the neuraminidase or EBGase treated ZP.

(1) It was theorized that the ligand for the egg binding protein present on the sperm is actually a peptide or protein epitope present on the ZP which is exposed due to the enzymatic removal of some of the terminal sugars;

(2) The egg binding protein of human sperm recognizes internal carbohydrate structures that are exposed due to the treatment of neuraminidase and EBGase.

Results obtained in this study, however, do not support the requirement of a protein-protein interaction for mediating initial human gamete binding. Studies with fucoidan, sialyl Lewis^x, AGP and the glycodelins clearly indicate that specific sugars can inhibit sperm-ZP binding. Furthermore, selective oxidation of the terminal sugars results in significant inhibition of sperm binding in the HZA. However, possibly the best data indicating the requirement of protein-carbohydrate interaction for mediating human sperm-ZP binding was obtained from the final experiment performed in this study. Mild oxidation of desialylated ZP inhibited sperm binding by 80% (Table 4). This result suggests that a majority (80%) of the sperm binding sites exposed after neuraminidase digestion are sensitive to mild periodate oxidation

The results of SMP treatment and neuraminidase digestion indicate that there may exist two modes for human sperm-ZP binding. The first mode depends in part upon the binding of terminal sialic acid. The second mode is independent of this terminal sugar. Binding in the presence of terminal sialic acid is weaker, either because of lower affinity or decreased numbers of binding sites of this type.

In this context it is interesting to note that Testart and co-workers have reported that desialylation of human sperm by *Arthrobacter ureafaciens* neuraminidase also leads to 3-fold increase in their ability to bind to the human ZP (Lassalle and Testart, 1994). Recent evidence indicates the presence of a 54 kDa sialic acid binding protein in the human uterine secretions (Banerjee and Chowdhury, 1997). This sialic acid binding protein has been shown to bind to a 23 kDa sperm plasma membrane protein (Banerjee and Chowdhury, 1997). The interaction of these two proteins results in the desialylation of the human sperm, possibly by the activation of a sperm surface associated neuraminidase. Thus desialylation of either the spermatozoa or the ZP may lead to a substantial increase in sperm-ZP binding.

The results obtained in this study combined with those reported by other investigators suggest a new carbohydrate dependent model for initial human gamete interaction. When the gametes encounter each other, the sperm plasma membrane associated neuraminidase interacts first with the sialylated oligosaccharides of the ZP, providing sufficient affinity for initial weak attachment of the spermatozoa. The neuraminidase activity then cleaves the ZP associated sialic acid enabling access of the other lectin-like egg binding proteins of the sperm to bind strongly to the glycan sequences located beneath this terminal sugar. This process may be facilitated by oviductin, a glycoprotein that binds to the ZP in the oviduct and greatly enhances this interaction (O'Day-Bowman et al., 1996). Thus the transition between loose sperm attachment and subsequent tight primary binding of the human gametes could be explained by this 'active digestion' model combined with other glycoproteins that promote adhesion.

There is some indirect evidence that supports this model. Optimal sperm binding to the ZP requires an extended incubation of 4 h (Burkman et al., 1988). The HZA when

applied as it is in our studies is considered to be a measure of tight binding (Burkman et al., 1988) that leads to the induction of the acrosome reaction (Franken, et al., 1991). Such an extended period of incubation of the hemizona with the spermatozoa may be required to enable the desialylation of the ZP glycans by the sperm associated neuraminidase activity resulting in the exposure of the internal oligosaccharide chains. The tight binding of the sperm to the hemizona in the HZA may hampered due to the lack of the adhesion facilitating glycoproteins like oviductin that are present *in vivo*.

The results obtained with EBGase are also significant. Litscher and Wassarman (1996) isolated a restricted 55 kDa lactosaminoglycan-containing glycopeptide from mouse ZP3 glycoprotein that blocked murine sperm-ZP binding. Digestion of this glycopeptide with EBGase resulted in a substantial loss of glycan mass but did not affect its capacity to inhibit gamete interaction. By contrast, the results of our EBGase digestion studies indicate that lactosaminoglycan type chains block potential binding sites for human spermatozoa. These rather bulky extensions of glycans obscure the binding sites either directly by being attached to the potential binding glycans or indirectly by being in close proximity to them.

Conclusion

The data obtained by studying the specific inhibition of sperm-ZP binding by glycoconjugates and by specific chemical and enzymatic manipulation of the ZP clearly indicates that the oligosaccharides expressed on the ZP are absolutely responsible for mediating initial human gamete binding. Considerable work will be needed to determine exactly how human spermatozoa bind to the ZP. However, the evidence indicates that this interaction may be considerably more complex than previously thought. The involvement of sialic acid, the evidence for a sperm-associated neuraminidase, and other considerations

discussed here support this conclusion.

Data obtained from experiments using sialyl Lewis^x, AGP, and the glycodelins have provided further proof that this initial interaction between the gametes may involve a selectin-like interaction. On-going research initiatives undertaken in our laboratory will focus on the characterization of the exact molecular events taking place during the initial human gamete interaction. The current study has raised the possibility of the requirement of a sperm surface neuraminidase in mediating this process. Further proof will be needed to prove this requirement.

Utilizing the latest technological advances in oligosaccharide structural analysis coupled with the experiments performed in the HZA, it is very likely that substantial information can be obtained about the initial human gamete binding events. Our future goals will be oriented to reach this objective.

CHAPTER III

INTRODUCTION

In our studies with the sperm-ZP binding system we observed that only those glycoconjugates that bear close resemblance to the selectin ligands are capable of inhibiting initial gamete interaction (Clark, et al., 1995). Fucoidan, sialyl Lewis^x and the fucosylated lacdiNAc terminals present on some of the Gd-A glycans have all been shown to be potent ligands for the selectins (Foxall, et al., 1992; Grinnell, et al., 1994; Varki, 1994). These studies suggest that the carbohydrate binding specificities of receptors mediating gamete recognition and lymphocyte/leukocyte adhesion may overlap at least to a certain extent. However, monoclonal antibodies directed against selectins do not bind to human spermatozoa, suggesting the existence of distinct egg binding proteins on the human spermatozoa that can bind to the selectin ligands. Why would it be beneficial for carbohydrate-dependent recognition systems to overlap in the human? What type of selective advantage(s) would be gained under such circumstances? A review of the literature in human reproduction and glycobiology strongly suggests that such a convergence could facilitate the exchange of information necessary to protect the human embryo/fetus during its development. The studies that lead us to this conclusion are discussed below. However, some of the essential aspects of human reproductive immunobiology are reviewed first.

The Ultimate Parasite

The human embryo formed after fertilization of the egg undergoes repeated cell divisions ultimately resulting in the formation of the blastocyst (Edwards, 1994). The blastocyst produces specialized cells known as the syncytiotrophoblasts. These cells invade and extravasate into the basement membrane of the uterine wall resulting in successful implantation of the embryo. Due to movement of the embryonic cells into the maternal endothelium the implantation is invasive resulting in close interactions between the embryonic cells like the trophoblasts and different components of the maternal immune system. Some of these embryonic cells express both the maternal and paternal antigens like the major histocompatibility complex (MHC) (Loke and King, 1991; Schmidt and Orr, 1993). The paternal antigens are usually foreign to the mother's immune system. The embryo is therefore usually referred to as a "semi-allograft". In some cases of surrogate pregnancy, the embryo is a complete allograft. The expression of foreign antigens on the embryonic cells should therefore evoke a strong maternal immune response against the implanting embryo (Loke and King, 1991; Wegmann and Guilbert, 1992; Schmidt and Orr, 1993). Evidence suggests that factors capable of producing a strong immune response are present throughout the period of pregnancy (Loke and King, 1991). The placenta expresses MHC class I antigens and some pregnant women also carry anti-HLA antibodies developed against the paternal antigens that theoretically should interfere with subsequent pregnancies with the same male (Regan et al., 1991). However, these women are usually capable of carrying the fetus to full term.

Human decidua of early pregnancy contains considerable numbers of natural killer (NK) cells (Loke and King, 1991). The NK cells are capable of killing cells that do not express the self MHC molecules. However, in most cases the presence of such factors that would normally launch allograft rejection of foreign tissues does not lead to fetal rejection. The fetus generally survives through the whole period of pregnancy. This situation is analogous to persistent infection with some parasites. Such parasites are capable of surviving immune challenge in the human hosts. These parasites are also adapted to obtain nutrients, and oxygen preferentially, characteristics shared by the fetus. It is for this reason that the embryo/fetus is sometimes referred to as the ultimate parasite.

It is generally accepted that specific suppressive factors are produced during the period of pregnancy inducing the immunotolerance that allows the fetal allograft to survive during its intrauterine development. Since pregnant women are not generally immunocompromised during or after pregnancy, it has been hypothesized that this immunosuppressive effect is highly localized to the uterus and expressed only during pregnancy (Hegde, 1991). Various factors like progesterone, α-fetoprotein, human chorionic gonadotrophin and the non-polymorphic MHC antigens such as HLA-G expressed on trophoblast at the fetomaternal interface have been implicated in maintaining this localized immunosuppressive effect (Hegde, 1991).

The Human Embryonic Defense System Hypothesis

Our recent findings and several other glycobiological observations suggest that specific glycoconjugates expressed by the endometrium in conjunction with those produced by the human embryo/fetus are responsible for manifesting significant contraceptive, anti-inflammatory, and immunosuppressive effects. This system is based upon specific

glycoconjugate interactions that abrogate initial sperm-egg binding, block selectin-mediated adhesions, and suppress lymphocyte-mediated responses. All of these activities expressed in a specific temporal fashion may serve to protect the developing embryo/fetus. We therefore refer to this model as the human feto-embryonic defense system hypothesis or the Hu-FEDS hypothesis (Clark, 1996). Known components of this system originate or are present in the glandular epithelium of the endometrium, the embryo/fetus, human amniotic fluid, and the human placenta.

The Different Components of the Hu-FEDS

Human amniotic fluid contains mucins of fetal or endometrial origin that inhibit E-selectin mediated adhesions (Walz et al., 1990). The mucins probably mediate this effect via their covalently linked sialyl Lewis^x terminated oligosaccharides. These mucins could therefore block acute inflammatory responses during pregnancy by inhibiting the primary adhesion of inflammatory cells. However, recruitment and activation of lymphocytes and other immune cells may require receptors other than the selectins. It is therefore likely that these mucins may not play a major role in potently inhibiting the lymphocyte-mediated responses.

Furthermore, the immune cell type that is found in the uterus during pregnancy is the $CD3^-CD56^+$ natural killer (NK) cells (Sato et al., 1990). However, these decidual cells do not attack the fetus under normal circumstances and have greatly reduced cytotoxicity. It is therefore likely that other components are expressed in the uterus during pregnancy that are specifically responsible for abrogating such lymphocyte responses.

We believe that Gd-A is one of the major component of this glycoconjugate based

immunosuppressive system. The expression of Gd-A is temporally and spatially regulated. During the menstrual cycle, Gd-A is not expressed in the proliferative endometrium but increases significantly from the fourth postovulatory day, peaking around day 12 (Julkunen, et al., 1986). Thus Gd-A expression is at a minimum during the peri-ovulatory period of the cycle. However, at the time of implantation of the embryo, Gd-A synthesis in the decidua is induced to very high levels (4-10% of total protein). This glycoprotein is also found in significant concentrations in the amniotic fluid, reaching a final concentration above 40 μg/ml by mid-trimester.

As mentioned in the discussion section of Chapter II, Gd-A manifests several suppressive activities when tested in the immunological assay systems. Gd-A has been shown to be involved in inhibiting the thymidine uptake in stimulated lymphocytes, suppression of the cytokines IL-1, IL-2 and IL-2 receptor synthesis, and to block the lysis of K562 cells by the NK cells at low concentrations (Bolton, et al., 1987; Pockley, et al., 1988; Pockley, et al., 1989; Pockley and Bolton, 1989; Pockley and Bolton, 1990; Okamoto, et al., 1991). Thus previous reports clearly suggest that Gd-A manifests potent immunosuppressive effects. Gd-A synthesis is highly localized. Although it is found in the blood of pregnant women, its maximal systemic concentration (2 μg/ml) is well below that required for the suppression of the T- and NK cell responses (Julkunen, et al., 1985). The highly regulated temporal and spatial expression and the potent immunosuppressive effects manifested by Gd-A clearly meet all of the necessary criteria that would be necessary for an agent responsible for mediating immunotolerance of the embryo/fetus during pregnancy.

α-Fetoprotein (AFP) a glycoprotein that is synthesized by the fetal liver and the yolk sac has been associated with mediating immunosuppression of the fetal and the

maternal immune responses (Deutsch, 1991). Suppression of the fetal immune response during pregnancy is also likely to be crucial for maintaining tolerance of the developing human. AFP is also synthesized by hepatomas and other ovarian cancer cells and is therefore referred to as an oncofetal antigen (Ruoslahti, 1979). Previous studies indicate that AFP expressed during early pregnancy carries bisecting type *N*-linked glycans (Figure 16). The expression of the bisecting type glycans on AFP peaks around week 16 of pregnancy and then slowly declines so that at the time of birth no bisecting GlcNAc sequence is present on this glycoprotein (Deutsch, 1991).

Recent studies indicate that glycans bearing the bisecting GlcNAc sequences (Figure 16) suppress NK cell response when expressed on the cell surface. Electroinsertion of glycophorin, a major erythrocyte glycoprotein, into membranes of K562 cells makes them resistant to NK cell attack (el Ouagari et al., 1995). K562 are human erythroleukemic cells which do not express any MHC Class I molecules and are therefore efficiently killed by human NK cells. Glycophorin expresses one *N*-linked glycan in addition to many other *O*-linked chains (el Ouagari et al., 1995). A substantial number of the *N*-linked glycans of glycophorin are of the bisecting type. Selective removal of these *N*-linked glycans associated with glycophorin by the enzyme PNGase F completely eliminates its ability to suppress NK cell mediated responses. In addition stable transfection of K562 cells with the enzyme that catalyzes the synthesis of the bisecting GlcNAc sequence (GlcNAc transferase III; Table 2) yields NK cell resistant transformants that show enhanced expression of this glycan on their cell surface (Yoshimura et al., 1996).

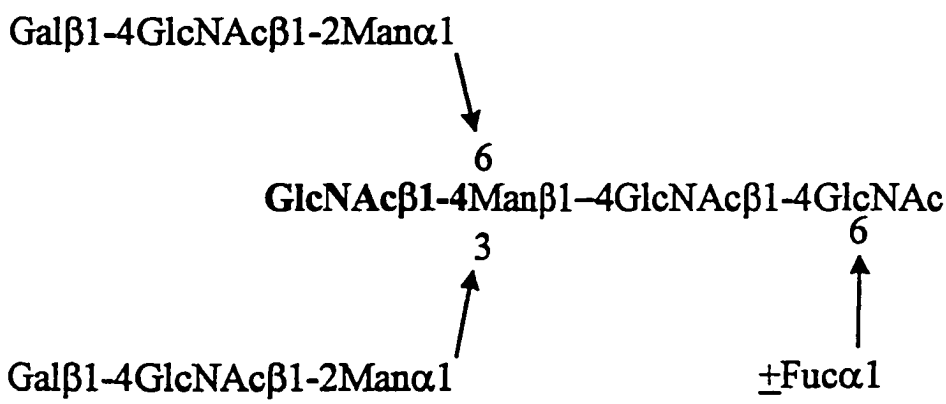


Figure 16: Structure of the bisecting type biantennary glycan associated with the suppression of NK cell mediated cytotoxicity.

The temporal expression of the bisecting type glycans on AFP during pregnancy points to an interesting feature. It is possible that expressing different type of glycans on the same carrier protein like AFP at different time points during pregnancy permits an additional control on the physiological events. Such dynamic changes in the glycosylation patterns of the same protein could possibly allow the same protein to perform different biological function(s). Thus such temporal variations in the glycosylation patterns probably makes the system extremely flexible to adapt to the ever changing physiological needs during pregnancy.

A Model For the Biological Actions of Gd-A

As discussed in Chapter II, Gd-A is a potent inhibitor of human sperm-ZP binding in the HZA. To our knowledge, this finding is the first demonstration that a human glycoprotein can manifest both immunosuppressive and contraceptive effects. It is very likely that the temporal regulation of Gd-A synthesis prevents this contraceptive glycoprotein from interfering with the fertilization of the human eggs. As mentioned above, Gd-A is not synthesized during the peri-ovulatory cycle, the period when fertilization occurs. Subsequent induction of Gd-A synthesis 4-5 days after ovulation possibly creates a contraceptive microenvironment in the uterine cavity. Thus a 'fertilization window' may extend from the time of ovulation to the induction of endometrial Gd-A synthesis. Any spermatozoa that migrate through the uterine cavity after the synthesis of Gd-A has been initiated would encounter this contraceptive glycoprotein in the uterine fluid and thus would be unable to fertilize eggs released outside the appropriate temporal window.

A large number of NK cells are recruited to the uterus just after fertilization. Unlike

the T- or B-cells, the NK cells do not require prior exposure to antigen to manifest cytolytic activity against their targets. The pre-embryo is therefore a potentially vulnerable target for the NK cell cytolytic responses. However, through these initial developmental stages the pre-embryo is still encapsulated in the ZP. It is probably at this time that the glycans expressed on the ZP provide a protective shield to successfully ward off any such immune responses. At about the same time, Gd-A is being increasingly synthesized by the decidual cells. Therefore, during the implantation process, when the embryo 'hatches' out of its protective ZP shield, Gd-A provides efficient protection against the NK cells. Implantation of the embryo therefore continues, unabated by the maternal immune system. It must be noted here that other glycoconjugates, as yet known or unknown, may also be produced during this process which also play a role in immunoprotection. However, it is true that Gd-A remains the best characterized immunosuppressive factor expressed during pregnancy.

The Gd-A Glycans

As mentioned in the discussion section of Chapter II, Gd-A carries highly unusual fucosylated lacdiNAc type sequences. Such sequences have been previously shown to be potent selectin ligands. It is intriguing to note that mature schistosomes and filarial worms, two highly persistent parasites known to infect humans, also carry the fucosylated lacdiNAc sequence (Srivatsan et al., 1992; Kang et al., 1993). It will be interesting to investigate if these parasites utilize the same glycoconjugate based system of evading immune responses that are used to protect the human embryo/fetus from the maternal immune response. If these parasites do use a similar system for protecting themselves from the host's immune response, the term parasite used for the human embryo/fetus could take on a new meaning.

It should be noted that tumor cells that express selectin ligands exhibit enhanced tumorigenicity, suggesting that such glycans may also be protecting the cancer cells from cell-mediated immune responses.

All of the NeuAc expressed on Gd-A is linked in the α2-6 configuration to the terminal Gal or GalNAc. CD22, a B-cell maturation receptor has been shown to bind α2-6 linked NeuAc. Although we have not been able to demonstrate binding of Gd-A to CD22 by using affinity chromatography or Western blot analysis, it must be noted that a potential role for the α2-6 linked NeuAc does exist. We are currently synthesizing the different glycans expressed on Gd-A to better characterize their roles in different immunological assay systems. Such studies would provide considerable information that would be vital to obtaining unambiguous evidence for the Hu-FEDS hypothesis.

Goals of the Current Study

The specific expression of Gd-A during pregnancy, the occurrence of highly unusual but potent selectin ligands on this glycoprotein coupled with the expression of these glycans on parasites like schistosomes and filarial worms strongly suggests that a glycoconjugate based system protecting the developing human embryo/fetus exists. Considerable evidence will no doubt be needed to prove the Hu-FEDS hypothesis. It would also be extremely useful to determine if such a system of immunoprotection also exists in other placenta-bearing mammals. If so, it could be possible to utilize lower mammals or primates as models to study the existence of such a system. In the present study we have attempted to determine the expression of immunosuppressive carbohydrates on the surface of the human gametes. The rationale behind the current study is briefly summarized below.

As mentioned previously, the glycans expressed on the ZP may also be playing a role in protecting the developing pre-embryo. A similar situation exists with the sperm. Insemination of the female results in recruitment of large number of neutrophils (Pandya and Cohen, 1985). Unless coated with anti-sperm antibodies, the neutrophils do not phagocytose the spermatozoa (D'Cruz et al., 1992). In most cases, the neutrophils kill the phagocytosed pathogens like bacterial and viruses by ingesting and then "bathing" them with reactive oxygen species like peroxides. Antibody coated sperm somehow manage to completely suppress the release of hydrogen peroxide and free radicals into the pahgosome of the neutrophils after phagocytosis (D'Cruz, et al., 1992). Furthermore, in most cases, the female immune system is recurrently exposed to the sperm antigens. However, usually no major immune response is produced against the sperm antigens. In those cases where anti-sperm antibodies are produced, the female usually experiences severe fertility problems.

Human gametes do not express any MHC class I molecules on their surface. The major immune cell type found in the uterus are the NK cells (70-80% of total immune and inflammatory cells). NK cells survey both immune and non-immune cells of the body for self Class I molecules and destroy those cells where such molecules are missing (Moretta et al., 1992). The NK cells are therefore continuously surveying for targets that lack the expression of MHC Class I molecules. The human gametes perfectly fit the profile for being good candidates for destruction by NK cells. In addition to the human gametes, the cells produced by the embryo, syncytiotrophoblasts, and the villous trophoblasts are all Class I negative (Vince and Johnson, 1995). The extravillous cytotrophoblasts express HLA-G, a restricted polymorphism MHC molecule known to suppress the NK-cell mediated

cytotoxicity (Chumbley et al., 1994; Pazmany et al., 1996). It is therefore very likely that these embryonic cells and the human gametes engage a system independent of MHC recognition to protect themselves against lysis by the NK cells.

Based on our previous studies with Gd-A, we hypothesized that the human gametes may also be evading immune recognition in the female reproductive tract by expressing specific glycoconjugates. Our goal for this study was therefore to determine if any immunosupressive glycans are expressed on the human ZP. The lack of sufficient numbers of human eggs precluded our ability to directly analyze the ZP glycans. Therefore in this study we attempt to probe the human ZP with specific lectins to determine if any of the glycans previously implicated in mediating immunosuppression are expressed on their surfaces. In a previous study several lectins were tested for their ability to bind to human ZP (Shalgi et al., 1991; Maymon et al., 1994). Three lectins that were not tested in this previous study were analyzed in this study to determine if they could bind to human ZP. These lectins were selected because they bind to glycan sequences associated with glycoconjugates that suppress NK cell-mediated cytotoxicity. *Sambucus nigra* agglutinin (SNA) was chosen because of its specificity for terminal α2-6 NeuAc linked to a lactosamine sequence (Galβ1-4GlcNAc) (Shibuya et al., 1987). As mentioned before, the α2-6 linked NeuAc is expressed on Gd-A. *Wisteria floribunda* agglutinin (WFA), a GalNAc binding lectin that is known to bind to the oligosaccharides carrying terminal fucosylated lacdiNAc type sequences (Nyame et al., 1989) that were also found on Gd-A. Both WFA and SNA have been shown to react with Gd-A in a carbohydrate dependent manner. Erythroagglutinating phytohemaglutinin (E-PHA) the third lectin used in this study has a very strict specificity for the biantennary and triantennary *N*-linked glycans that bear a

bisecting GlcNAc linked β1-4 to the β-linked mannose (Figure 17) (Cummings and Kornfeld, 1982; Yamashita et al., 1983). This molecular probe was chosen because the bisecting type glycans recognized by this lectin have been shown to suppress NK cell activity when they are expressed on cell surfaces as discussed above.

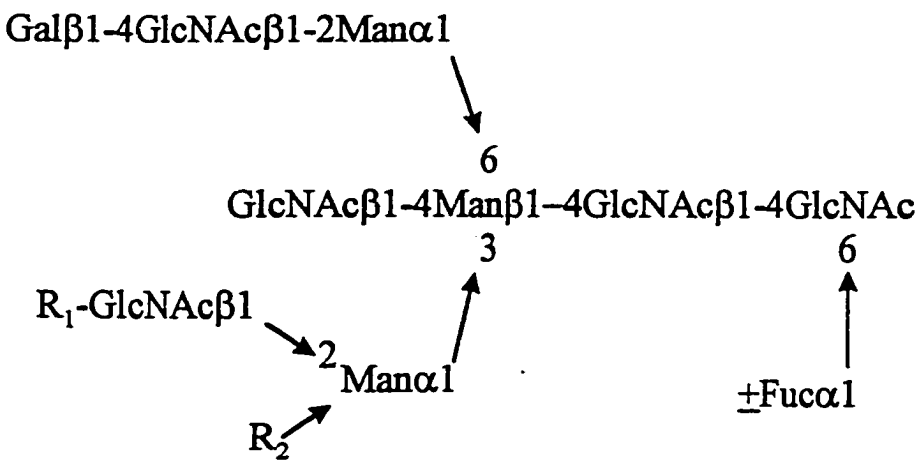


Figure 17: Oligosaccharide sequences recognized by E-PHA. R_1 and R_2 are either a hydrogen or a carbohydrate group.

MATERIAL AND METHODS

Materials and Human Samples

Fluorescein isothiocyanate (FITC)-labeled E-PHA, SNA and WFA were purchased from E-Y laboratories (San Mateo, CA, USA). Conalbumin, bovine milk galactosyltransferase, trypsin and uridine 5'-diphosphogalactose were purchased from Sigma (St. Louis, MO, USA). Human gametes were obtained and manipulated as explained in the materials and methods section of Chapter II.

Synthesis of Inhibitors of FITC-E-PHA Binding

Conalbumin was digested with trypsin after derivatization as previously described (Dell, et al., 1995) to yield glycopeptides (3 mg) that were enzymatically galactosylated by incubation with bovine milk β-galactosyltransferase (5 units) and uridine-5'-diphophogalactose (5 mg) for 24 h under optimal conditions (Barker, et al., 1972). A sample of the glycopeptides (100 mg) was digested with peptide N-glycosidase F to yield oligosaccharides that were analyzed by FAB-MS as previously described (Dell, et al., 1995). This analysis confirmed complete galactosylation of the conalbumin glycans to biantennary and triantennary bisecting type N-linked oligosaccharides that would react with E-PHA (Cummings and Kornfeld, 1982; Yamashita, et al., 1983).

Lectin Binding Studies

The lectin binding to the human eggs and hemizona was performed as described in the materials and methods section of Chapter II. The swim-up sperm obtained as described in the methods and materials section of Chapter II were used for labeling with FITC-E-

PHA. Non-acrosome reacted swim-up sperm were air dried overnight on a glass slide, methanol-fixed for 15 min and rehydrated in PBS as described previously (Cross and Overstreet, 1987). The slide was incubated in PBS containing FITC-E-PHA for 15 min, washed five times in PBS and inspected for fluorescence as described for the human egg and hemizona.

Labeling of sperm with FITC-E-PHA in Solution

Swim up sperm at a final concentration of 0.5 million cells/ml in HAMS-HSA were incubated for 10 min at room temperature. The effect of the lectin on the sperm was visually observed under a phase contrast microscope.

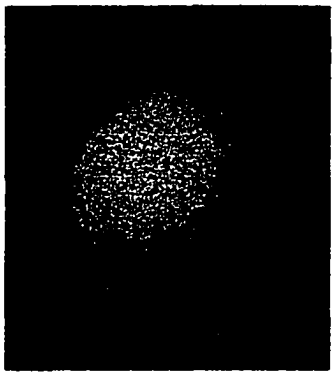
RESULTS

No detectable binding of FITC-SNA or FITC-WFA to whole human eggs was observed even when relatively high concentrations of the lectins (50 μg/ml) was used (data not shown). Extremely strong binding of FITC-E-PHA to the whole human egg was detected (Figure 18). This binding was detectable even at low lectin concentrations of 1 μg/ml. Optimal binding was seen at 10 μg/ml concentration of FITC-E-PHA. Hemizona obtained by microbisection of the eggs and removal of the cellular contents of the ooplasm were also found to bind strongly to FITC-E-PHA. For the hemizona the binding was observed even at 1 μg/ml concentration, however, optimal binding was observed at 5 μg/ml concentration of the lectin (Figure 18). There was a universal homogeneous binding of FITC-E-PHA to all eggs and hemizona tested. The binding of FITC-E-PHA was carbohydrate specific. A mixture of biantennary and triantennary bisecting type N-linked glycans obtained from conalbumin significantly reduced FITC-E-PHA binding to the hemizona (Figure 18).

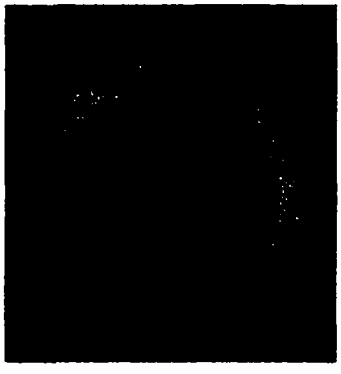
In a previous study it was shown that immobilized human spermatozoa also bind E-PHA in a carbohydrate dependent manner (Cross and Overstreet, 1987). We were able to reproduce this result. Substantial labeling of immobilized spermatozoa was observed when incubated with 20 μg/ml FITC-E-PHA (Figure 18). Labeling was found all over the surface of the sperm, although the acrosomal region was found to be more highly labeled. In other experiments we have also observed that live swim-up sperm bind to E-PHA in solution. In solution E-PHA has been shown to form tetramers. The lectin can therefore

Figure 18: Binding of FITC-E-PHA to human gametes. FITC-E-PHA strongly binds to the whole human egg (Panel A). Panel B shows that the lectin binding to hemizona, indicating that the E-PHA binding sites exist on the ZP. The binding of this lectin can be considerable reduced by adding a bisecting type glycan bearing glycopeptides isolated and synthesized from conalbumin (Panel C). Panel D shows binding of the lectin to sperm.

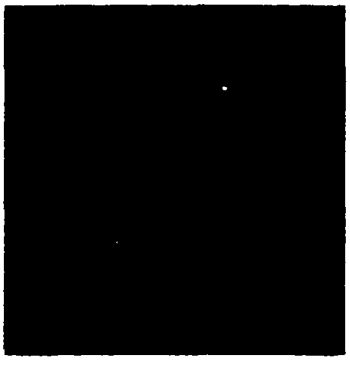
A



B



C



D

bind four moles of the bisecting type *N*-linked glycans. We have observed that incubation of the live sperm with low amounts of E-PHA results in rapid agglutination of the sperm. When the sperm were incubated with 10 and 20 μg/ml of the lectin at room temperature an almost instant aggregation of the sperm was observed (data not shown). Incubation with 1 and 5 μg/ml required 5 min and 2 min respectively for the spermatozoa to aggregate (data not shown).

DISCUSSION

In the present study we were unable to show binding of WFA and SNA to the human ZP. It is therefore unlikely that the fucosylated lacdiNAc or α2-6 linked NeuAc would be expressed on the human ZP. As mentioned earlier these sugars were found on the Gd-A glycans. We have however demonstrated that E-PHA binds strongly to the human ZP (Figure 18). E-PHA has been previously shown to be highly specific for bisecting type biantennary and triantennary *N*-linked glycans.

As mentioned earlier, the human gametes do not express MHC class I antigens. The expression of bisecting type N-linked glycans may therefore be crucial for the suppression of NK cell-mediated responses especially after the egg is fertilized and the paternal contribution to the embryonic genome becomes detectable. Spermatozoa may also encounter NK cells during their movement through the uterus to reach and fertilize the egg. How spermatozoa are protected from maternal NK cells is currently unknown. It is however possible that the spermatozoa are also protected due to the presence of specific oligosaccharide sequences like the bisecting type *N*-linked glycans.

Conclusion

Using specific lectin probes we have clearly demonstrated the presence of glycans that may be involved in suppression of NK cell mediated responses. The results obtained in the next chapter clearly indicate that the bisecting type *N*-linked glycans can inhibit the NK cell function. These results therefore strongly suggest that glycoconjugate dependent

suppressive system, as outlined in the Hu-FEDS hypothesis, plays a major role in protecting the human gametes and the developing embryo/fetus from the maternal immune responses.

CHAPTER IV

INTRODUCTION

Natural killer (NK) cells comprise approximately 5-10 % of the total lymphocyte population in the human body. The NK cells are known to provide immune surveillance by killing tumor and some virally infected cells that do not express self MHC molecules (See et al., 1997). Their physiological role therefore complements that played by the T-lymphocytes in mediating cellular immunity in mammals. The T-cells specifically recognize the foreign peptides presented on the self MHC molecules. Detection of such foreign peptides results in cytolysis of the target cells. However, in some virally infected or tumor cells the expression of MHC molecules on their surface is either lost or severely down regulated. The NK cells have the ability to recognize the absence of self MHC molecules and resulting in the killing of such targets. In essence, therefore the NK cells are involved in the recognition of "missing self". This hypothesis was first put forward by Karre (1991).

The major phenotypic difference between the T- and the NK cells is the lack of the T-cell receptor (TCR) complex in the latter (Leiden et al., 1988). The T-cells are capable of identifying the various foreign peptides due to the presence of TCR. Similar to the gene recombination events that occur with the immunoglobulin molecules leading to the formation of antibodies with different antigen specificities, gene rearrangements lead to the various T-cell clones carrying TCR with different peptide specificities. A vast repertoire of T-cell clones is therefore generated with each clone capable of identifying a specific foreign peptide sequence when presented on the MHC molecules of the antigen presenting cells.

The mechanism for target recognition utilized by the NK cells is however very different. Similar to the T-cells, different clones of the NK cells have been reported. Each of these clones has a strict specificity to a particular MHC allele. For example based on their specificity for the HLA-C alleles, the human NK cells have been classified as Group 1 and Group 2 clones (Moretta, et al., 1997). The group 1 clones recognize the Cw2, Cw4, Cw5, and Cw6 alleles whereas the group 2 clones recognize the Cw1, Cw3, Cw7, and Cw8 alleles (Moretta, et al., 1997). Presence of these alleles on a normal cell inhibits their killing by the respective clones. Such alleles are therefore termed as "protective alleles". NK cell clones specific for different alleles of the HLA-A and HLA-B molecules have also been reported (Moretta, et al., 1997). The allelic specificities of the different NK cell clones has been elucidated by transfecting HLA class I negative cells with the different HLA class I alleles and the ability of such cells to evade NK cell cytolysis. Experiments performed with HLA-C alleles have indicated the existence of a dimorphism in the amino acid residues at positions 77 and 80 in the α_1 domain of the HLA-C molecules. The protective alleles recognized by the Group 1 clones have a asparagine at position 77 and lysine at position 80. Alleles that inhibit lysis mediated by the Group 2 NK cell clones have a serine at position 77 and asparagine at position 80 (Moretta, et al., 1997).

Recognition of the specific HLA class I alleles is mediated by different inhibitory receptors expressed on the surface of the NK cells. Such receptors are expressed clonally on the NK cells. One such inhibitory receptor is termed as p58, a 58 kDa protein (Moretta, et al., 1997). Two different molecular forms of p58 have been identified to be characterized on two different NK cell clones. The p58.1 inhibitory receptor has been found to recognize Cw4 as the protective HLA-C allele whereas NK cells expressing p58.2 require the Cw3

allele to suppress their cytolytic responses (Moretta, et al., 1997).

Other known NK cell inhibitory receptors are Ly49 in the mouse, and p70, or p140 in the human. The intracytoplasmic tails of all of these NK cell inhibitory receptors (also known as killer cell inhibitory receptors, KIR) carry the immunoreceptor tyrosine-based inhibitory motif (ITIM) which is used for cell signaling. Binding of the receptor to its specific HLA allele results in the activation of intracellular phosphatases like the SHP-1 in the mouse resulting in a signaling cascade which leads to inhibition of the cytolytic response (Mason et al., 1997). It is significant that truncated forms of all of these inhibitory receptors lacking the ITIM motifs serve as activating receptors (also known as killer cell activating receptors, KAR) (Ciccone et al., 1996). There appears to be a delicate balance between the responses mediated by the activating and the inhibitory receptors that controls the cytolytic activity of the NK cell against a target cell.

Suppression of NK cell Responses by Glycoconjugates

In addition to the mechanisms discussed above, the cytolytic responses mediated by NK cells have also been shown to be inhibited by various glycoconjugates. Gd-A potently inhibits lysis of K562 erythroleukemic cells by NK cells (Okamoto, et al., 1991). The bisecting type GlcNAc sequence (Figure 16) expressed on glycophorin has also been suggested to block NK cell responses (el Ouagari, et al., 1995; Yoshimura, et al., 1996). It is as yet very unclear how such glycoconjugates mediate their inhibitory effects. However, many KIR like CD94, NKG2, and NKR-P1A in the human and NKR-P1 in the rat have been shown to possess C-type lectin domains (Moretta, et al., 1997). Furthermore, truncated forms of all of these lectin receptors that do not carry the ITIM motifs have been shown to serve as KAR's. The presence of the C-type lectin domains on these receptors

strongly suggests a role for glycoconjugates in mediating NK cell recognition. The role of these lectin receptors is currently under intense scrutiny.

Focus of the Current Study

In our previous study (Chapter III) we have shown that the bisecting type *N*-linked glycan is expressed on the human gametes. It is therefore likely that expression of this type of glycan may be responsible for suppressing the anti-embryo/fetus responses that could be mediated by the maternal NK cells that are recruited in high numbers during the implantation process. However, concrete evidence for the involvement of the bisecting type *N*-linked glycan in suppressing the NK cell responses has not been clearly demonstrated. In this study we have therefore attempted to determine the effect of the bisecting type glycans on NK cell function. Inhibition of the NK cell cytolytic responses by such glycans could provide valuable evidence for the existence of a glycoconjugate based suppressive system responsible for protecting the human embryo/fetus from the maternal immune system as outlined in the Hu-FEDS hypothesis.

It must however be mentioned at the outset that all of the studies shown here should be considered with a restriction. Due to ethical considerations uterine NK cells could not be obtained from pregnant women. Therefore all of the experiments were conducted using peripheral blood NK cells. Uterine NK ($CD2^+$, $CD3^-$, $CD16^+$, $CD56^+$, $CD94^+$) cells are of a different phenotype than those found in the peripheral blood ($CD2^+$, $CD3^-$, $CD16^+$, $CD56^{+/-}$, $CD94^{+/-}$). It is therefore possible that the effect of the glycans tested on the peripheral blood NK cells may not be similar to that on the uterine NK cells. Considering the constraint placed due to the unavailability of the correct biological materials, we believe that the peripheral blood NK cells can serve as an excellent model.

Ovomucoid A Source for Bisecting Type Glycans

In Chapter III we utilized the glycopeptides enzymatically synthesized and isolated from conalbumin, a chicken egg albumin protein, as a source to obtain the bisecting type glycans used to block the binding of FITC-E-PHA to the hemizona. However, conalbumin was found to express very low level of the bisecting type biantennary glycan (5-10%) and high levels of the bisecting type triantennary glycan that also reacts with E-PHA (Corradi da Silva et al., 1994). As mentioned earlier, the bisecting type biantennary glycans expressed on glycophorin were found to be essential for inhibiting NK cell function. Conalbumin would therefore have yielded low amounts of the bisecting type biantennary glycans. Ovomucoid is a 23 kDa glycoprotein that is present in significant amounts in the chicken egg white. This glycoprotein has four major *N*-glycosylation sites (Asn-10, Asn-53, Asn-75 and Asn-69) with one partial glycosylation site (Asn-175) (Yet et al., 1988). A summary of the different carbohydrate chains expressed on the four major glycosylation sites is shown in Figure 19. Table 5 lists the amount of each glycan present on the four major glycosylation sites. A majority of the glycans expressed on ovomucoid are non galactosylated (Figure 19). However small amounts of these glycans bear 1-2 moles of galactose on each glycan chain (Table 5). The bisecting type biantennary glycan is present in either the non- or partially galactosylated form on 26, 33, 36 and 40% of the glycans at Asn-10, Asn-53, Asn-75 and Asn-69 respectively (Table 5) (Yet, et al., 1988). Overall, the non- or partially galactosylated forms of bisecting type biantennary glycan constitutes approximately 34% of the total glycans associated with ovomucoid. In addition, the bisecting type glycans with 3, 4, and 5 antennae (Figure 19) could be used as control glycans to test the specificity of the biantennary bisecting type glycans in affecting NK cell

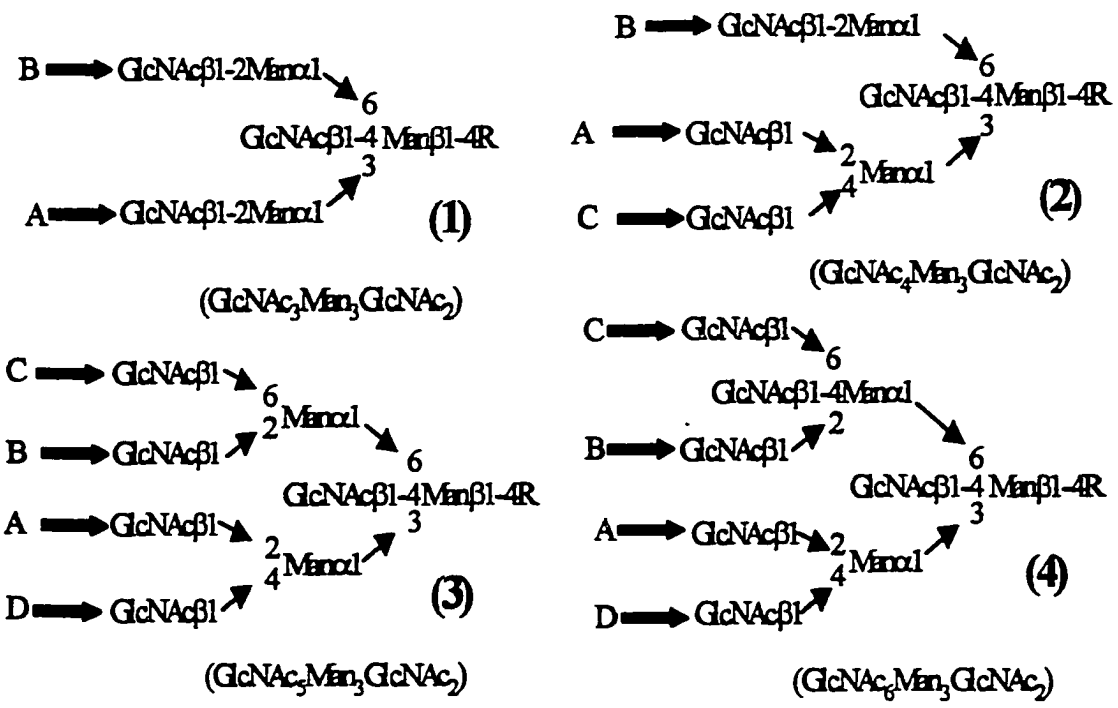


Figure 19: Major oligosaccharide sequences found in ovomucoid and their abbreviations which will be used in Table 5. The arrows indicate the preference shown by Gal-T for the Gal residues.

Table 5: Percent distribution of the different ovomucoid glycans on its four major glycosylation sites

Structures	% Glycan at Each Glycosylation Site			
	Asn-10	Asn-53	Asn-75	Asn-69
$GlcNAc_3Man_3GlcNAc_2$	19	24	23	31
$GalGlcNAc_3Man_3GlcNAc_2$	2	5	8	6
$Gal_2GlcNAc_3Man_3GlcNAc_2$	5	4	5	3
$GlcNAc_4Man_3GlcNAc_2$	21	25	19	20
$GalGlcNAc_4Man_3GlcNAc_2$	5	10	8	4
$Gal_2GlcNAc_4Man_3GlcNAc_2$	3	2	4	3
$GlcNAc_5Man_3GlcNAc_2$	14	10	3	6
$GalGlcNAc_5Man_3GlcNAc_2$	3	4	3	3
$Gal_2GlcNAc_5Man_3GlcNAc_2$	2	-	1	-
$GlcNAc_6Man_3GlcNAc_2$	11	8	4	2
$GalGlcNAc_6Man_3GlcNAc_2$	3	-	1	2
$Gal_2GlcNAc_6Man_3GlcNAc_2$	-	-	-	-

function. We have therefore used ovomucoid to synthesize and isolate the different types of glycans required in this study.

Another point worth noting here is that we have used glycopeptides isolated by trypsin treatment of ovomucoid instead of free oligosaccharides. The glycopeptides were specifically used for the following reasons:

(1) As mentioned in the discussion section of Chapter II, only appropriately presented oligosaccharide chains seem to be active in biological assay systems. Extensive research performed with the selectins strongly supports this hypothesis. Therefore, it was very likely that the use of glycopeptides instead of the free oligosaccharides could help present these glycans in a more physiological configuration whereby they could better elicit their actual biological effects in the NK cell assays.

(2) Another advantage in using the glycopeptides was that the peptide sequence could be tagged with reagents like fluorescein to allow for sensitive detection during the enzymatic synthesis and isolation of the glycopeptides and possibly also during the NK cell assays if the need arises.

Trypsin has a fairly strict specificity for proteolytically clipping peptide sequences. This enzyme preferentially cleaves at the carboxyl terminal end of lysines or arginines present in the protein sequence. Analysis of the ovomucoid peptide sequence as listed in the Swiss-Prot database indicates that only four different glycopeptides could be obtained after trypsin treatment of ovomucoid (Figure 20). The smallest glycopeptide is GP-1 which carries the first glycosylation site (Asn-10) attached to a 7-mer. The glycosylation site at Asn-53 is attached to a 32-mer peptide. This glycopeptide is designated as GP-2. GP-3

constitutes a 19-mer peptide which carries the glycosylation sites at Asn-69 and Asn-75. The partially glycosylated site at Asn-175 is present in GP-4 (minor glycopeptide) which is a 22-mer peptide. Since each glycosylation site carries glycan sequences as listed in Table 5, different glycoforms of each of these glycopeptides exist. We have attempted to partially purify the bisecting type biantennary and triantennary glycans using E-PHA lectin affinity chromatography and analyzed them for their effect in the NK cell assays.

MAJOR GLYCOPEPTIDES

FPNATDK
(GP-1 at Asn-10; mass range 2114 - 3047 Da)

DLRPICGTDGVTYTNDCLLCAYSIEFGTNISK
(GP-2 at Asn-53; mass range 4950-5883)

ETVPMNCSSYANTTSEDGK
(GP-3 at Asn-69 and Asn-75; mass range 4459-6361)

MINOR GLYCOPEPTIDE

CNFCNAVVESNGTLTLSHFGKC
(GP-4 partially filled at Asn-175; precise mass range not yet determined)

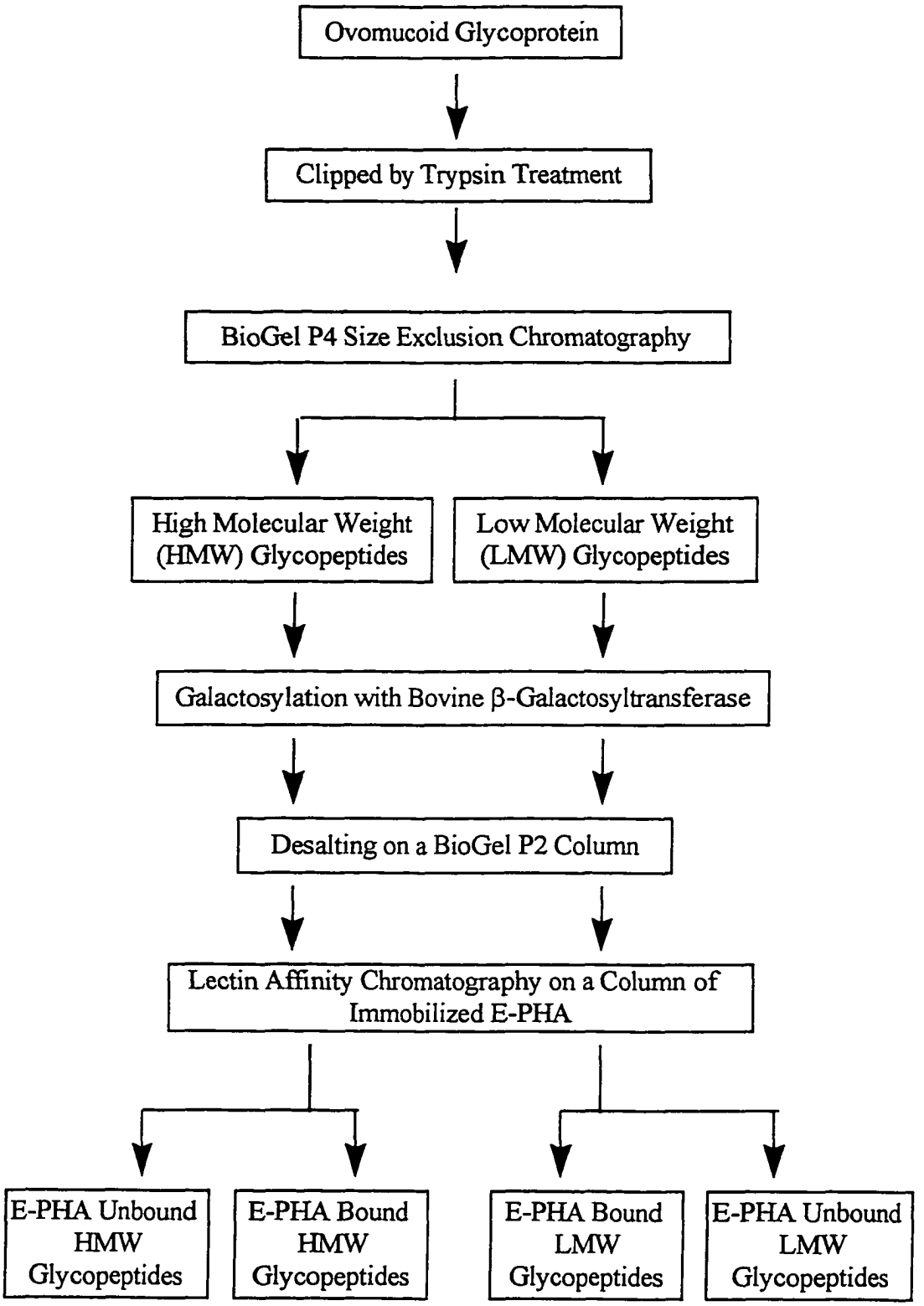
Figure 20: The structures of the peptide sequences attached to the three major (GP-1, GP-2 and GP-3) and the minor (GP-4) glycopeptide.

MATERIALS AND METHODS

Isolation of Ovomucoid Glycopeptides

The general scheme used to isolate and enzymatically synthesize the galactosylated glycopeptides is given in Figure 21. Ovomucoid (2 gm) was dissolved in 20 mL of 8 M guanidine hydrochloride and reduced for 30 min at room temperature by adding 1.5 gm of dithiothrietol (Corradi da Silva, et al., 1994). The reduced protein was acetylated with iodoacetamide (3.5 gm) for 1 hr at room temperature. The reduced and acetylated protein was dialyzed for 24 hr using 1000 molecular weight cut-off dialysis membrane against 2 mM tris-hydrochloride pH 8.0. The retained protein was lyophilized and resuspended in 2 ml 8 M guanidine hydrochloride. This solution was diluted with 20 mM Tris-hydrochloride pH 8.0 to make the final concentration of guanidine hydrochloride 2 M. TPCK-treated trypsin (20 mg) was added to the solution that was subsequently incubated at 37°C for 12 hr. The reaction was terminated after 4 hr by heating the solution on a boiling water bath for 3 min. The solution was dried on a SpeedVac apparatus (Savant) and resuspended in 2 ml of 0.1 M pyridine acetate (pH 5.4) buffer. Insoluble precipitate was removed by centrifugation. The precipitate was primarily composed of insoluble peptides as indicated by the carbohydrate analysis using the phenol-sulfuric acid assay (Dubois et al., 1956). The supernatant was desalted on a BioGel 2 (2.5 cm X 60 cm) column. Elution of the glycopeptides was monitored by phenol-sulfuric acid assay and by absorbance at 280 nm. Approximately 93% of the glycopeptides eluted in the void volume of the column. Fractions containing the glycopeptides were pooled and dried completely at low pressure.

Figure 21: Schematic diagram of the strategy utilized for isolation and synthesis of the different ovomucoid glycopeptides.



The dried sample was separated on the basis of size on a BioGel P4 (2.5 cm X 60 cm) column. The column was eluted with 0.1 M pyridine acetate (pH 5.4) and small aliquots (10 µl) were drawn for carbohydrate analysis using the phenol sulfuric acid assay. The high molecular weight (HMW) and low molecular weight (LMW) glycopeptides were pooled separately and dried completely. The amount of glycopeptide obtained was determined by phenol-sulfuric acid assay. 6 µmoles of HMW glycopeptides (assuming an average molecular weight of 3000 Da) and 8 µmoles of LMW glycopeptides (assuming an average molecular weight of 2500 Da) were obtained.

Galactosylation of Ovomucoid Glycopeptides

The ovomucoid glycopeptides (5 µl) were dissolved in 600 µl of Gal-T buffer (20 mM Na-cacodylate, pH 7.0, containing 1 mM magnesium chloride and 1 mM calcium chloride and 0.02% sodium azide). 300 µl of Gal-T buffer containing 30 µmoles of UDP-Gal and 1 X 10^7 cpm of UDP-[^{3}H]Gal was added followed by 10 units of Gal-T (Sigma) in 100 µl Gal-T buffer. The reaction mixture was incubated for 12 h at 37°C. Another aliquot of Gal-T (10 units) and 10 more µmoles of UDP-Gal in 100 µl Gal-T buffer were added to the reaction which was incubated for another 12 h at 37°C. The galactosylated glycopeptides were separated by desalting on a BioGel P2 column as before. The elution of the radiolabelled glycopeptides was followed by liquid scintillation. Fractions containing the glycopeptides were pooled and dried under low pressure. A 50 µg sample of the galactosylated glycopeptides was forwarded to Dr. Anne Dell for FAB-MS analysis.

Separation of Galactosylated Glycopeptides by E-PHA Chromatography

To avoid over loading, 30-45 nmol of the glycopeptides were separated on the E-

PHA-Affigel 10 (5 ml column; 6 mg lectin/ ml of gel) per run. The lectin column was washed thoroughly with phosphate buffered saline (PBS; 20 mM sodium phosphate pH 7.4 containing 150 mM sodium chloride and 0.02% sodium azide). The glycopeptides (200 µl) in PBS loaded on the column. The column was washed with 25 ml of PBS and fractions (2.5 ml) were collected. The bound glycopeptides were eluted with PBS containing 0.4 M GalNAc. The elution of the unbound and bound glycopeptides was determined by liquid scintillation counting of a small aliquot (20 µl) of the total fraction. After performing several runs on the column, 750 nmoles (1.5 mg) of the glycopeptides were isolated from 3 µmoles of the galactosylated. Approximately 10-20 mg of the glycopeptides obtained after E-PHA chromatography were analyzed by FAB-MS.

Coupling of E-PHA to AffiGel-10

AffiGel-10 (BioRad; 6 ml) was transferred to a 10 cm column and washed thoroughly to remove the isopropanol used to store the gel. The gel was then equilibrated with 0.1 M morpholinosulfonic acid buffer (MOPS), pH 5.0. 40 mg of E-PHA (EY Laboratories) was dissolved in MOPS (3 ml) and the protein solution was added to the gel slurry. The column was capped tightly and rotated end over end at 4°C overnight. To cap any unreacted reactive groups on the gel, 1 M ethanolamine was added to make the final of ethanolamine 100 mM. The column was again capped and rotated for 2 h at 4°C. The column was then washed extensively with PBS followed by 1 M sodium chloride to remove any non specifically bound protein. The washings were collected and analyzed for unbound protein by determining the absorbance at 280 nm. Protein analysis indicated that 30 mg of E-PHA (5 mg/ml of gel) was bound to the AffiGel-10. The column was reequilibrated in PBS.

Isolation of Peripheral Blood NK Cells

The peripheral blood NK cells were isolated as described earlier. Briefly, 60 ml of heparinized blood was obtained from a healthy male individual. The mononuclear cells were separated from the red blood cells by density gradient centrifugation using Histopaque 1077 (Sigma). The mononuclear cells (10 ml) were diluted with an equal volume of RPMI-1640 media containing penicillin-streptomycin-amphotericin cocktail obtained from Gibco and 2% fetal calf serum (RPMI-2) and centrifuged for 10 min at 250 X g using a swinging bucket type rotor at room temperature. The pelleted cells were washed one more time with RPMI-1640 media containing penicillin-streptomycin-amphotericin cocktail obtained from Gibco and 10% fetal calf serum (RPMI-10) under the same conditions. The pelleted cells were resuspended in 5 ml of RPMI-10 and loaded on 20 ml nylon wool columns presoaked in RPMI-10 at 37°C. The nylon wool columns were prepared by taking 1.8 gm of well combed nylon wool in a sterile 60 ml column. The nylon wool was washed with 40 ml of 37°C RPMI-2 followed by 30 ml of 37°C RPMI-10. The column were capped and incubated at 37°C in a 5% CO_2 incubator for at least 1 hr before loading the cells.

After loading the cells on the columns, some of the media was allowed to flow through the column to ensure that all of the cells were in contact with the nylon wool. After approximately 5 ml of RPMI-10 was slowly added on the column, the columns were capped and incubated at 37°C in the 5% CO_2 incubator for 1 h. After incubation the non-adherent cells highly enriched in NK and T-cells were obtained by washing the nylon wool column with 40 ml of 37°C RPMI-10. The eluted cells were collected in a 50 ml sterile centrifuge tube. The cells were pelleted by centrifugation at 250 X g for 10 min. Pelleted cells were washed with RPMI-2 as described before and were then resuspended in 2 ml of

RPMI-2. Typically, approximately 80-90 million cells were obtained at this point. The cell counting was performed by using a hemacytometer. Viability of cells was checked by the trypan blue dye exclusion assay. On an average more than 90% of the cells were found to be viable.

The cells were then subjected to negative antibody selection. A cocktail of mouse antibodies directed against the T-and B-cell epitopes was added to the cell suspension. The antibodies used were anti-CD3, anti-CD14, and anti-CD19. The cells were incubated with these antibodies on ice for 30 min. The cell suspension was gently shaken every 5 min to enable proper binding of the antibodies to the contaminating T- or B-cells. The antibody bound contaminating cells were separated from the NK cells by incubating the cell suspension with 1 ml of magnetic beads coated with goat anti-mouse IgG (BioDesign) on ice for 30 min. Prior to use the magnetic beads were thoroughly washed five times with RPMI-2 in a round bottom sterile plastic tube. After each wash, the beads were isolated by holding the tube against a strong magnet (BioDesign). The media was gently pipetted out and fresh RPMI-2 media was immediately added. The tube was taken apart from the magnet and the beads were gently resuspended in the media.

Beads attached to the contaminating cells were removed by placing the tube against a strong magnet as above. The media containing the NK cells was carefully pipetted out and a fresh 1 ml aliquot of the magnetic beads to the cell suspension. The separation was performed as described above. The cell suspension highly enriched in the NK cells was centrifuged at 250 X g to pellet the cells. The pelleted cells were washed with RPMI-1640 media containing penicillin-streptomycin-amphotericin cocktail obtained from Gibco and 10% human AB serum (RPMI-10-AB). The pelleted cells were finally suspended in 2-3 ml

of RPMI-10AB. Typically 15-18 million cells were obtained at this point in the isolation. The phenotype of the cells was checked by incubating approximately 100,000 cells in a 500 µl microcentrifuge tube with a cocktail of mouse anti human CD16 and mouse anti-human CD56 antibodies (5 µg/ml concentration of each antibody) on ice for 30 min. The cells were briefly pelleted and washed three times with 400 µl of RPMI-10. The cells were finally incubated with 5 µg/ml concentration of FITC labeled goat anti-mouse IgG for 30 min on ice. The FITC labeled antibody was washed off by centrifugation as above. The cells were then placed on a microscope slide and the number of FITC labeled cells in different fields of view were counted. On an average more that 85% of the total cells were fluorescent, indicating a high number of NK cells in the isolate.

Labeling of K562 Cells with ^{51}Cr

The K562 target cells were obtained from American Type Tissue Culture (ATCC). The cells were cultured in RPMI-10 for 3 weeks before labeling. 2 million cells were used each time for labeling. The cell counts were performed using a hemacytometer. Viability of the cells was determined by the trypan blue dye exclusion assay. The cells used for labeling were resuspended in 2 ml of RPMI-10 and incubated at 37°C for 1 hr in the presence of 100-200 mCi of $Na_2[^{51}Cr]_2O_7$. After incubation the cells were washed 4 times with 10 ml of RPMI-2 and finally once with 10 ml of RPMI-10. The pelleted cells were finally resuspended in 4 ml of RPMI-10.

Cytolytic Assays

The NK or LAK cytolytic assays were performed as described in an established protocol. General paradigm used for performing these assays is shown in Figure 22.

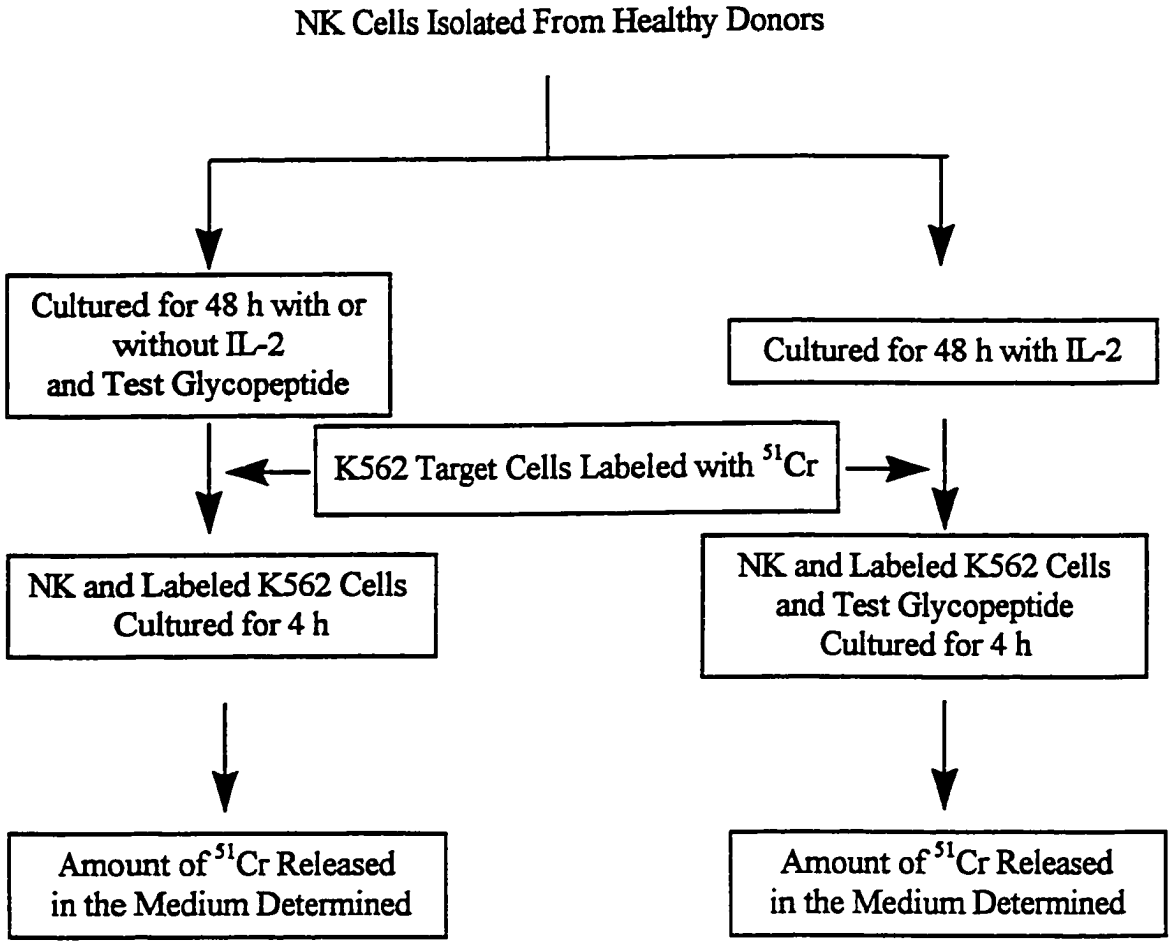


Figure 22: Schematic representation of the general procedure for performing the NK cell assays in the presence of the test glycopeptides.

The NK cells (approximately 2 million cells/ml media final concentration) were incubated in 12-well tissue culture plates (Falcon) in the presence or absence of appropriate amounts of the test glycopeptides for 48 h at 37°C in a 5% CO_2 incubator. For the LAK assays, the NK cells (approximately 1 million cells/ ml media, final concentration) were cultured in 12-well tissue culture plates in the presence of 6000 IU of IL-2 (R&D Labs) in the presence or absence of the appropriate amounts of the test glycopeptides. Cells were cultured for 48 hr in a 5% CO_2 incubator. Cytolytic assays were then performed using these cultured NK or LAK cells. In some cases, the treated LAK cells were transferred to a 15 ml sterile centrifuge tube and washed three times with RPMI-10 to remove the glycopeptide.

Viability of these treated NK or LAK cells was checked by the trypan blue dye exclusion assay. The cytolytic assays were performed in 96-well round bottomed tissue culture plates (Falcon). The total volume of each assay was 200 µl. Appropriate numbers of the treated or untreated NK or LAK cells were added to the wells. The total volume in each well was made 195 µl by adding the necessary amount of RPMI-10 media. This step was followed by adding 5 ml of the ^{51}Cr-labeled K562 (5000 cells total) targets. All of the assay were performed in triplicates. To determine the amount of spontaneous release of the ^{51}Cr activity from the targets, 5 µl of the labeled K562 cell suspension was added to 195 µl of media in six separate well. For calculating the maximal release, 5 µl of the ^{51}Cr-labeled target cell suspension was added to six separate wells containing 195 µl of 5% Triton-X100 in PBS. The plates were briefly centrifuged for 2 min at 1000 rpm and were incubated at 37°C in a 5% CO_2 incubator for 4 hr.

The plates were again centrifuged briefly as before. A 100 µl aliquot was carefully pipeted out from each of the wells and was assayed for the amount of ^{51}Cr in a gamma counter (Beckmann). The amount of cytolysis was determined by the following calculation-

% Cytolysis= (Amount of ^{51}Cr released in test) - (Amount of Spontaneous ^{51}Cr release)/(Amount of maximum ^{51}Cr release)-(Amount of Spontaneous ^{51}Cr release) X 100.

RESULTS

Isolation of Ovomucoid Glycopeptides

The general scheme used to isolate and enzymatically synthesize the galactosylated glycopeptides is given in Figure 21. Size exclusion chromatography of the ovomucoid glycopeptides on a BioGel P4 column resulted in the isolation of HMW and LMW glycopeptide fractions (Figure 21). It was realized that these glycopeptide fractions were substantially contaminated with peptides. It has been previously reported that immobilized E-PHA could be used to purify biantennary and the triantennary bisecting type glycans that have Galβ1-4GlcNAc attached via a β1-2 linkage to the α1-6 linked mannose of the core (Figure 17). Such a purification step could also lead to removal of the peptide sequences from the glycopeptides expressing these glycans. However, as mentioned before, a majority of the glycans expressed on ovomucoid are either non- or partially galactosylated. Both the HMW and LMW glycopeptides were therefore enzymatically galactosylated and their glycans were analyzed by FAB-MS.

The FAB-MS profiles of the oligosaccharides linked to HMW and LMW glycopeptides are shown in Figure 24. The structures of the glycans associated with these glycopeptides is shown in Figure 25. Both the HMW and LMW glycans showed similar major ions with only minor variations (Figure 24). Such minor variations are usually observed in FAB-MS spectra due to protonation or addition of a sodium ion. Fragmentation is also not perfectly reproducible by FAB-MS below 0.1 Da. The FAB-MS profiles indicated that substantial galactosylation of both the glycopeptide fractions had

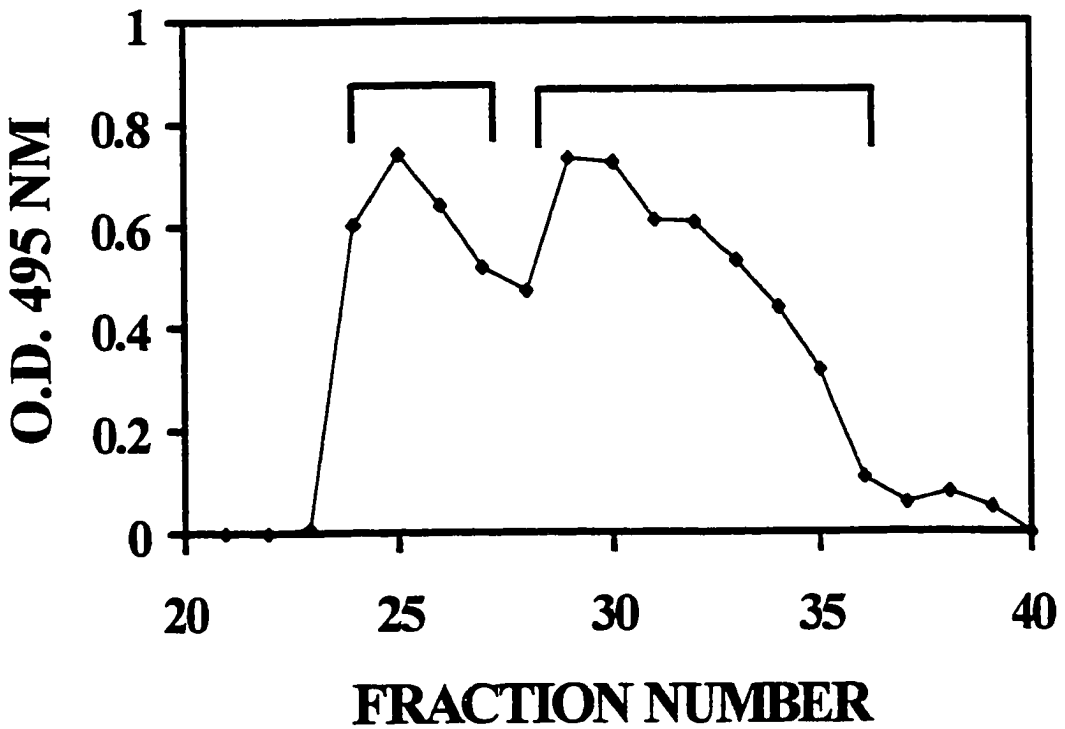
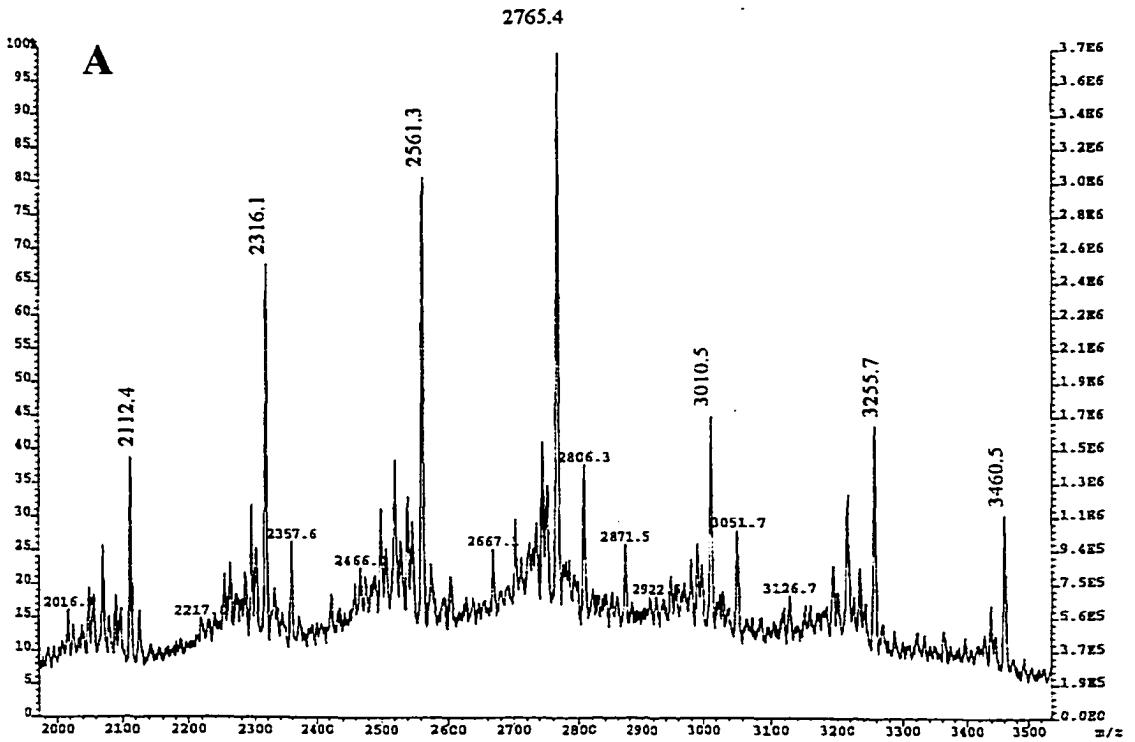
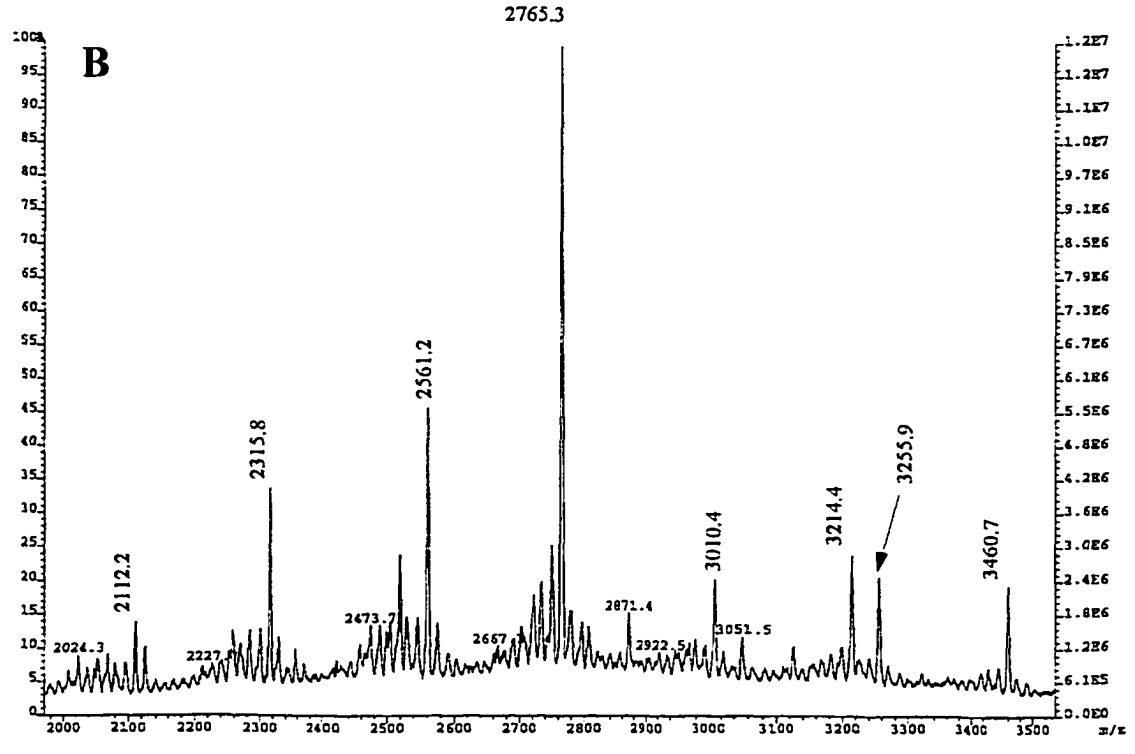


Figure 23: BioGel P4 elution profile of the trypsinized ovomucoid glycopeptides. Ovomucoid glycopeptides were applied to a column of BioGel P4 (60 X 2.5 cm) and eluted in 0.1 M pyridine acetate, pH 5.4. A 5 ml aliquot was obtained from each fraction (2.8 ml) and subjected to phenol-sulfuric acid assay (Dubois et al., 1953). Fractions 24-27 and fractions 28-37 were pooled separately and designated as HMW and LMW respectively.

Figure 24: FAB-MS profile of oligosaccharides derived from glycopeptides in the LMW (Panel A) and HMW (Panel B) after reaction with Gal-T. The major peaks identified in the text have been labeled.





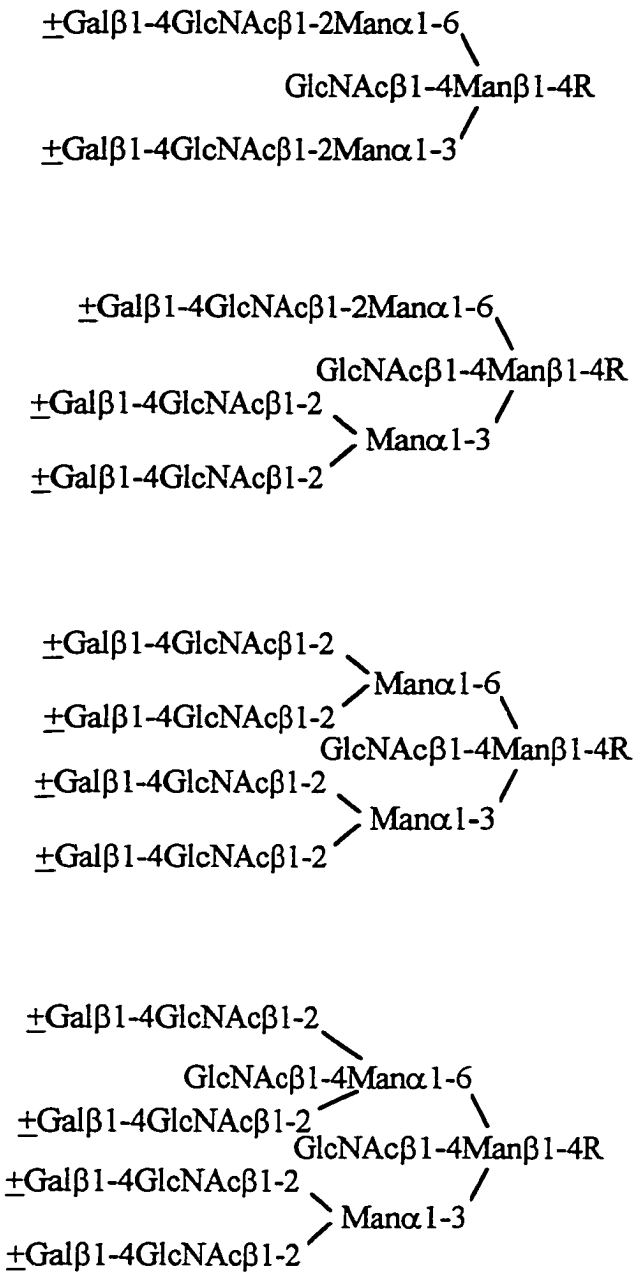


Figure 25: Structures of glycans associated with the HMW and LMW glycopeptides

been achieved. Comparison of the abundance of the molecular ion peaks for the different glycans indicated that approximately 60% of the HMW and 50% of the LMW glycopeptides had been galactosylated.

Separation of the Glycopeptides by E-PHA Column Chromatography

The biantennary and triantennary bisecting type glycans present in the HMW and LMW fractions were isolated chromatography on a immobilized E-PHA column. On an average 20-25% of the total [^{3}H]Gal labeled glycopeptides loaded were found to bind to the column (Figure 26). To obtain sufficient amounts of the material the E-PHA chromatography was performed several times. Each run was found to be highly reproducible. A representative profile of the separation is shown in Figure 26. The bound (E-PHA$^+$) and the unbound (E-PHA$^-$) HMW and LMW glycopeptide fractions from all of the runs were pooled and their glycans analyzed by FAB-MS. Analysis of the E-PHA$^+$ HMW and LMW glycopeptides indicated a significant enrichment of the biantennary bisgalactosylated (m/e ~2316) and the triantennary tris-galactosylated (*m/e* ~2765) bisecting type glycans (Figure 27). Other significant peaks at *m/e* ~2560 and ~2118 represent the triantennary bisgalactosylated and the biantennary monogalactosylated bisecting type glycans respectively, obtained due to under galactosylation of the glycopeptides (Figure 27). FAB-MS spectra of the E-PHA$^-$glycopeptides indicated the presence of the tetraantennary (*m/e* ~3214) and pentaantennary (*m/e* ~3460) bisecting type glycans in addition to the biantennary and triantennary bisecting type oligosaccharide sequences (Figure 28). Undergalactosylated forms of the tetra and pentaantennary oligosaccharides were also found to be present (*m/e* ~3010 and 3255) (Figure 28). Presence of biantennary and triantennary bisecting type glycans in the E-PHA$^-$ fractions

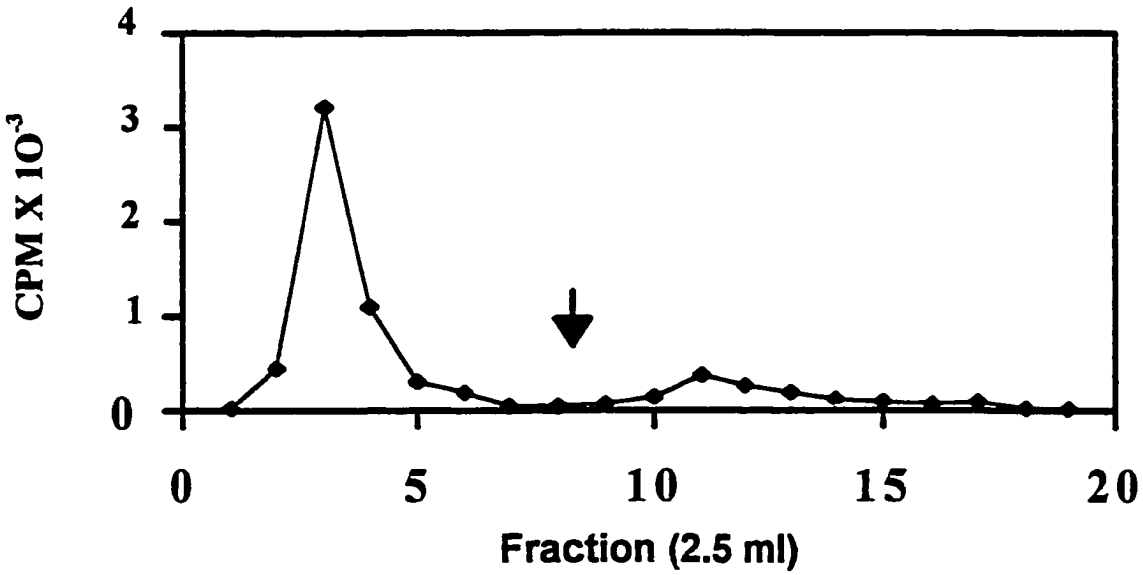
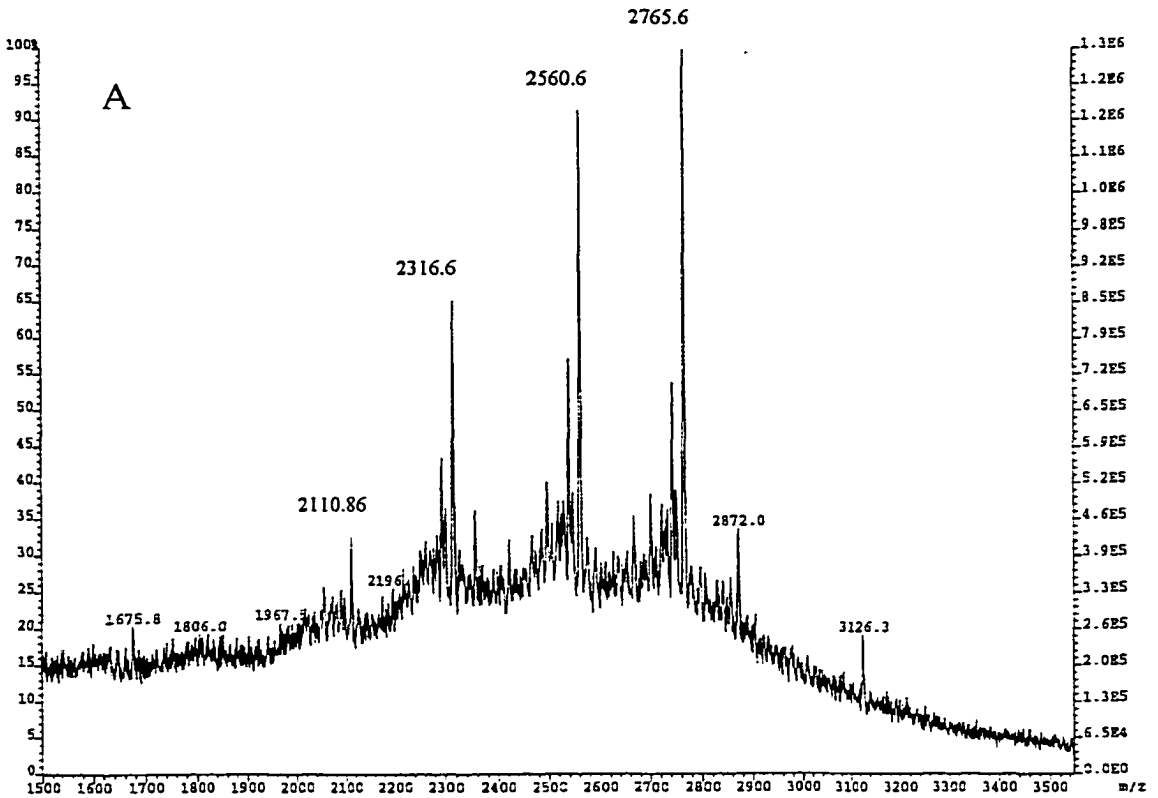
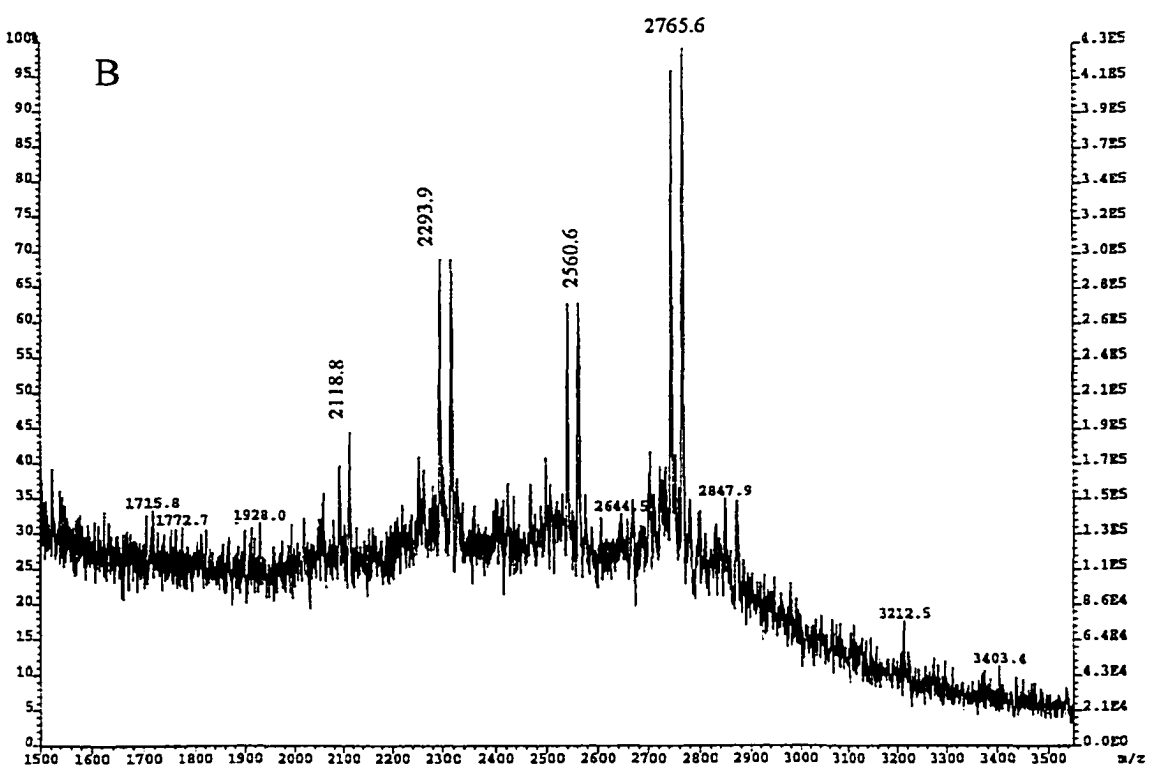


Figure 26: Separation of HMW glycopeptides on a column of E-PHA-agarose (6.0 X 1.0 cm; 5 mg E-PHA/ ml gel). The arrow indicates the point where elution with buffer containing 0.4 M GalNAc was initiated to remove bound glycopeptides.

Figure 27: FAB-MS analysis of oligosaccharides obtained from LMW (Panel A) and HMW (Panel B) glycopeptides that were bound to the E-PHA column. The doublets observed in Panel B are due to Na^+ and H^+ forms being in almost equimolar amount.





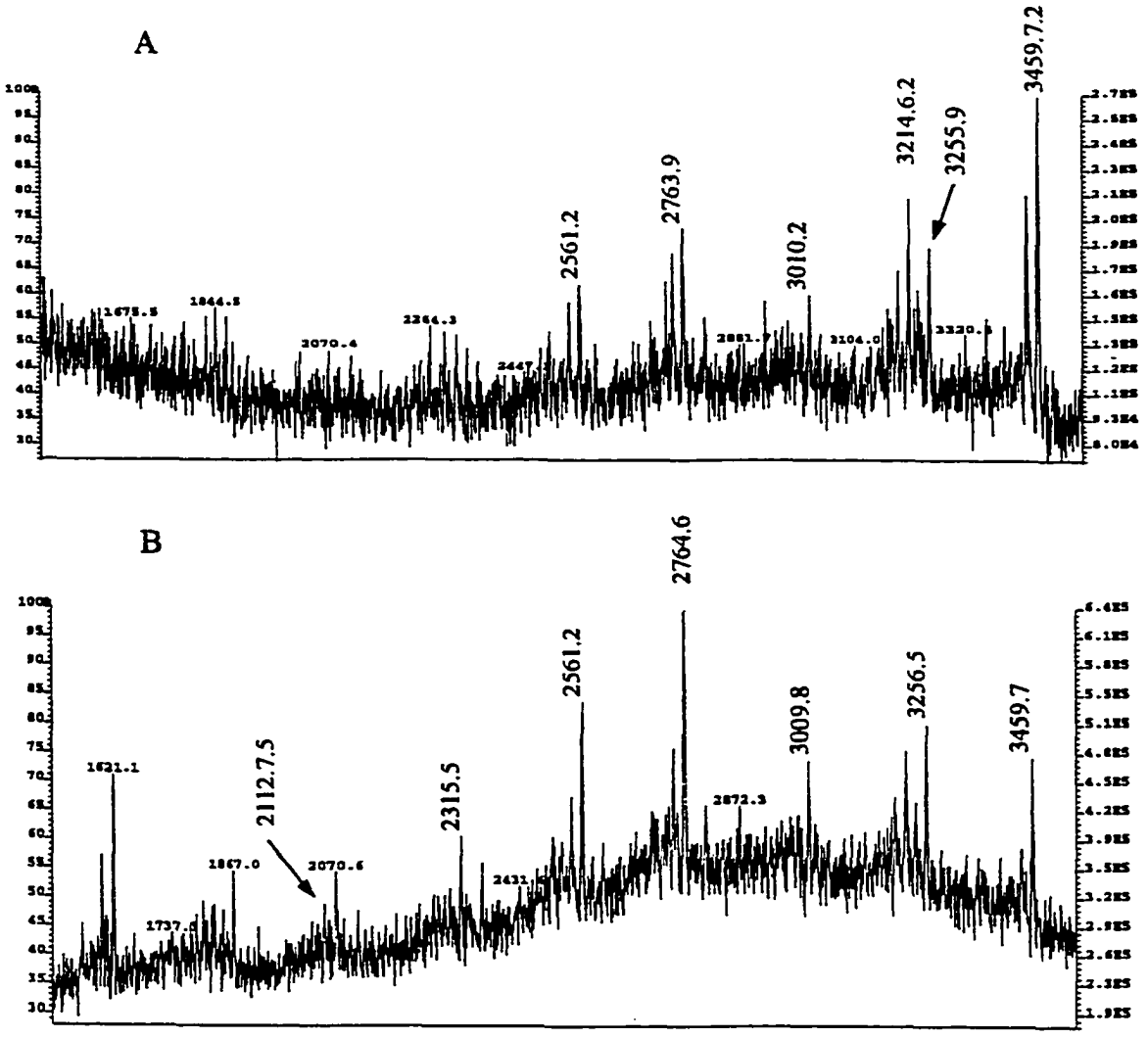


Figure 28: FAB-MS profiles of the LMW (Panel A) and HMW (Panel B) that did not bind to the E-PHA column.

indicated that not all of these glycans were selectively removed from the mixture. However a substantial depletion of these glycans in the unbound fractions was definitely achieved. Unfortunately during the isolation process the LMW E-PHA$^-$ fraction was contaminated by bacteria and could therefore not be used in the NK cell assays. Figure 29 shows the structures of the glycans associated with the E-PHA$^+$ HMW and LMW glycopeptides.

Structural Identities of the HMW and LMW Glycopeptides

Glycopeptides used in this study were isolated after trypsin treatment of ovomucoid. The glycopeptides obtained after this treatment were separated into two peaks according to their size on a BioGel P4 column. It was reasoned that the glycopeptides in the LMW fraction primarily should consist of GP-1 based on their molecular weight as shown in Figure 20. The HMW fraction should be mainly composed of GP-2 and GP-3 (Figure 20). This distribution of the glycopeptides was confirmed by biophysical analysis of the peptide sequences obtained after N-glycanase treatment (data not shown).

It is very likely that the E-PHA$^+$ HMW glycopeptides primarily composed of GP-2 and not GP-3. This reasoning is based on the following arguments. GP-3 carries two glycosylation sites. Therefore presence of biantennary or triantennary bisecting type glycan at even one of these two sites should be sufficient for GP-3 to bind to the E-PHA column. If GP-3 bound to E-PHA column via one of its glycans, it is very likely that glycans present on the other glycosylation site of this glycopeptide would be tetraantennary or pentaantennary glycans (Table 5). However, FAB-MS analysis of the E-PHA$^+$ glycans did not indicate the molecular ions for such tetraantennary or pentaantennary glycans (Figure

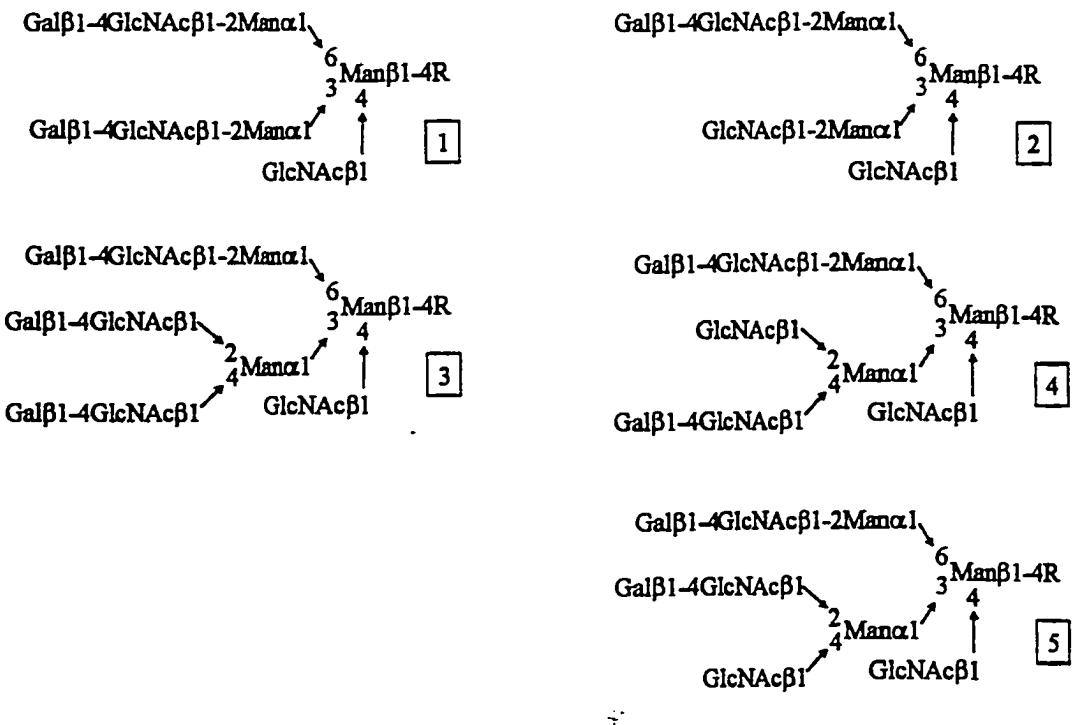


Figure 29: Structures of the glycans associated with the HMW and LMW glycopeptides that were bound to the E-PHA column.

27 and 28). Unfortunately, due to lack of sufficient material, peptide analysis of this fraction could not be performed.

The elution profile of the E-PHA$^+$ HMW glycopeptides on the RCA_{120} column however, also indicates that GP-3 is not present in this fraction (Figure 30). The tetraantennary and pentaantennary glycans terminated with 4 and 5 Gal residues are expected to bind tightly to the RCA_{120} column, eluting off only in the presence of 0.1 M lactose. The elution profile of the E-PHA$^+$ HMW glycopeptides on the RCA_{120} column shows three peaks (Figure 30). Peak 3 which was the most retarded is consistent with a triantennary trisgalactosylated oligosaccharide. Peak 2 is consistent with an oligosaccharide bearing two Gal terminals, which can be present on a triantennary bisgalactosylated or a biantennary bisgalactosylated oligosaccharide (Figure 30). Peak 1 can be obtained from a biantennary monogalactosylated oligosaccharide. These results are consistent with the FAB-MS analysis of the glycans present in the E-PHA$^+$ HMW fraction. No radioactivity was eluted upon washing the column with 0.1 M lactose indicating that the tetraantennary or the pentaantennary glycans were not present in this fraction (Figure 30).

If GP-3 was present in this fraction at least a small amount of the tetraantennary or pentaanntennary glycans should have eluted in the 0.1 M lactose wash. However, no radioactivity was eluted in the 0.1 M lactose wash (Figure 30). Therefore the FAB-MS analysis taken together with the RCA_{120} affinity chromatography results clearly indicate that the E-PHA$^+$ HMW fraction is mainly composed of GP-2.

FAB-MS analysis of the E-PHA$^-$ HMW fractions indicated the presence of both the triantennary and biantennary bisecting type glycans. Since GP-3 did not bind to the E-PHA

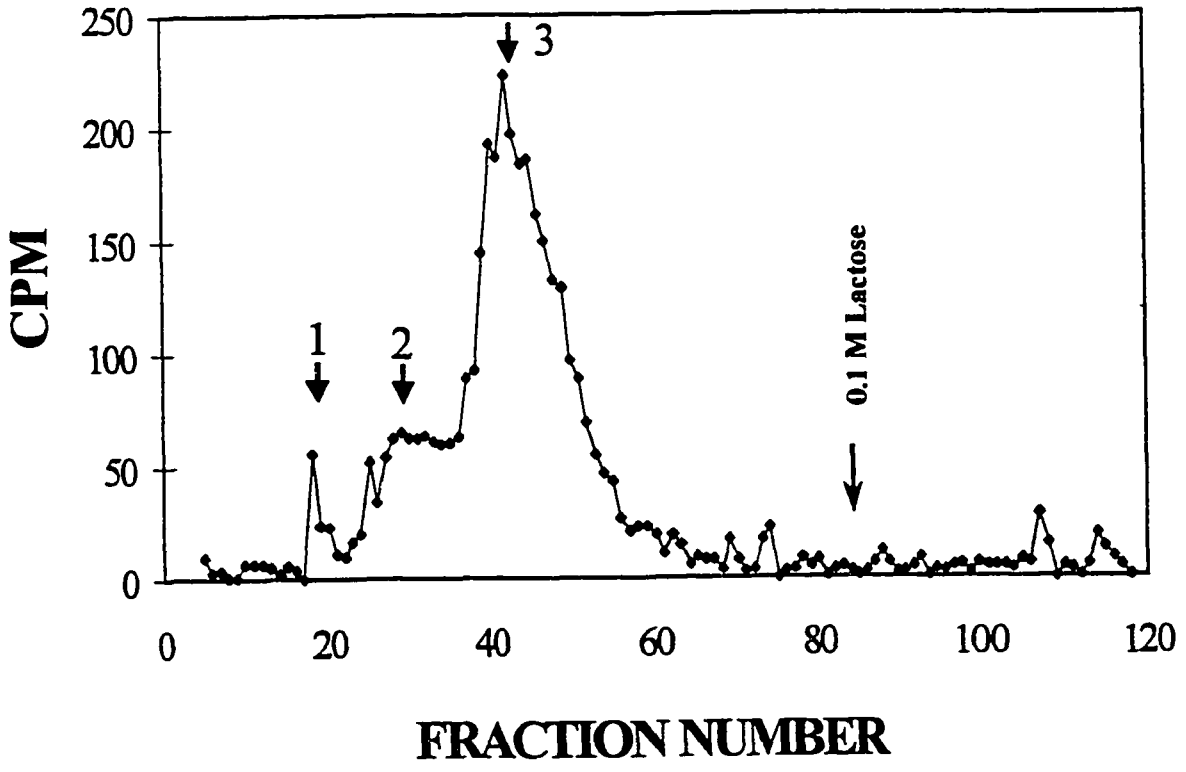


Figure 30: Separation of HMW E-PHA bound glycopeptides on a column of immobilized RCA_{120}. The glycopeptides were applied to a column (1 X 50 cm) of lectin-agarose (1 mg lectin/ml gel). Labeled oligosaccharides with 1, 2, and 3 Gal terminals eluted at the same numbered positions indicated by arrows. Specific elution with 0.1 M lactose was performed to remove all bound glycopeptides.

column it should all have been selectively eluted in the flow through fraction. A significant percentage of the glycans present on both the glycosylation sites of this glycopeptide should bear triantennary and biantennary bisecting type glycans. Both the glycosylation sites are separated by only five amino acid residues. Such close spatial arrangement of the glycans probably results in a peculiar conformational orientation of both the glycan chains in solution. Due to such a configuration it is possible that E-PHA is not able to recognize the triantennary or biantennary glycans present on either one of these two glycosylation sites. The GP-3 glycopeptide therefore remain unbound and is found predominantly in the E-PHA$^-$ HMW fraction

Inhibition of NK Cell And Lymphokine Activated Killer Cell Cytotoxicity By Glycopeptides

It has been reported earlier that culturing the isolated peripheral blood NK cells with IL-2 (interleukin-2) results in their activation. These activated cells are referred to as the lymphokine activated killers (LAK). In this study we have determined the effect of the E-PHA$^+$ and the E-PHA$^-$ glycopeptides on both NK and LAK cell functions. The general experimental paradigm used for these *in vitro* assays is shown in Figure 20.

E-PHA$^+$ HMW Glycopeptides Inhibit NK and LAK Function

In the first experiment, the NK cells were cultured with the different glycopeptides (50 μM final concentration of each glycopeptide) for 48 hr in the presence or absence of IL-2. Trypan blue assays conducted on the cells after the 48 hr incubation period indicated that more 90 % of the cells were viable. The labeled K562 target cells were then co-cultured with the effectors (NK and LAK) at a effector: target ratio of 50:1 for the NK

cells and 24:1 for the LAK cells.

The results obtained indicated that the E-PHA$^+$ HMW significantly inhibited NK and LAK cell function (Figure 31). Marginal inhibition was observed when the effector cells were co-cultured with E-PHA$^+$ LMW glycopeptides (Figure 31). E-PHA$^-$ HMW glycopeptides exhibited virtually no effect on the NK and LAK cell function.

Concentration Dependent Inhibition of LAK Activity by E-PHA$^+$ HMW Glycopeptides

Having established that the E-PHA$^+$ HMW glycopeptides suppressed NK and LAK cell cytolytic responses it was necessary to determine the concentration dependence of this inhibitory effect at different effector cell: target cell ratios. However, as mentioned before only a small amount (1.5 mg) of the E-PHA$^+$ HMW glycopeptide could be isolated. Not enough material was available to perform experiments with both the NK and LAK cells. We therefore focused on obtaining data using LAK cells. The LAK's were cultured for 48 hr with 20, 10, 5, and 1 μM final concentration of the E-PHA$^+$ HMW glycopeptide. Assays were performed at effector cell:target cell ratios of 24:1, 12:1, 6:1, and 3:1. The data obtained clearly showed a concentration dependent inhibition of the LAK responses by the E-PHA$^+$ HMW glycopeptides (Figure 32). These results suggest that 50% inhibition of the LAK cytolysis can be achieved between 5 μM and 10 μM concentration of the E-PHA$^+$ HMW glycopeptides (Figure 32).

E-PHA$^+$ HMW Glycopeptides Inhibit The Activation Events Of LAK Cells

In all of the experiments mentioned above, the LAK or NK cells were cultured with the glycopeptides for 48 hr prior to incubation with the target cells. The next experiment

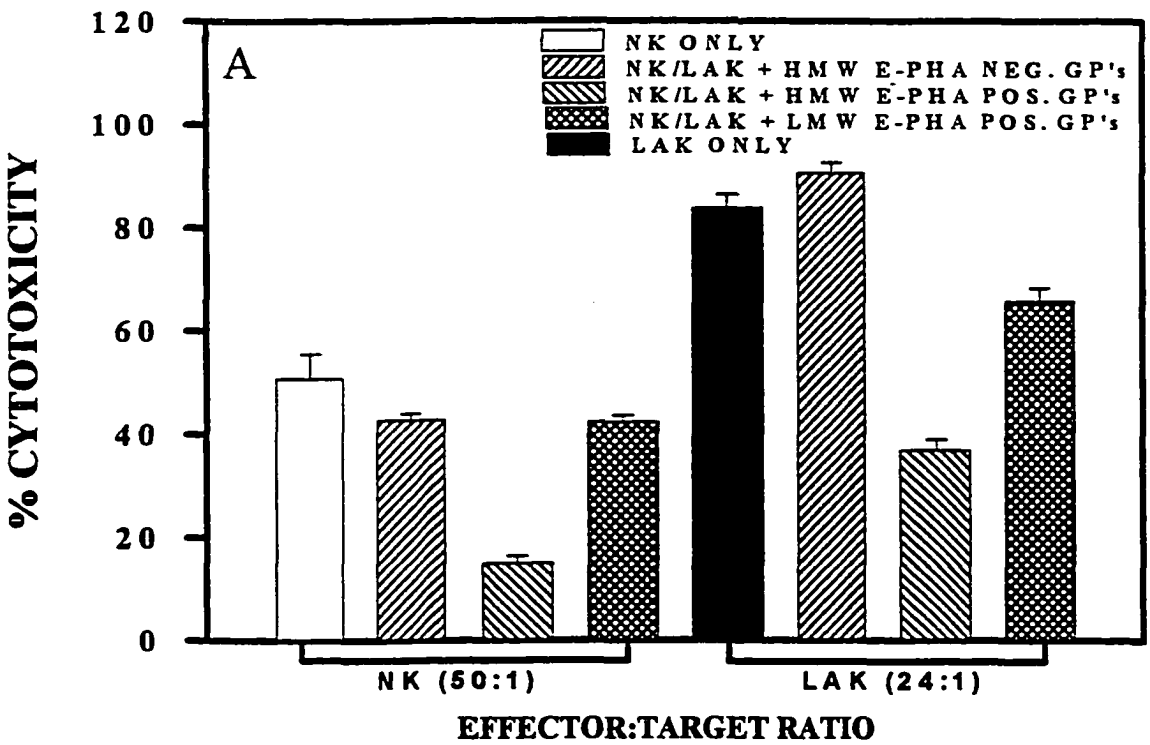


Figure 31: Inhibition of NK and LAK responses at maximal EC:TC ratios tested (50:1 for NK and 24:1 for LAK). The concentration of added glycopeptides was 50 μM. Each assay was performed 6 times.

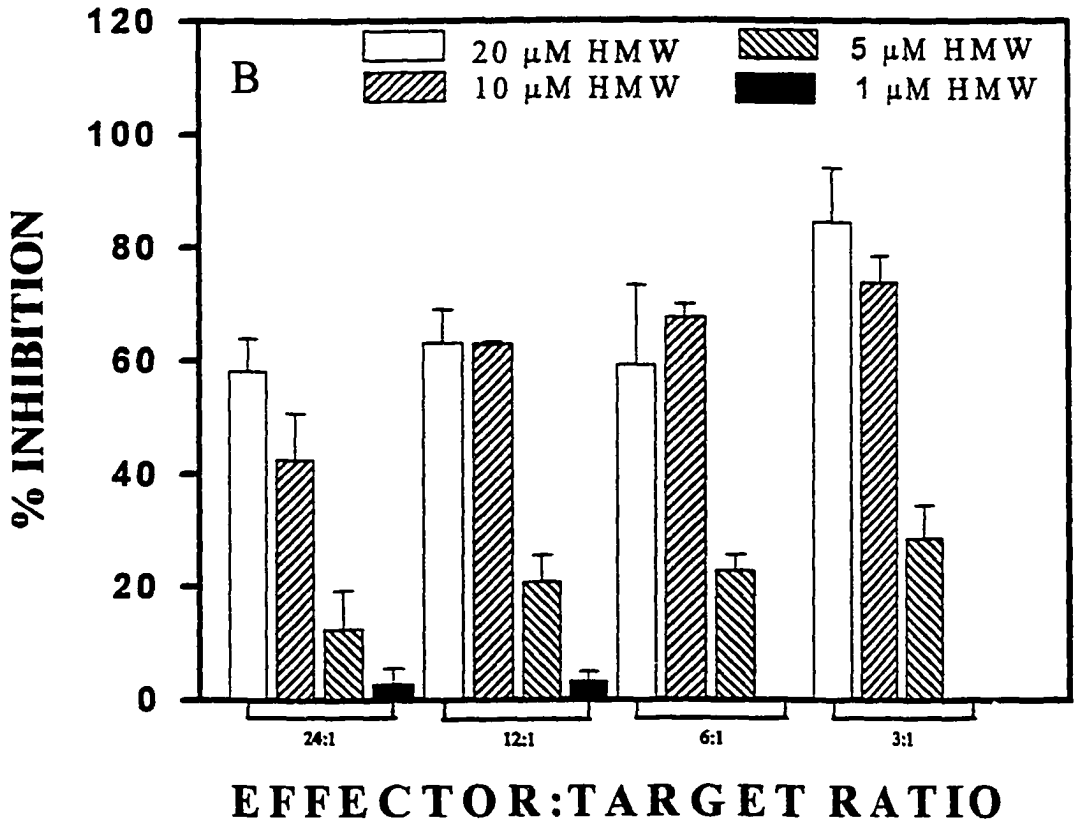


Figure 32: Concentration dependence of the inhibitory effect of the HMW E-PHA bound glycopeptides on LAK cells at different EC:TC ratios. Each assay was repeated 6 times.

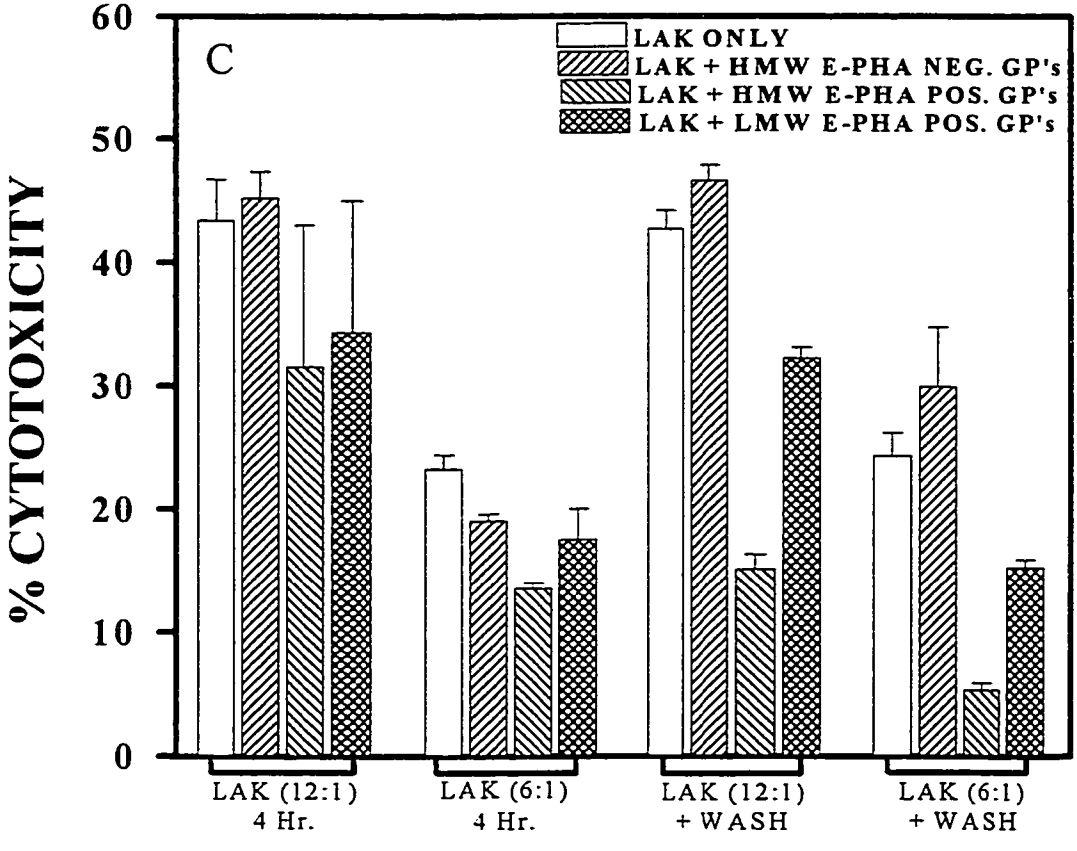
was therefore designed to study the effect of the glycopeptides when added during the 4 hr cytolytic assay. The experimental paradigm used for this experiment is briefly shown in Figure 22. Assays were performed with LAK cells at effector:target cell ratios of 12:1 and 6:1. The effector and target cells were co-cultured for 4 hours in the presence of the E-PHA$^+$ HMW, E-PHA$^-$ HMW, and E-PHA$^+$ LMW glycopeptides at a final concentration of 20 mM. In separate experiments, the LAK cells were also incubated with these glycopeptides for 48 h. The glycopeptides were removed by centrifuging the LAK's and washing them twice with RPMI-10 media. The washed LAK cells were mixed with the target cells at effector:target cell ratios of 12:1 and 6:1.

Glycopeptides tested had very little effect on the LAK responses when added during the 4 hr cytolytic assay (Figure 33). Washing of the LAK's prior to performing the cytolytic assay had no significant effect on difference in the inhibition profiles of the glycopeptides (Figure 33). The E-PHA$^+$ HMW glycopeptides were again found to potently inhibit LAK mediated cytolysis under these conditions (Figure 33).

Ovomucoid and Conalbumin Have No effect On NK Cell Mediated Cytolysis

As a control experiment we determined if incubation with either conalbumin or ovomucoid had any effect on NK cell function. These glycoproteins had no inhibitory effect on NK cell function at this concentration when incubated for 48 hr with the NK cells at 100 μg/ml concentration (Figure 34). In fact there appeared to be a slight stimulatory effect with conalbumin (Figure 34). Another control experiment would have been to determine if galactosylated ovomucoid or conalbumin was capable of affecting NK cell function. However, incubation of 1 mg of each glycoprotein with 2 units of bovine milk

Figure 33: Addition of the E-PHA$^+$ HMW glycopeptides during the 4 h cytolytic assay does not result in inhibition of NK cell function. For 4 h comparison points, NK cells were stimulated with IL-2 for 48 h. Target cells were added to obtain the appropriate EC-TC ratio along with glycopeptides at a final concentration of 20 μM for the assay. In the +Wash comparisons, NK cells were incubated with IL-2 for 48 h with glycopeptides. The cells were washed 2 times to remove glycopeptide and IL-2 and subjected to the cytolytic assay at the given target ratios. Each assay was performed in triplicates.



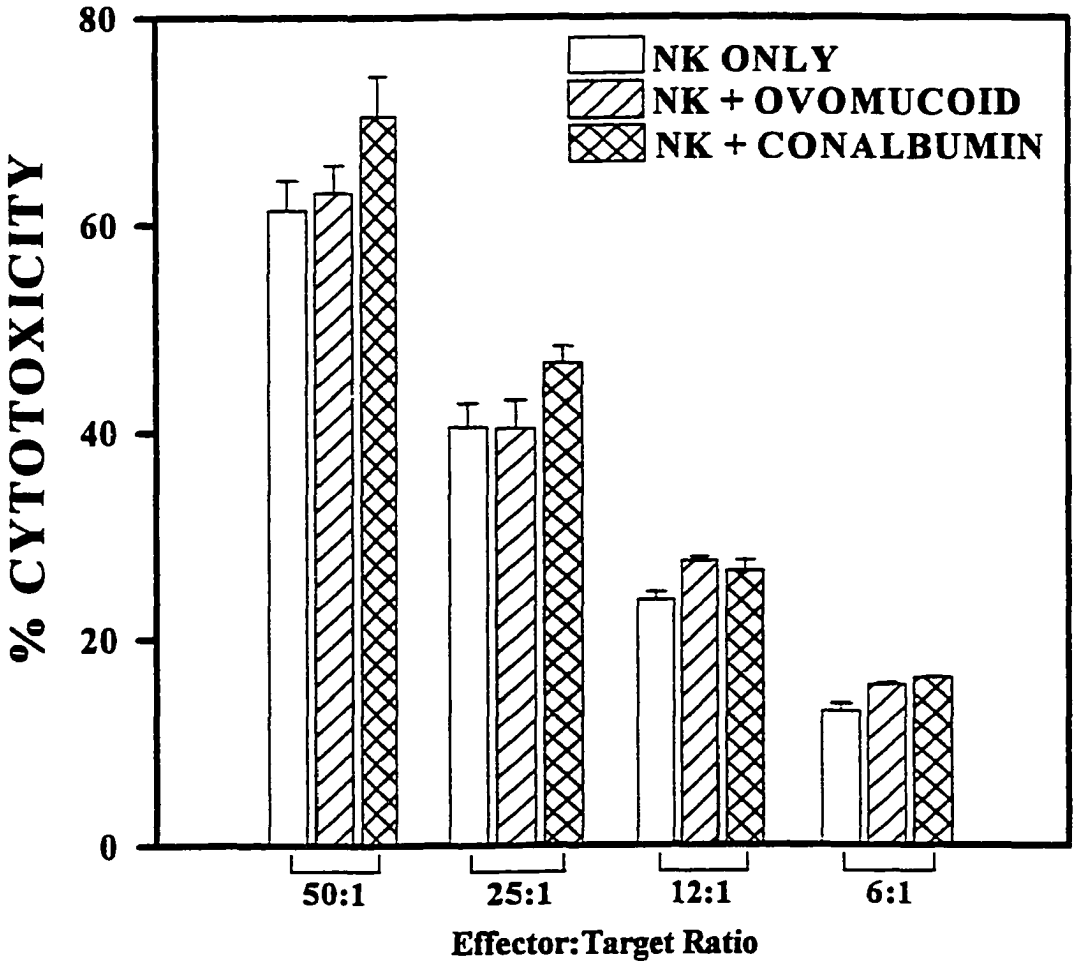


Figure 34: Effect of ovomucoid and conalbumin on NK cell cytotoxicity. This experiment was carried out in triplicate.

galactosyltransferase, 3 mM UDP-Gal and 1 X 10^6 cpm of UDP-[^{3}H]Gal under optimal conditions for galactosylation (Barker, et al., 1972) did not lead to any detectable galactosylation of either ovomucoid or conalbumin. Therefore the potential effect of galactosylated ovomucoid and conalbumin on NK cell activity could not be tested.

DISCUSSION

Protection of the developing human from the maternal immune response is a vital function in reproduction (Hegde, 1991). Similarly, defending the human spermatozoa and eggs from potential immune responses would also be beneficial for the propagation of the species. As described in Chapter III, we have recently proposed the Hu-FEDS hypothesis which states that specific glycoconjugates expressed during pregnancy and on the surface of the male and the female gametes are responsible in suppressing the female's immune responses thereby allowing fertilization and implantation to occur. In the current study we provide preliminary evidence that specific glycans can inhibit immune cell function.

Only E-PHA$^+$ HMW Glycopeptides Inhibit NK/LAK Cell Function

The NK and LAK cell assays indicated that the E-PHA$^+$ HMW glycopeptides were potent inhibitors of cytolytic responses. These results indicated that either the biantennary or triantennary bisecting type glycans or both were able to inhibit NK or LAK activities. The E-PHA$^-$ HMW fractions which contained a mixture of the tetraantennary and pentaantennary glycans in addition to triantennary and biantennary bisecting type glycans did not have any effect on NK or LAK cell mediated cytolytic responses. As mentioned before, the triantennary and the biantennary bisecting type glycans detected in the E-PHA$^-$ HMW fraction are probably present on the GP-3 glycopeptide. The close proximity of the two glycosylation sites present in this glycopeptide probably results in these glycans not being recognized by E-PHA. It is likely that due to similar reasons, the triantennary and biantennary glycans present on GP-3 are unable to interact with the inhibitory cell surface

receptors expressed on the NK or LAK cells. Therefore, although the triantennary or biantennary bisecting type glycans are present the E-PHA$^-$ HMW fraction is not capable of inhibiting NK or LAK cell mediated cytolytic responses. The inability of the E-PHA- HMW glycopeptides to suppress NK or LAK cell activities also strongly suggests that the tetraantennary or pentaantennary bisecting type glycans present in this fraction are not inhibitory.

Another interesting observation made in these studies was that although the E-PHA$^+$ LMW fraction contained the triantennary and biantennary bisecting type glycans, these glycopeptides were not as potent in inhibiting the NK or LAK cell responses as the E-PHA$^+$ HMW glycopeptides. The oligosaccharides of the LMW glycopeptides are carried on a 7 amino acid peptide. the E-PHA$^+$ HMW glycans are presented on a 32 amino acid peptide sequence. It is therefore likely that the inhibitory receptors present on the NK or LAK cells recognize the bisecting type glycans in the context of their peptide sequences. Presentation of these glycans on a longer rather than short peptide sequence results in a better recognition of the glycans by these receptors leading to a more potent inhibition of NK or LAK cell function.

The results presented in this study therefore indicate that the inhibitory receptors expressed on the NK or LAK cells have a strict specificity for the triantennary or biantennary bisecting type glycans. These receptors are not affected by the tetraantennary or pentaantennary bisecting type glycans. Furthermore, it is also likely that presentation of the inhibitory glycans on an appropriate peptide platform may be absolutely essential for potently abrogating the NK or LAK cell responses. Further studies are absolutely required for clearly characterizing the details of the inhibitory effects exhibited by these

glycopeptides.

It is likely that glycoconjugates expressing the bisecting type may play an important role in inhibiting the maternal immune responses. During pregnancy, 2-3 weeks prior to term, a clinical development known as cervical ripening is observed (Romero, 1994). This process is accompanied by a substantial influx of inflammatory cell types into the cervix and decidua. the cause of this influx is not known. It is however interesting to note that this recruitment of the immune effector cells correlates temporally to the loss of Gd-A synthesis and also the changes in the glycosylation pattern of AFP. It is therefore also possible that removal of these suppressive factors plays a role during child birth. It would therefore be significant to study if an untimely removal of such glycoconjugates during pregnancy could lead to premature labor in some clinical cases.

Expression of the Bisecting Type Sequences on HIV

There is evidence indicating that the human immunodeficiency virus (HIV) may also be acquiring the biantennary bisecting type glycans. Infection of human H9 lymphoblastoid cells with HIV yields the viral glycoprotein gp120. Analysis of the *N*-linked glycans associated with this form of gp120 indicates that approximately 11% of the glycans carry the bisecting type biantennary glycans (Mizouchi et al., 1990). It would be interesting to study if the expression of this glycan sequence helps the virus to evade NK cell mediated responses.

Glycosylation of HIV Glycoproteins

The gp120 glycoprotein is a heavily glycosylated protein with up to 20 total *N*-linked and an undetermined number of *O*-linked glycosylation sites. Thus a vast diversity of

oligosaccharide sequences can be expressed on this glycoprotein. A significant question that can be asked here is does the expression of such diverse glycans help HIV to induce immunosuppressive effects in the human? We have recently proposed a model that could possibly explain role of glycoconjugates expressed on the HIV glycoproteins in mediating the potent immunosuppressive effects observed in infected individuals.

HIV is unique because of its ability to preferentially infect the T-lymphocytes (Barre-Sinoussi et al., 1983). The virus therefore has an uncontrolled access to the glycosylation machinery of the T-cells. HIV glycoproteins could therefore acquire glycan sequences that may be exclusive to the T-cell glycoproteins. A growing number of receptors expressed on the immune cells have been shown be lectins. Some of these lectin receptors like the selectins bind only to the carbohydrate ligands (Varki, 1994)whereas others like CD22 recognize the carbohydrates in the context of the protein structures to which the glycans are attached (Hanasaki et al., 1994; Powell and Varki, 1994).

Thus after infection, the HIV glycoproteins could acquire the oligosaccharide sequences that are usually found on the T-cell glycoproteins. Expression of such oligosaccharides could therefore enable the virus to inhibit or evoke a particular immune or inflammatory response via lectin receptors that only require recognition of the carbohydrate. Several reports also indicate that the HIV proteins share significant structural similarities with some of the T-cell proteins (Golding et al., 1988; 1989; Imberti et al., 1991; Dalgleish et al., 1992; Levy, 1993). Glycosylation of such HIV proteins in the T-cell could therefore also result in their recognition by receptors that require both the carbohydrate and the protein structures together.

Such molecular mimicry could be extremely important for HIV virus during the initial stages of infection when the major goal for the virus is establish itself in the T-cells. Because of its high rate of mutation, infected T-cells could produce mutant viruses that could either be destroyed or propagate depending on their ability to infect other cells and evade or misdirect the immune response. The high mutational rate in the viral genome coupled with the selective pressures applied by the host's immune system could therefore lead to the production of mutant virions that could completely defeat the host's immune system.

Our hypothesis therefore predicts that by acquiring the T-cell glycans at least a fraction of the HIV infected cells that closely mimic the T-cell glycoproteins could evade immune responses directed against them. The viruses that survive the immune attacks could then be able to utilize their glycans to interact with the carbohydrate receptors of other cells, enabling them to infect a variety of different immune and non-immune cell types. Finally, soluble or cell surface associated viral glycoproteins carrying the appropriate chains could inhibit normal immune function or induce aberrant immune responses. These mechanisms could eventually lead to the generation of a variant set of viruses that could overwhelm the immune system, leading to the development of the acquired immunodeficiency syndrome (AIDS).

Glycoconjugate Mediated Recognition of Self

Finally the results obtained in our experiments with the NK and LAK cells have clearly indicated that the bisecting type biantennary or triantennary glycans can inhibit their cellular responses. However, the exact mechanisms by which these glycopeptides are able to abrogate the NK or LAK cell activities are not known at this point. However, as

mentioned earlier, many different KIR and KAR expressed on the surfaces of the NK cells carry C-type lectin domains (Moretta, et al., 1997). It is possible that the some KIR may bind to the bisecting type glycans thereby triggering a signaling cascade that results in the inhibition of NK cell function. As mentioned earlier, NK cells recognize the absence of MHC molecules on the surface of their target cells (Moretta, et al., 1992). However, we believe it is likely that the NK cells may also be looking for the expression of the appropriate glycoconjugates on the targets. The lack of expression of such glycoconjugates may result in induction of the cytolytic responses against the target cells. Therefore, the expression of these appropriate glycoconjugates may in turn be a signal to the NK or LAK cells to recognize the target cell as self. It would be interesting to study if specific glycoconjugates could also suppress responses mediated by other immune cell types. If so, recognition of self could be mediated not only by the MHC system but also by the expression of cell surface associated glycoconjugates.

Conclusion

In this chapter we have provided preliminary evidence suggesting that specific exposure of the NK or LAK cells to specific glycoconjugates could inhibit their cellular responses. More work will definitely be needed to characterize the exact mechanism involved in mediating such glycoconjugate mediated suppressive effects. However the occurrence of the bisecting type glycans on glycoproteins like AFP and on HIV gp120 strongly suggests that suppression of cellular immunity by glycoconjugates may be playing a significant physiological role in pregnancy and also in various pathological states. These preliminary studies may lead to new approaches for understanding the human immune and reproductive systems and may help in providing better avenues to combat infections.

CHAPTER V

CONCLUSION

Many previous reports have suggested that glycoconjugates could play important roles in human gamete binding interactions and also in various immune cell functions (Gahmberg et al., 1992). Substantial evidence obtained in the mouse system clearly indicates that the oligosaccharides expressed on the surface of the ZP are absolutely essential for mediating sperm binding (Wassarman, 1990). Unambiguous evidence for the involvement of the human ZP oligosaccharides in mediating gamete adhesion however has not been available so far. The experiments conducted in this study strongly support such a role for the ZP oligosaccharides. We have performed carefully controlled studies to demonstrate that glycoconjugates or oligosaccharides bearing restricted structures are able to potently block human sperm-ZP binding in an *in vitro* assay system. Such a specificity for the oligosaccharide structure clearly indicates that the egg binding protein expressed on the surface of the sperm is recognizing a oligosaccharide ligand present on the ZP. Using subtle chemical and enzymatic modification that affect only the ZP oligosaccharides, we have shown that sperm binding to these treated ZP can be significantly affected. Periodate treatment of the ZP under mild conditions that affect only the terminal sialic acid residues results in approximately 30% inhibition of sperm binding. Similarly periodate oxidation of the ZP under conditions that affects all of the terminal sugars, results in a significant 40% inhibition of sperm binding. Both these experiments indicate that chemical modifications of the terminal ZP oligosaccharides substantially affects human sperm-ZP binding. We have

also shown that removal of the terminal sialic acids and also some of the terminal polylactosamine chains significantly increases sperm binding to the ZP indicating that the egg binding receptor present on the human sperm may be recognizing some internal oligosaccharide motifs. Removal of the outer glycans helps the receptor to better recognize the internal oligosaccharides.

Periodate oxidation experiments conducted on neuraminidase treated ZP perhaps provide the most compelling evidence supporting the role of the ZP oligosaccharides in mediating sperm binding. Mild periodate treatment of neuraminidase treated ZP results in a almost complete loss of sperm binding. It is likely that the removal of the terminal sialic acids results in complete exposure of the internal glycan residues. However treatment of these residues with periodate results in their total chemical modifications which are not recognized by the egg binding protein present on the sperm.

Collectively all of this data unambiguously demonstrates the role of the ZP oligosaccharides in mediating initial human sperm-egg binding. We are pursuing different approaches to clearly identify the glycan structures that are required to mediate this adhesion event. Identification of such glycan sequences would greatly help in identification of the egg binding protein expressed on human sperm. A better understanding of these processes would lead to the development of novel contraceptives and also in the devising better strategies for treatment of at least some causes of infertility.

Finally our studies have also indicated that a considerable overlap likely exists between the carbohydrate ligands involved in human gamete binding and in the mediating various immune cell functions. On this basis we have proposed that specific expression of immunosuppressive glycoconjugates occurs during pregnancy. Such temporal expression of

these glycoconjugates may be absolutely required to protect the human embryo/fetus from the maternal immune responses. This paradigm is referred to as the Human fetoembryonic defense system hypothesis (Hu-FEDS). It is proposed that the human gametes and other glycoconjugates expressed during pregnancy specifically acquire glycans that potently suppress the cell mediated cytolytic responses of the maternal immune system. The first preliminary evidence for the existence of such a glycoconjugate based immunosuppressive system is provided in this study. First we show that the human gametes express the bisecting type GlcNAc sequences which have been previously suggested to inhibit NK cell mediated responses. In chapter IV we have synthesized and isolated the bisecting type glycopeptides and shown that they can potently inhibit NK cell mediated cytolysis of the target K562 cells.

AFP and other glycoproteins expressed during pregnancy have been previously shown to carry the bisecting type glycans (Tsuchida, et al., 1984). We therefore believe that specific expression of such glycans on these glycoproteins may play an important role in the immunoprotection of the embryo/fetus. In addition many different studies have indicated that the expression of the bisecting type and other unusual glycans on the surfaces of parasites (Mizouchi et al., 1990; Kang, et al., 1993). Expression of the bisecting type glycans has also been demonstrated on the HIV glycoprotein gp120. It may therefore be very likely that these different pathogens may be co-opting the same glycoconjugate based system of immunosuppression that is used to protect the fetus to evade the hosts immune responses. Further studies are currently in progress to determine the exact mechanisms by which these glycopeptides inhibit the NK cell responses. However, the data obtained from these studies may have paved a way for developing new strategies to understanding

fetomaternal tolerance and the ability of different pathogens to circumvent the immune response of the host.

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Publications:

1. Ozgur, K., Patankar, M. S., Oehninger, S., Clark, G. F. (1998) Direct evidence for the involvement of carbohydrate sequences in human sperm-zona pellucida binding. *Mol. Hum. Reprod.*, **4**, (in press).
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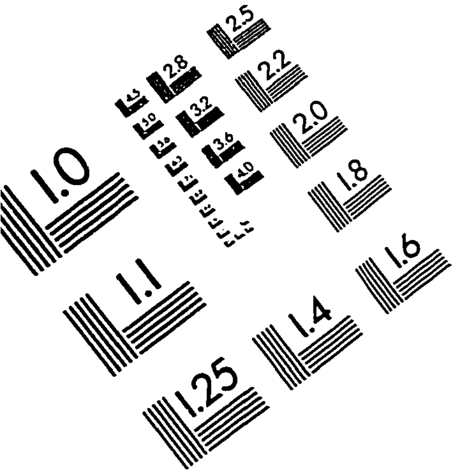
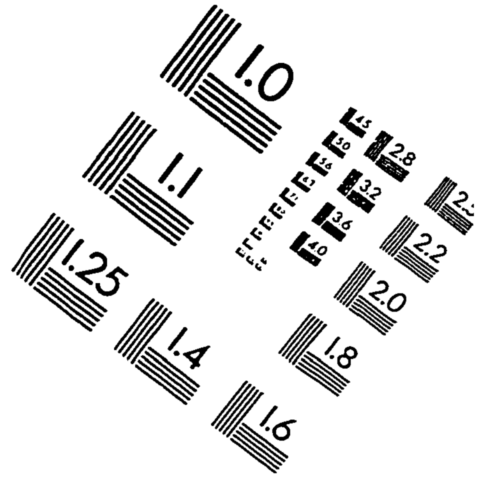
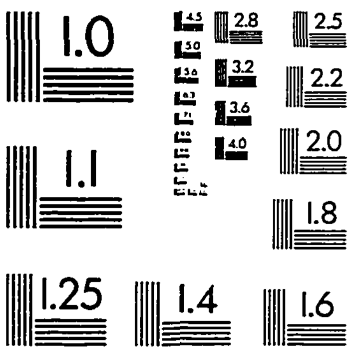
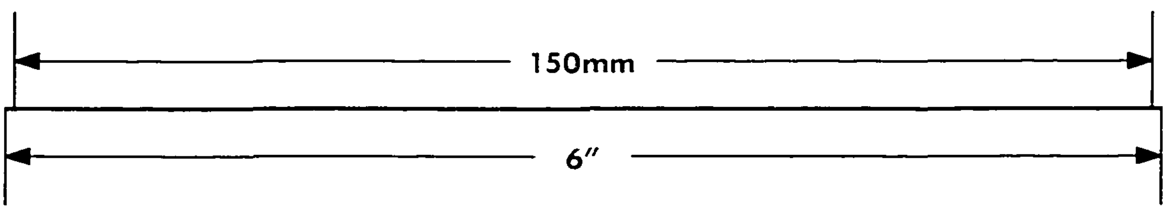
11. Patankar, M. S., Oehninger, S., Barnett, T. B., Williams, R. L., and Clark, G. F. (1993) A revised structure for fucoidan may explain some of its biological activities. *J. Biol. Chem.* **268**, 21770-21776.

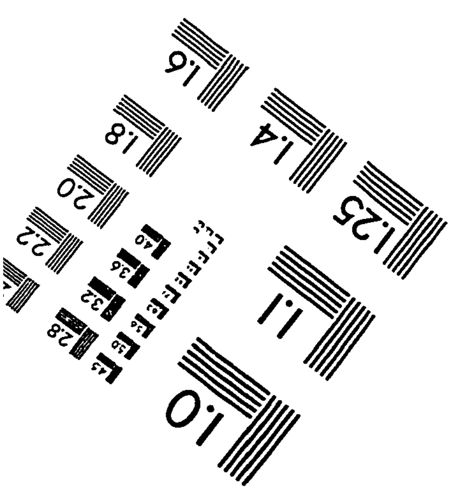
IMAGE EVALUATION TEST TARGET (QA-3)

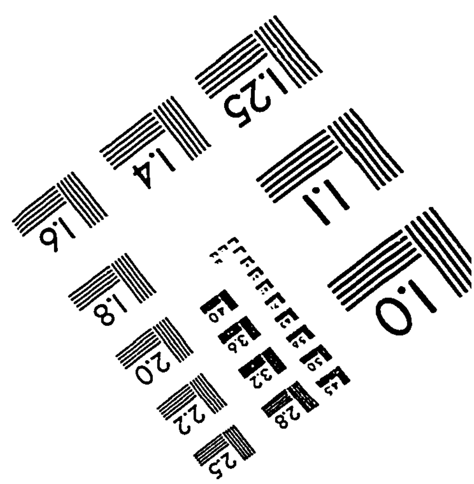












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